THE RELATIONSHIP OF THE NEUROKININ-1 RECEPTOR TO REWARD AND LEARNING AND MEMORY BEHAVIOURS IN THE MOUSE

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ABSTRACT

Mice lacking the neurokinin-1 (NK1) receptor, the preferred receptor for the neurotransmitter substance P, appear to be insensitive to the rewarding properties of morphine, suggesting a role for this system in opiate abuse. The primary aim of this work was to extend these findings in the mouse.

Following confirmation that the distribution of the NK1 receptor in the mouse brain was similar to that in the rat, the reduction in sensitivity of NK1 receptor knockout (NK1−/−) mice to morphine reward was verified. Using the locomotor sensitisation model, it was further demonstrated that mice lacking NK1 receptors do not exhibit the behaviours associated with the adaptive changes in response to repeated opiate exposure, suggesting that the NK1 receptor is crucial for the development of opiate addiction.

Subsequently, two approaches were used to attempt to identify the locus for these effects. Firstly, c-Fos immunohistochemistry was used to 'map' regions of the brain differentially activated by morphine in NK1−/− mice. Perturbations in c-Fos expression were observed in the basolateral nucleus of the amygdala, the dentate gyrus and cortical areas in response to the morphine conditioned place preference task. Secondly, using the specific neurotoxin substance P-saporin, mice with bilateral ablation of neurones expressing the NK1 receptor in the amygdala, but not in the nucleus accumbens, showed similar reductions in morphine reward-related behaviours to NK1−/− mice. The amygdala is therefore an important area for the NK1 receptor's effects in mediating behaviours related to morphine reward in mice.

Finally, the behaviour of NK1−/− mice was assessed in a range of learning and memory tasks. Despite having higher levels of hippocampal neurogenesis and brain-derived neurotrophic factor, NK1−/− mice displayed very similar behaviours to wild type mice, suggesting that the NK1 receptor and these hippocampal adaptations have only a weak influence on learning behaviour and memory.
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CHAPTER ONE

INTRODUCTION
1. INTRODUCTION

1.1. Introduction

Since its discovery in the 1930s, the neuropeptide substance P (SP), the preferred ligand for the neurokinin-1 (NK1) receptor, has been studied intensively. The majority of the work carried out on this molecule has concentrated on its role in the transmission of pain signals and in inflammation. However, more recent work has begun to illustrate its role in emotional and motivational behaviours, including depression, anxiety and stress.

One aspect of these more recent discoveries is the involvement of SP and its receptor in the rewarding or pleasurable aspects of morphine, an opiate drug widely used in the alleviation of pain. The majority of the experimental work presented in this thesis is based upon these findings, in an attempt to understand more fully the role of this system in opiate reward and addiction in mouse models. A further set of experiments was also carried out which examined the behaviour of mice lacking the NK1 receptor in a range of learning and memory tasks, in order to assess the contribution of this system to mnemonic processes.

This chapter contains a brief overview of the history of research into SP and its receptor and the known functions of this system in mammals. This is followed by a summary of the neuroanatomical processes known to underlie reward processes and drug addiction, which form a basis for most of the studies described in the remainder of this thesis. Within each subsequent chapter, the background to the problems being addressed is described in more detail, along with descriptions and explanations of the experimental approaches used.

1.2. Substance P and the neurokinin-1 receptor

1.2.1. History of substance P research

SP was first isolated in 1931 as a crude extract from the brain and gut of the horse. It was found to have potent hypotensive and smooth muscle contractile properties (von Euler & Gaddum 1931), being named three years later: ‘P’ simply refers to the powder obtained in the extraction process (Gaddum & Schild 1934). It was soon found to be peptidergic in nature (von Euler 1936, 1942), and subsequently became the subject of intense
investigation. By the mid 1950s it was known that SP was a neuronal sensory transmitter associated with the transmission of pain signals, and that it was found in high concentrations in the dorsal horn of the spinal cord (Lembeck 1953). The basic pharmacology of SP was also understood, in that its actions were not blocked by cholinergic, histaminergic, serotonergic or any other known antagonists (Pernow 1953). In the 1960s, SP was included in the tachykinin family, since it possesses similar hypotensive and smooth muscle stimulant properties to this family of peptides, which are mainly derived from amphibian skin (Erspamer 1981; Erspamer & Anastasi 1966). However the structure of SP was not identified until 1971, when it was found to be an undecapeptide with the amino acid structure H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ (Chang et al. 1971). This discovery allowed the production of synthetic peptide and a subsequent explosion in SP research (Tregear et al. 1971).

Around ten years after SP’s sequence was identified, the discovery of other mammalian peptides with similar properties led to their inclusion in the tachykinin family: all mammalian tachykinins, including these newly characterised peptides, now termed neurokinin A and neurokinin B, share the same carboxy terminal sequence of Phe-X-Gly-Leu-Met-NH₂, where X is Phe or Val (Kanagawa et al. 1983; Kimura et al. 1983, 1984; Maggio et al. 1983; Minamino et al. 1984; Nawa et al. 1983).


1.2.2. Distribution and actions of substance P

1.2.2.1. Nervous system

Although suggested in the 1950s, the excitatory nature of SP on central neurones was not demonstrated until 1972, in frog spinal motor neurones (Otsuka et al. 1972). This, along with the observation that SP’s concentration was around 20 times higher in dorsal than
ventral root extracts from bovine spinal cord (Lembeck 1953; Takahashi et al. 1974), led to the suggestion that SP was located in primary afferent neurones. This was supported by observations that a dramatic decrease in SP was seen in the superficial spinal cord following rhizotomy or nerve section (Jessell et al. 1979; Takahashi & Otsuka 1975), and that SP immunoreactivity and PPT-A mRNA were observed in the cell bodies of primary afferent cells in the dorsal root ganglia, which possessed unmyelinated or thinly myelinated axons (Cuello & Kanazawa 1978; Hökfelt et al. 1975, 1976; Ljungdahl et al. 1978a,b).

The localisation of SP to the unmyelinated C-fibres was confirmed using the neurotoxin capsaicin. In adult animals, chronic capsaicin administration results in an insensitivity to painful stimuli, due to the reversible depletion of SP from the terminals of primary afferent C-fibres (Gamse et al. 1979; Jancsó et al. 1977; Jessell et al. 1978). In neonatal animals, capsaicin causes the selective degeneration of C-fibres resulting in the irreversible loss of SP from nerve terminals in the dorsal horn of the spinal cord (Gamse et al. 1980; Jancsó et al. 1977; Nagy et al. 1981, 1983; Nagy & Hunt 1983; Scadding 1980). Furthermore, the release of SP from primary afferent nerve terminals is induced by sciatic nerve stimulation, but only at stimulus intensities high enough to activate C-fibres (Gamse et al. 1979; Theriault et al. 1979; Yaksh et al. 1980). These findings have subsequently been verified in ultrastructural studies, demonstrating the presence of SP-containing vesicles within nerve terminals located in the superficial dorsal horn (Barber et al. 1979; Chan-Palay & Palay 1977; Cuello et al. 1977; Pickel et al. 1977; Ribeiro-da-Silva et al. 1989). SP application brings about a slow, long-lasting depolarisation of dorsal horn neurones both in vivo and in culture, which is excitatory and often accompanied by a fast excitatory post-synaptic potential (EPSP) due to the co-release of glutamate (Henry 1976; Konishi & Otsuka 1974; Krnjevic & Morris 1974; Nowak & Macdonald 1982; Randic & Miletic 1977; Zieglgänsberger & Tulloch 1979).

In the brain, SP is found in a wide range of structures. Immunohistochemical studies have shown that the area with the highest levels of SP is the substantia nigra, where SP terminals derive from the ipsilateral striatum (Davies & Dray 1976). SP release in this brain region causes a long-lasting excitation of the nigrostriatal dopaminergic neurones that are under inhibitory control by dopamine and γ-aminobutyric acid (GABA; Glowinski et al. 1980, 1982; Iversen 1982; Minabe et al. 1996; Reubi et al. 1978).
In the striatum, SP is found in the dynorphin-positive population of medium-sized GABAergic spiny neurones of the caudate putamen (CPU) and the nucleus accumbens (NAcc), which project to the substantia nigra, the globus pallidus and the ventral pallidum (Anderson & Reiner 1990; Besson et al. 1990; Haber & Nauta 1983; Napier et al. 1995; Penny et al. 1986). These neurones also possess SP-positive collaterals that project back onto the large cholinergic interneurones of the striatum (Bolam et al. 1983, 1986). The release of SP in this region increases the firing rate of these cholinergic interneurones (Le Gal La Salle & Ben Ari 1977), and leads to the release of dopamine and acetylcholine within the striatum (Anderson et al. 1993; Arenas et al. 1991; Boix et al. 1992; Galarraga et al. 1999; Petit & Glowinski 1986; Starr 1978; Steinberg et al. 1995; Tang et al. 1998). The ventral tegmental area (VTA), which sends a dopaminergic projection to the NAcc, also possesses SP fibres, which originate from the medial habenular nucleus (Emson et al. 1977). This mesolimbic dopaminergic projection (see below) is involved in the control of locomotor activity, learning, arousal and reward processes, and is regulated by SP (Bannon et al. 1983; Elliott et al. 1992; Minabe et al. 1996; Stinus et al. 1978).

Elsewhere in the brain, SP is found in low levels in the cortex and the hippocampus, whilst in the hypothalamus, it is believed to be involved in the regulation of hormone release from the pituitary gland (Aronin et al. 1986). In the amygdala, SP fibres have been observed primarily in the medial and central subnuclei, where it is involved in the control of emotional behaviour (Emson et al. 1978; Kramer et al. 1998; Steinberg et al. 2001). SP is also found in the parabrachial nucleus and in the locus coeruleus, where SP terminals contact the noradrenergic neurones, whose firing rate is increased by SP application (Bert et al. 2002; Guyenet & Aghajanian 1977). The nucleus tractus solitarius possesses high levels of SP, where it plays a role in respiratory, cardiovascular and emetic reflexes (Gillis et al. 1980; Tattersall et al. 1996), whilst the distribution and action of SP in the trigeminal nucleus (and trigeminal ganglia) is homologous to that in the dorsal horn of the spinal cord (and dorsal root ganglia), with painful stimuli causing its release onto nociception-specific neurones (Henry et al. 1980; Yonehara et al. 1986).

1.2.2.2. Periphery

In the periphery, SP is found in most tissues, where it is usually derived from the peripheral endings of primary afferent neurones. Additionally, in the eye, SP is found in the ganglion
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cell layer of the retina, where it acts as a neurotransmitter (Brecha et al. 1987), and in the
innervation of the iris, where it causes contraction (Andersson 1987; Tornqvist et al. 1982;
Ueda et al. 1986). SP is also a potent stimulator of salivation, being found at high levels in
the parasympathetic innervation of the salivary glands (Ekström et al. 1988). In the urinary
bladder and the respiratory system, SP mediates smooth muscle contraction, resulting in
micturition or bronchoconstriction respectively (Carruette et al. 1992; Falconieri-Erspamer
et al. 1973; Sjögren et al. 1982). Furthermore, exposure to irritants in the airways leads to
the release of SP and other tachykinins resulting in coughing, sneezing, vasodilation,
plasma extravasation and the secretion of mucus: SP release may therefore be an important
factor in asthma (Barnes 1987; Barnes et al. 1988, 1990; Bertrand & Geppetti 1996; Delay-
Goyet & Lundberg 1991; Frossard & Advenier 1991; Lei et al. 1992). In the circulatory
system, SP induces vasodilation and hypotension via a nitric oxide-dependent mechanism
(Fiscus et al. 1992; Pernow & Rosell 1975; von Euler & Gaddum 1931; Whittle et al. 1989).

Within the gastrointestinal tract, SP is found both in primary afferent terminals and in
intrinsic neurones (Franco et al. 1979; Sternini et al. 1995). SP causes smooth muscle
contraction in the gut, coupled with an increase in motility and secretion of water and
electrolytes, mediated both by the direct action of SP on muscle cells, and by a cholinergic
mechanism (Bauer & Kuriyama 1982; Gaddum & Schild 1934; Holzer & Lembeck 1980;
Holzer & Petsche 1983; Huidobro-Toro et al. 1982; Kachur et al. 1982). Blocking the SP-
mediated inflammatory response may be of use in the treatment of inflammatory gut
diseases such as colitis and Crohn’s disease, as well as irritable bowel syndrome. Dramatic
increases in the number of NK1 receptors have been observed in the colon of patients
with these diseases, while administration of NK1 receptor antagonists to rats decreases
gastric emptying, slows down gastrointestinal contractions, and reduces inflammation,
suggesting that they might control the overactivity of the gut musculature observed in these

1.2.3. The neurokinin-1 receptor

The tachykinin receptors, including the NK1 receptor, were discovered in the early 1980s.
Following comparison of the potencies of SP and other non-mammalian tachykinins in
various bioassays, it was found that they displayed dramatic differences in potencies in
different tissues (Erspamer 1981; Falconieri-Erspamer et al. 1980). Two tachykinin
receptors were therefore proposed: an SP-P type which was more selective for SP and physalaemin, and an SP-E type which was more selective for eldeoisin and kassinin (and, following its later discovery, neurokinin A; Lee et al. 1982; Watson et al. 1983). A third receptor was added to these following the discovery of neurokinin B, and was termed SP-N (Laufer et al. 1985). It was noted that all three mammalian tachykinins were able to act as full agonists at each of the three receptor types, but with different affinities (Ingi et al. 1991; Mantyh et al. 1989; Regoli et al. 1987; Saffroy et al. 1988). In 1987, tachykinin receptor classification was standardised at the Substance P and Neurokinin meeting in Montreal, Canada, and SP-P, SP-E and SP-N were renamed NK1, NK2 and NK3, the preferred receptors for SP, neurokinin A and neurokinin B respectively (Henry 1987). These three receptors were cloned from the cow and the rat in the late 1980s, and were found to be very similar in amino acid sequence and in structure, as guanine nucleotide binding protein (G-protein)-coupled rhodopsin-like seven transmembrane domain proteins (Hershey & Krause 1990; Masu et al. 1987; Nakanishi 1991; Shigemoto et al. 1990; Yokota et al. 1989). The three receptors differ primarily in the third intracellular loop and the carboxy terminus, which may confer their differential coupling to G-proteins (Nakanishi 1991). The human NK1 receptor was cloned in 1991, and was found to be 94.8% homologous to the rat receptor (Gerard et al. 1991).

The carboxy terminal hexapeptide of SP is essential for binding to and activation of the NK1 receptor (Bury & Mashford 1976; Cascieri et al. 1992). Activation of the NK1 receptor results in the activation of G-proteins ($G_{q,11}$, $G_\alpha$ and $G_\alpha_c$), which induce the formation of inositol 1,4,5-trisphosphate ($IP_3$) and diacyl glycerol (DAG) via phospholipase C$_q$ activation (Nakajima et al. 1992; Roush & Kwatra 1998; Seabrook & Fong 1993; Takeda et al. 1992; Taylor et al. 1986). The local increase in $IP_3$ results in the release of calcium ions from intracellular stores and the subsequent activation of calcium-calmodulin-dependent kinases, as well as the influx of extracellular calcium, whereas DAG activates protein kinase C (Merritt & Rink 1987; Seabrook & Fong 1993; Sugiya et al. 1987; Womack et al. 1988). NK1 receptor activation also stimulates arachidonic acid mobilisation via phospholipase A$_2$ and cyclic adenosine monophosphate (cAMP) accumulation via adenylyl cyclase (Garcia et al. 1994; Mitsuhashi et al. 1992; Nakajima et al. 1992; Seabrook & Fong 1993; Takeda et al. 1992). These effects on second messenger pathways are followed by a slow excitatory response mediated by increases in membrane sodium conductance and decreases in

1.2.3.1. Distribution of the neurokinin-1 receptor

The distribution of the NK1 receptor in the brain is reviewed in Chapter 3. Briefly, it is expressed in many regions of the brain, particularly in the olfactory bulbs, the striatum, the dentate gyrus, the amygdala, the periaqueductal grey, the superior colliculus, the parabrachial nucleus and the locus coeruleus. Elsewhere in the central nervous system, it is found in high levels in the superficial laminae of the spinal cord, where it is expressed on the neurones that are activated by SP released from nociceptive C-fibres (Nakaya et al. 1994). Peripherally, the expression of the NK1 receptor is similar to that of SP, particularly in the salivary glands and in the small and large intestines, but also in the dorsal root ganglia (Andoh et al. 1996; Dray & Pinnock 1982; Li & Zhao 1998; Stermini et al. 1995).

1.2.3.2. Neurokinin-1 receptor antagonists

NK1 receptor antagonists were first produced in the 1960s (Schroder & Lubke 1964). These compounds, most of which were developed in the 1980s, such as spantide, GR 71 251, FR 113 680 and FK 888, were generally analogues of SP with various amino acid substitutions or rearrangements. However, they often had low potency and specificity, were often neurotoxic, did not cross the blood-brain barrier easily, and were unstable in vivo as they were susceptible to peptidase activity (Bjorkroth et al. 1982; Devillier et al. 1985; Engberg et al. 1981; Folkers et al. 1984; Hagiwara et al. 1992, 1994; Hökfelt et al. 1981; Holmdahl et al. 1981; Leander et al. 1981; McLean et al. 1996; Ward et al. 1990). The more recent production of highly selective non-peptide NK1 receptor antagonists with good central nervous system penetration has, however, enabled the more thorough analysis of the contribution of the receptor in various processes and provided some promising therapeutic leads (Emonds-Alt et al. 1993; Garret et al. 1991; McLean et al. 1991, 1993; Snider et al. 1991).

Non-peptidergic NK1 receptor antagonists often display dramatic differences in affinity between species because of differences in primary structure between rodents and other mammals. The differences at amino acid residues 116 and 290 are particularly important: in the human receptor these contain Val and Ile, but Leu and Ser in the rat and mouse
(Appell et al. 1992; Beresford et al. 1991; Fong et al. 1992; Gitter et al. 1991; Jensen et al. 1994; Pradier et al. 1995; Sachais et al. 1993). For example, CP 96 345 antagonises the human, guinea pig and rabbit receptors at concentrations of less than 1 nM, but it is 100 times less potent against the mouse and rat receptors (Snider et al. 1991). Conversely, RP 67 580 is a better antagonist in the mouse and rat (Gitter et al. 1991). Importantly, the use of concentrations of CP 96 345 at concentrations sufficiently high to antagonise the NK1 receptor in rodents often interferes with calcium channel function (Guard et al. 1993; Schmidt et al. 1992). Although newer antagonists with fewer non-specific activities in rodents have been produced, their primary developmental goal as potential therapeutic agents for use in man has led to the majority of available NK1 antagonists showing low affinities for the rodent receptor. Since mice and rats are the most commonly used preclinical species, this has complicated the study of NK1 receptor function, necessitating the use of species such as the gerbil and guinea pig, which have more human-like NK1 receptor pharmacology, in most preclinical studies.

1.2.3.3. Neurokinin-1 receptor knockout mice

The analysis of the functions of the NK1 receptor was greatly enhanced by the development of ‘knockout’ mouse lines, which were engineered to lack functional NK1 receptors. Three lines of NK1 receptor knockout (NK1⁻⁻) mouse have been produced separately by homologous recombination (Bozic et al. 1996; De Felipe et al. 1998; Santarelli et al. 2001), and have generally produced similar findings. The strategy adopted by De Felipe et al. (1998) is explained and illustrated in Chapter 2.

1.2.4. Functions of substance P and the neurokinin-1 receptor

1.2.4.1. Nociception

The most widely studied physiological role of SP is as a transmitter of nociceptive information. Since painful stimuli bring about the release of SP onto dorsal horn neurones (Randic & Miletic 1977) and based on SP’s distribution in the primary afferent C-fibres, which are mostly polymodal in the rat (Lynn & Hunt 1984), SP was hypothesised to play an important role in pain transmission. Behavioural studies supported this hypothesis, since intrathecal and intravenous administration of SP elicit scratching and biting of the abdomen (Gamse & Saria 1986; Hylden & Wilcox 1981; Piercey et al. 1981), behaviours
which are blocked by an NK1 receptor antagonist (Yamamoto & Yaksh 1991; Yashpal et al. 1993), and by systemic morphine injections (Hylden & Wilcox 1981). Opiates also inhibit the release of SP in the trigeminal nucleus induced by high potassium concentration and in the dorsal horn after stimulation of the primary afferents (Jessell & Iversen 1977; Yaksh et al. 1980). Additionally, noxious stimulation brings about an increase in PPT-A and NK1 receptor mRNA expression in the dorsal root ganglion (McCarson 1999; Noguchi et al. 1988), further supporting SP's involvement in the detection of pain.

However, early conclusions that SP was the 'primary' pain transmitter have not been upheld by more recent findings. Despite the anatomical evidence for a role of SP and the NK1 receptor in pain, they have little influence on acute pain sensation, although the responses to intense or inflammatory pain seem to engage NK1 receptor-dependent processes. Hence, in the mouse, rat and guinea pig, NK1 antagonists have been shown to reduce SP-induced plasma extravasation and hyperalgesia in models of chronic inflammatory and neuropathic pain, but with relatively few effects on acute tests (Birch et al. 1992; Campbell et al. 1998, 2000; Chapman & Dickenson 1993; Garret et al. 1991; Laird et al. 1993; Lecci et al. 1991; Ma & Woolf 1995, 1997; Nagahisa et al. 1992; Yashpal et al. 1993). Similarly, NK1-/- mice show few alterations in acute pain-related behaviour, with normal behavioural responses to acute thermal, mechanical and chemical painful stimuli. However, they do exhibit a decrease in the behavioural response to the second phase of the formalin test, and a lack of the amplification of C-fibre responses ('wind-up') that normally occurs with repeated noxious stimulation (De Felipe et al. 1998), thereby confirming previous work with NK1 receptor antagonists (Ma & Woolf 1995, 1997; Xu et al. 1992b). NK1-/- mice also fail to display intensity coding for noxious stimuli, a reduction in neurogenic inflammation (see below) and a reduction in capsaicin-evoked secondary hyperalgesia (Bozic et al. 1996; De Felipe et al. 1998; Laird et al. 2001). Similar results were observed in mice lacking the gene for PPT-A (Cao et al. 1998), suggesting that SP and the NK1 receptor are of limited importance in acute pain responses, but that they are necessary for the responses to chronic and inflammatory pain, possibly by mediating central hyperexcitability and increasing sensitivity to painful stimuli.

Surprisingly, NK1 receptor antagonists have been ineffective in the majority of clinical trials for analgesia (Hill 2000). It is possible that this apparent difference between humans
and preclinical species may reflect a difference in NK1 receptor distribution (at supraspinal sites). However an intriguing possibility is that the reductions in pain-related behaviours observed in preclinical trials with NK1 receptor antagonists may reflect reductions in stress, rather than pain per se. NK1 receptor antagonists are known to attenuate the response to stressful stimuli (see below), of which pain may be one factor. Nevertheless, there is some evidence to suggest a therapeutic role for NK1 receptor antagonists in migraine and arthritic pain, possibly by blocking SP-mediated plasma extravasation (Beattie et al. 1993; Binder et al. 1999; Moussaoui et al. 1993; O'Shaughnessy & Connor 1993; Shepheard et al. 1995), as well as a single trial showing that CP 99 994 reduces postoperative pain after dental extraction (Dionne et al. 1998).

1.2.4.2. Inflammation

The release of SP from the peripheral terminals of sensory neurones in response to injury or infection brings about vasodilation, plasma extravasation, histamine release, smooth muscle contraction and the activation and adhesion of inflammatory cells, coupled with the excitation of postganglionic neurones (Birch et al. 1992; Gamse et al. 1980; Garret et al. 1991; Lembeck et al. 1992; Lembeck & Holzer 1979; Nagahisa et al. 1992; Nicolau et al. 1993; Piedimonte et al. 1993; Saria 1984; Xu et al. 1992a). This 'neurogenic inflammation', which is characterised by itching, flare and wheal, occurs by the direct actions of SP on capillary endothelial cells and via SP's action on mast cells, resulting in histamine release (Bowden et al. 1994; Devillier et al. 1986; Foreman et al. 1983; Fuller et al. 1987). The spread of vasodilation from the site of injury occurs via the axon reflex, whereby noxious stimulation travels along the branches of the terminal nerve to the point of ramification, where it descends into another branch, leading to the release of inflammatory agents from this nerve ending. SP and NK1 receptor expression is increased in the somata of dorsal root ganglion neurones during acute and chronic inflammation, coupled with increased release of SP in the superficial spinal cord (Marlier et al. 1991; McCarson 1999; Noguchi et al. 1988; Schaible et al. 1990). SP also regulates the release of cytokines and induces lymphocyte proliferation at the site of injury (Payan et al. 1983).
1.2.4.3. Neurotrophic actions

Both SP and the NK1 receptor agonist GR 73 632 have been shown to induce neurite outgrowth \textit{in vitro} (Iwasaki \textit{et al.} 1989; Narumi & Fujita 1978; Narumi & Maki 1978; Whitty \textit{et al.} 1993), as well as increase the rate of reinnervation following 6-hydroxydopamine (6-OHDA) lesions within the central nervous system (Iwasaki \textit{et al.} 1989; Jonsson & Hallman 1982a; Jonsson & Hallman 1982b; Mattioli \textit{et al.} 1992; Nakai & Kasamatsu 1984; Narumi & Fujita 1978; Narumi & Maki 1978; Whitty \textit{et al.} 1993). SP is also neuroprotective, and may play an important role in certain neurodegenerative diseases (Barker 1991). Indeed, SP is depleted from the brains and spinal cords of patients with disorders including Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease (Beal & Martin 1986; Beal & Mazurek 1987; Crystal & Davies 1982; Gillberg \textit{et al.} 1982; Mauborgne \textit{et al.} 1983; Quigley & Kowall 1991; Tenovuo \textit{et al.} 1984).

1.2.4.4. Emesis


1.2.4.5. Emotional behaviours

1.2.4.5.1. Depression and anxiety

In humans, the efficacy of the NK1 receptor antagonist MK 869 has been demonstrated in patients with major depressive disorder and moderately high anxiety (Kramer \textit{et al.} 1998). In this randomised, double-blind, placebo-controlled study, single daily doses of MK 869 (300 mg) were found to reduce patients' scores on the 21-item Hamilton depression scale,
to a similar level to paroxetine (20 mg per day), as well as having a pronounced anxiolytic effect. In preclinical studies, chronic administration of NKP 608 reversed the development of anhedonia following chronic mild stress to a similar degree to imipramine (Papp et al. 2000). NK1 antagonists also bring about increases in the firing rate of dorsal raphe serotonergic neurones, in common with the effects observed with established antidepressant drugs (Conley et al. 2002; Haddjeri & Blier 2001).

NK1 receptor antagonists also show an anxiolytic profile in preclinical tests. MK 869 has been shown to inhibit maternal separation-induced vocalisations in guinea pig pups (Kramer et al. 1998), as have the antagonists L 733 060 and GR 205 171 in an enantioselective manner (Rupniak et al. 2000). Recently, a novel NK1 receptor antagonist which is more readily soluble in water than MK 869 has been developed, and found to cause similar reductions in guinea pig vocalisations (Harrison et al. 2001). Furthermore, SP is released in the amygdala following aversive stimuli such as immobilisation stress or maternal separation, and blockade of NK1 receptor in the amygdala prevents the vocalisations associated with these stimuli (Boyce et al. 2001; Kramer et al. 1998; Smith et al. 1999; Steinberg et al. 2002). NK1 receptor antagonists reduce anxiety in the social interaction test in the rat (File 1997, 2000; Vassout et al. 2000) and the gerbil (Cheeta et al. 2001; Gentsch et al. 2002). In mice, intracerebroventricular administration of FK 888 brought about an anxiolytic profile on the elevated plus maze test (Teixeira et al. 1996), an effect that was also seen with oral administration of a range of NK1 antagonists in the gerbil (Varty et al. 2002). In the same species, a range of NK1 receptor antagonists have also been shown to reduce anxiety-related foot-tapping behaviour (Ballard et al. 2001; Duffy et al. 2002). Intracerebroventricular SP administration or microinjection into the dorsal periaqueductal grey brings about an increase in anxiety-related behaviour in mice and rats (Aguiar & Brandão 1996; De Araújo et al. 1999; Teixeira et al. 1996), but it has an anxiolytic effect when injected systemically or into the nucleus basalis magnocellularis (Hasenöhrl et al. 1998b; Nikolaus et al. 2000). SP signalling is therefore of importance in the mediation of anxiety-related behaviour, in a region-specific manner.

NK1\(^{-/-}\) mice have been shown to exhibit alterations in brain function similar to those observed after chronic antidepressant treatment, notably down-regulation and desensitisation of inhibitory presynaptic 5HT\(_{1A}\) receptors in the dorsal raphe nucleus.
(Froger et al. 2001; Santarelli et al. 2001). Behaviourally, NK1<sup>-/-</sup> mouse pups emit fewer isolation-induced vocalisations than their wild type littermates (Rupniak et al. 2000; Santarelli et al. 2001). They also exhibit more struggling in the forced swim and tail suspension tests than wild type littermates (Rupniak et al. 2001), as do mice lacking the PPT-A gene (Bilkei-Gorzo et al. 2002), providing further support for a role of NK1 receptor blockade in the effective treatment of depressive illness. However, the decreases in anxiety-related behaviour observed by Santarelli et al. (2001) and Bilkei-Gorzo et al. (2002) have not been replicated in the strain of NK1<sup>-/-</sup> mouse produced by Hunt and colleagues (De Felipe et al. 1998; Murtra et al. 2000b).

1.2.4.5.2. Stress

Central injection of NK1 receptor agonists produces a range of defensive behavioural and cardiovascular responses, which are consistent with reactions to stressful stimuli (Aguiar & Brandão 1996; Elliott 1988; Krase et al. 1994). Similarly, NK1 receptor antagonists reduce the stress-induced activation of ascending neural pathways originating in the locus coeruleus (Hahn & Bannon 1999; McLean et al. 1993), as well as the cardiovascular and behavioural responses to the stress associated with chronic formalin injection (Culman et al. 1997). The facilitation of defensive rage behaviours in the cat induced by stimulation of the amygdala is also reduced with NK1 receptor antagonists (Shaikh et al. 1993), suggesting an important role for the NK1 receptor in modulating the responses to stressful stimuli. This is supported by findings in NK1<sup>-/-</sup> mice, which have reduced stress-induced analgesia and aggressive responses to territorial challenge (De Felipe et al. 1998). The NK1 receptor therefore seems to be necessary for the orchestrated response to a variety of stressors, including pain, injury, invasion of territory or psychological stress, which may partly explain their antidepressant efficacy.

1.2.4.5.3. Reward

A potential role for SP and the NK1 receptor in reward processes was suggested by the observations that systemic injections of SP, as well as focal injections into the nucleus basalis magnocellularis, induce a place preference in rats (Hasenöhrl et al. 1998a; Holzhäuer-Oitzl et al. 1988), which is dependent upon NK1 receptor activation (Nikolaus et al. 1999). These focal injections are coupled with an increase in dopamine turnover in the
contralateral NAcc, a brain region known to be of importance in the execution of reward-related behaviours (see below; Boix et al. 1995). Furthermore, administration of SP intracerebroventricularly or into the VTA, NAcc or ventral pallidum brings about increases in locomotor behaviour in a dopamine-dependent manner, in a similar manner to rewarding stimuli such as drugs of abuse (Elliott et al. 1992; Elliott & Iversen 1986; Iversen 1982; Kalivas et al. 1993; Napier et al. 1995; Piot et al. 1995). Rats will self-administer SP into the ventromedial CPu, although repeated injections can have aversive properties (Krappmann et al. 1994). Furthermore, the cocaine-induced increases in dopamine release in the striatum are reduced with local infusion of NK1 receptor antagonists (Noailles & Angulo 2002), and self-administration of cocaine causes upregulation of PPT-A mRNA in the shell region of the NAcc (Arroyo et al. 2000). A further link between drugs of abuse and the NK1 receptor was provided by work demonstrating that some of the behavioural responses to morphine withdrawal were reduced in rats following intracerebroventricular injection of the NK1 receptor antagonist RP 67 580 (Maldonado et al. 1993), and following systemic injection of the SP amino terminal metabolite SP₁₇ (Kreeger & Larson 1996), suggesting that NK1 receptors may also be involved in the aversive aspects of drug administration. This was supported by the finding that conditioned taste aversions induced by either apomorphine or amphetamine were blocked by the NK1 receptor antagonist GR 205 171 (McAllister & Pratt 1998).

However, the clearest indication for a role for the NK1 receptor in drug reward was the demonstration that NK1⁻/⁻ mice do not show a conditioned place preference (CPP) to morphine at a dose of 3 mg.kg⁻¹, a dose at which wild type mice showed a clear preference for a morphine-associated context (Murtra et al. 2000b). NK1⁻/⁻ mice also failed to show CPP to amphetamine (Murtra et al. 2000a). They are therefore insensitive to the pleasurable, or rewarding aspects of morphine and amphetamine. NK1⁻/⁻ mice also failed to show the hyperlocomotor response to acute morphine administration in the open field paradigm at doses of 10 and 20 mg.kg⁻¹: such locomotor responses to drugs of abuse are believed to be mediated by the same neural mechanisms as reward (Wise 1987; Wise & Bozarth 1987). However, the mice did not show a general disruption of reward-related behaviours, since CPP to food (a natural reinforcer) and to the psychostimulant cocaine were unaffected by the genetic manipulation. In addition to a loss of the rewarding response to morphine, the mice did not show many of the physical withdrawal signs
following naloxone-precipitated withdrawal from chronic morphine, and did not demonstrate a conditioned place aversion to naloxone, suggesting a loss of both some of the physical as well as the motivational aspects of opiate withdrawal. De Felipe et al. (1998) found that the analgesic properties of morphine were not impaired in NK1−/− mice in the hot-plate test, indicating that the substance P / NK1 system is not critically involved in opiate analgesia. The NK1 receptor therefore plays an essential and specific role in the motivational, but not analgesic, properties of opiates (and amphetamine), whereas it is not critical for the motivational properties of cocaine.

1.2.4.6. Learning and memory

The role of SP and the NK1 receptor in learning and memory processes is reviewed in Chapter 7. Briefly, there have been a large number of studies examining the effects of SP infusion, either systemically or into various brain structures on learning and memory tasks, the majority of which have demonstrated that SP brings about a facilitation of avoidance learning and can prevent the age-induced deficits in spatial learning ability or the deficits in spontaneous alternation behaviour (a model of working memory) brought about by scopolamine. These effects seem to be dependent on the brain region into which the injections are made: infusion of SP into the nucleus basalis magnocellularis or the medial septum bring about a facilitation of memory, whereas injections into the substantia nigra or amygdala can produce retrograde amnesia.

1.3. Reward and drug addiction

Murtra et al.'s (2000b) findings provided novel information concerning the involvement of NK1 receptors in the motivational properties of morphine administration. Since the majority of the work presented in this thesis is based on these findings, this section summarises the psychological basis of reward, its relationship to drug addiction and the neuroanatomical basis of the effects of drugs of abuse.

1.3.1. Reward and addiction in man

Reward is the positive subjective effect of a stimulus, which is elicited by 'natural' stimuli in the environment, such as those associated with survival, such as food, water and sexual contact. However, drugs that are abused by man bring about the same emotional and
neurochemical responses elicited by natural rewards. Administration of these drugs gives rise to subjective feelings of pleasure and well-being. Indeed, it is the desire to elevate mood and achieve a ‘high’ that often motivates initial drug use. However, addictive drugs are not only rewarding, but they are also reinforcing, in that they stimulate the user to repeat the behaviours associated with them (White 1996). They therefore stimulate a user to seek and take the drug again. In vulnerable people, the repeated administration of an addictive drug can bring about changes in the brain that promote continued drug taking, which subsequently becomes progressively harder to control. This compulsive use of drugs can eventually become out of control, forcing the life of the user to become progressively focused on obtaining, using and recovering from the effects of drugs, despite their serious negative consequences (O’Brien & McLellan 1996). Once such an addiction has taken hold, it tends to follow a chronic course: even after long periods of abstinence, and perhaps over a lifetime, drug addicts find that a return or relapse to active drug use can easily be precipitated. Addiction is therefore a chronic, relapsing medical illness, whose pathophysiology is characterised by complex and long-lasting molecular, cellular and system-level changes in the brain (Goldstein 1994).

The repeated administration of drugs of abuse not only brings about compulsive use, but also tolerance, sensitisation and dependence. Tolerance is the decrease in the effects of a drug despite a constant dose, which is often manifest in the need for a larger dose to be administered to maintain the same effect (Mendelson et al. 1998). In terms of human drug abuse, tolerance often develops to a drug’s rewarding or pleasurable effects, encouraging the user to increase their ingested dose, which exacerbates the changes that are occurring in the brain to bring about addiction. Sensitisation of a drug’s effects can also occur: in human addicts, craving of or desire for a drug may undergo such an enhancement (Robinson & Berridge 1993). Both tolerance and sensitisation can exist in the same individual: however, the two phenomena occur to different aspects of a drug’s effects, such as reward or ‘liking’, which tends to become tolerant, and craving or ‘wanting’, which tends to sensitise. Dependence refers to the phenomenon whereby, upon drug cessation, cognitive, emotional or physical withdrawal symptoms are experienced, and is mediated by the adaptation of cells, circuits or organ systems in response to drug administration (Goldstein 1994).
Early psychological theories of drug addiction assumed the avoidance of the aversive properties of drug withdrawal was the primary motivation for compulsive drug ingestion in addicts (see Lyvers 1998; Wise 1987). However, the alleviation of withdrawal symptoms cannot explain persistent drug seeking behaviour since some drugs of abuse which are highly addictive, such as cocaine and amphetamine, have relative mild emotional withdrawal symptoms, and do not produce physical withdrawal (Gawin 1991). Moreover, the discomfort associated with withdrawal cannot be the major obstacle to recovery, since addicts often relapse to drug-taking behaviour long after withdrawal symptoms have dissipated, even for drugs with severe physical withdrawal symptoms such as heroin and alcohol (O'Brien et al. 1992). Furthermore, pleasure-seeking alone cannot explain the persistence of drug-taking behaviour in addicts: the rewarding properties of drugs often habituate with repeated administration, as is often seen in alcoholics, for whom alcohol no longer brings pleasure but often elicits feelings of depression (Weiss & Porrino 2002), and in cocaine addicts, in whom the ‘high’ is reduced following repeated drug administration (Volkow et al. 1997b).

Addiction is now understood to be a more complex phenomenon than the maintenance of hedonic homeostasis: drugs of abuse seem to bring about a sensitisation of the brain systems that are involved in incentive motivation (Robinson & Berridge 1993, 2000). Thus, the ‘wanting’ of a drug increasingly becomes the dominant psychological response, at the same time as tolerance develops to the reward-mediated ‘liking’ of the drug. These psychological alterations are extremely stable and long lasting, and may explain the persistence of susceptibility to relapse in recovering addicts, which is usually the major obstacle to their rehabilitation. Drug-conditioned cues are of particular importance in relapse, and illustrate the associative learning that is believed to underlie the development and maintenance of addiction: in human addicts, the risk of a return to craving and drug-seeking behaviour is elevated upon exposure to people, places or paraphernalia associated with previous drug use, suggesting that the brain stores specific information about drugs and that drug addiction can ultimately can been viewed as a form of aberrant association of drug-associated cues with reward or the sensitised ‘wanting’ response (Childress et al. 1986; Ehrman et al. 1992; Shiffman et al. 1996). Furthermore, the neurochemical consequences of drug administration share a striking number of similarities with the processes that occur during associative learning (White 1996). Drug addiction is therefore a consequence of
long-term changes in brain structure and function, which can exert full control over an addict’s life.

1.3.2. Drugs of abuse

Although the properties that predict whether a given drug will give rise to addiction are not well defined, a wide variety of drugs can induce the compulsive use characteristic of addiction. These include drugs with varying routes of action, including opioids and psychostimulants, as well as ethanol, barbiturates, benzodiazepines and cannabis.

1.3.2.1. Opiates

Opiate drugs include morphine, an extract of the opium poppy, its derivative heroin (diacetylated morphine) and various other drugs such as fentanyl. They are agonists at $\mu$-, $\delta$- and $\kappa$-opioid receptors: $\mu$- and $\delta$-receptors mediate the drugs' rewarding and addictive properties, while $\kappa$-receptors mediate their aversive actions (Bals-Kubik et al. 1993; Devine & Wise 1994; Mucha & Herz 1985). They are usually injected, and bring about an overwhelming sense of well-being and euphoria in the user. However, withdrawal symptoms from opiates are severe, consisting of dysphoria (depression), muscle cramps, tear production, diarrhoea, sweating, anxiety and fever (Nestler & Aghajanian 1997). Opiates are also widely used as analgesics, particularly in the relief of severe or chronic pain. However, their clinical use is often limited by their ability to depress respiratory activity and to induce a dependence state in patients. Indeed, patients often receive inadequate control of their pain with opioids such as morphine for fear of the development of dependence, although the production of a true addiction characterised by compulsive drug-seeking behaviour is rare (Van Ree et al. 1999).

1.3.2.2. Psychostimulants

Psychostimulant drugs include cocaine, amphetamines and methylphenidate. Cocaine, which is produced from the leaves of the *Coca* plant, blocks the dopamine, 5-hydroxytryptamine (5-HT) and noradrenaline transporter molecules, resulting in increases in extracellular monoamine levels in a wide variety of brain regions and subsequent feelings of alertness, well-being, euphoria and confidence (Breiter et al. 1997; Hurd et al. 1989; Pettit & Justice 1989; Rocha et al. 1998; Volkow et al. 1997a). The
artificially produced drug amphetamine acts primarily at the dopamine transporter to bring about similar subjective effects (Aboul-Enein 1971). Cocaine and amphetamine are widely abused, are highly addictive, and represent a common social problem. Withdrawal symptoms from psychostimulant use are less severe than those with opiates, but can include dysphoria, fatigue, sleep disturbances, increased appetite and anxiety (Gawin & Ellinwood 1989; Markou & Koob 1991). Although ±3,4-methylenedioxymethamphetamine (MDMA; ecstasy) also falls into this class, it acts primarily through the 5-HT system, and is not thought to produce overt dependence or addiction (Gowing et al. 2002; McGuire 2000; Montoya et al. 2002; Morgan 2000; Mørland 2000; Murray 1998).

1.3.2.3. Ethanol

Ethanol is a natural product of fermentation mechanisms. Although its pharmacology is complex and not well understood, it is known to facilitate the GABA<sub>A</sub> receptor and inhibit N-methyl-D-aspartate (NMDA) glutamate receptors, bringing about sedative and amnesic effects. Alcohol addiction is very common, perhaps due to the drug’s legality and social acceptability in most cultures. Withdrawal effects can be severe, including autonomic hyperreactivity, nausea, hand tremor, anxiety and hallucinations (Tabakoff & Hoffman 1996; Weiss & Porrino 2002).

1.3.2.4. Other drugs of abuse

Nicotine is another widely abused and legal drug. It is inhaled by smoking the dried leaves of the tobacco plant: indeed, smoking is the biggest single cause of premature death in the Western world. Nicotine acts as an agonist of the nicotinic acetylcholine receptor. Cessation of nicotine administration can give rise to a withdrawal syndrome consisting of dysphoria, insomnia, anxiety, restlessness, decreased heart rate and weight gain (Dani & De Biasi 2001; Mansvelder & McGehee 2002).

Cannabis (hashish; marijuana), derived from the Cannabis sativa plant, can produce a dependence syndrome and mild cognitive impairment. Its main active constituent is Δ²-tetrahydrocannabinol (THC), which, in the brain, acts as an agonist at the cannabinoid-1 (CB1) receptor (Dennis et al. 2002; Maldonado & Rodríguez de Fonseca 2002; Miller & Gold 1989).
Benzodiazepines and barbiturates are often prescribed as anxiolytics. They act at the GABA_A receptor, activating chloride channels in their target neurones, and can cause dependence and addiction with long-term use (Juergens 1993; Lader 1999; Longo & Johnson 2000; Salzman 1998). Further addictive drugs of abuse include phencyclidine (PCP), an NMDA receptor blocker (Carlezon & Wise 1996), hallucinogens, such as lysergic acid diethylamide (LSD) and inhalants.

1.3.3. Animal models of drug abuse and addiction

Drug abuse and addiction are complex human phenomena, which involve compulsive drug-taking behaviour despite negative consequences, with psychological, physical and social consequences. Although it is not possible to model all the aspects of addiction using animal models, a number of techniques have been established that allow some of the individual components of drug abuse and addiction to be studied. The use of experimental animals is useful in probing the neurobiological processes underlying these processes, since they can be made subject to pharmacological, anatomical or genetic manipulation. Additionally, they allow study from the drug naïve state, whereas clinical studies usually involve the use of already addicted individuals who are usually seeking help to maintain abstinence.

Conditioned place preference (CPP) is a widely used technique to assess the rewarding properties of stimuli, including drugs of abuse (see Chapter 2). Briefly, it involves the development of a preference for a location that is repeatedly paired with a rewarding stimulus, such as cocaine. A stimulus is considered to be rewarding if it encourages the animal under test to increase the amount of time spent in the drug-associated location in a choice session, usually carried out in a drug-free state. This method therefore assesses the degree to which contextual stimuli previously paired with a drug exert control over behaviour.

In the self-administration task, an animal is trained to make an operant response, such as pressing a lever, in return for presentation of the stimulus, such as an intravenous infusion of cocaine (Schuster & Thompson 1969). A reinforcing stimulus will result in the animal under test making more responses and is hence a model of drug-seeking or -taking behaviour. Since the self-administration procedure allows animals to exhibit behaviour
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reminiscent of compulsive drug seeking, this model is possibly the closest correlate of the consummatory behaviours seen in human addicts. Furthermore, following cessation of drug infusion and extinction of responding, factors that bring about a return to responding can then be tested, as a model of relapse to drug-seeking behaviour or craving (Self 1998).

Animals will also, after sufficient training, respond in return for drug-associated cues alone: such second-order schedules allow the study of conditioned reward, drug-seeking behaviour and the systems underlying drug reinforcement whilst in a drug-free state and hence without any potential behavioural alterations induced by systemic drug exposure (Everitt & Robbins 2000; Schindler et al. 1988).

Tolerance, sensitisation and physical dependence can also be reproduced in animal models. Tolerance is most clearly observed in terms of analgesia, as in the reduction in morphine's analgesic properties with repeated administration (Harrison et al. 1998; Trujillo & Akil 1991). Sensitisation to a drug's effects is commonly studied by assessing locomotor activity: the repeated administration of a drug causes an enhancement of its locomotor stimulant properties, and has been demonstrated in numerous studies, especially with cocaine and amphetamine (see Chapter 4). Finally, physical and emotional withdrawal symptoms associated with cessation of drug treatment can be seen in animals treated chronically with drugs (Markou & Koob 1991). In opiate studies, withdrawal is often precipitated with a μ-opioid receptor antagonist such as naloxone, which brings about a set of species-specific physical withdrawal signs, which, in rodents, include jumping, diarrhoea and ptosis (Nestler & Aghajanian 1997). The aversive emotional aspects of withdrawal can be assessed in the conditioned place aversion (CPA) paradigm, in which an animal develops an aversion to a location paired with such an antagonist.

1.3.4. Neuroanatomy of drug abuse and addiction

Although the effects of drugs are mediated by multiple neurotransmitters acting in various brain regions, all addictive drugs tend to share the common neuroanatomical effect of stimulating the mesolimbic dopaminergic system. This system is therefore believed to be of central importance in drug reward, reinforcement and addiction. This and other important pathways in these processes are illustrated in Figure 1.1.
The repeated administration of addictive drugs brings about a wide range of responses in the brain. These can broadly be classified into neuronal adaptations, which tend to be homeostatic responses exhibited by neurones in response to excessive stimulation, and synaptic plasticity, which allows drug-associated stimuli to become associated with specific learned behaviours (Berke & Hyman 2000). While drug dependence and withdrawal, and possibly tolerance, may result from the neuronal adaptations occurring within specific brain regions, including receptor desensitisation and alterations in signal transduction cascades (Bohn et al. 1999, 2000; Whistler et al. 1999), it is likely that synaptic plasticity underlies the changes that occur in the transfer from drug-taking behaviour to the compulsive behaviour observed in addicts, and the long-lasting susceptibility to relapse. Indeed, the molecular mechanisms known to underlie associative learning seem to be engaged following
administration of addictive drugs, including activation of dopamine D1 receptors and the cAMP / protein kinase A (PKA) / cAMP response-element-binding protein (CREB) signal transduction cascade, altered gene expression and synaptic rearrangements (Berke & Hyman 2000; Nestler 2001). Furthermore, plasticity probably occurs in response to drug administration in regions of the brain known to be involved in associative learning, such as the hippocampus and amygdala, are also involved in the development of drug addiction (White 1996).

1.3.4.1. The mesolimbic dopaminergic system

There is strong evidence to suggest that the mesolimbic dopaminergic system that projects from the VTA to the NAcc, the CPu and the prefrontal cortex (PFC) is the major substrate for natural rewards and reinforcers, as well as addictive drugs (Olds 1956; Olds & Milner 1954; Wise 1998). The NAcc is involved in orchestrating behavioural responses to motivational stimuli, acting as a site of convergence of cortical and subcortical information, and controlling behaviour through its outputs to the brain stem via the ventral pallidum (Mogenson et al. 1980), whereas the CPu seems to be more important for 'habit' learning and executing learned sequences of behaviours (Graybiel 1998; White 1997). Under normal circumstances, this pathway is involved in controlling responses to stimuli associated with survival, such as food and opportunities for reproduction. However, most addictive drugs also bring about increases in these neurones' activity and dopamine release in the NAcc, especially in its medial 'shell' region (Di Chiara & Imperato 1988; Hemby et al. 1995; Hurd et al. 1989; Imperato et al. 1986; Kiyatkin et al. 1993; Pettit & Justice 1989; Pothos et al. 1991; Wise et al. 1995; Wise 1998). Opioids achieve this by inhibiting the GABAergic neurones that normally inhibit the dopaminergic cells in the VTA (Devine et al. 1993; Johnson & North 1992; Leone et al. 1991), whereas psychostimulants seem to cause local increases in the release of dopamine, primarily by preventing its reuptake into nerve terminals (Gold et al. 1989).

Lesions of the NAcc, or pharmacological blockade of dopamine receptors here prevent the execution of reward and reinforcement-related behaviours. Thus, rats will self-administer amphetamine into the NAcc (Hoebel et al. 1983), but 6-OHDA lesions of this system prevent the self-administration of amphetamine and cocaine (Gerrits & Van Ree 1996; Lyness et al. 1979; Pettit et al. 1984; Roberts & Koob 1982). Furthermore, actions that
increase dopamine release in this brain region tend to be repeated: indeed, the upregulation of this pathway is postulated to underlie the sensitisation observed with repeated drug exposure (see Chapter 4). Withdrawal from a several drugs of abuse, including alcohol, nicotine, opiates and psychostimulants, reduces the extracellular concentrations of dopamine in the NAcc (Imperato et al. 1992; Parsons et al. 1991; Paulson et al. 1991; Pothos et al. 1991; Robertson et al. 1991; Rossetti et al. 1992). The central role of dopamine is further supported by work with mice that lack dopamine D2 receptors. These mice consume less alcohol than their wild type littermates (Phillips et al. 1998), and also fail to demonstrate a CPP to morphine, although they exhibit normal physical signs of withdrawal (Maldonado et al. 1997). Similarly, mice lacking the dopamine transporter, one of the putative targets of cocaine, show reduced self-administration of the drug (Rocha et al. 1998). Paradoxically, however, the physical signs of opiate withdrawal are reduced following local infusion of a dopamine D2 receptor agonist into the NAcc (Harris & Aston-Jones 1994). The net effect of dopamine release, as well as the direct actions of addictive drugs such as PCP and cannabinoids within the NAcc is to bring about an inhibition of the medium spiny neurones (Hernández-López et al. 2000; Yasumoto et al. 2002) — however the way in which this actually leads to reinforcement is not clear.

However, dopamine release in the forebrain is probably not equivalent to 'pleasure'. Unpleasant noxious stimuli also cause release of dopamine in this brain region, as do stimuli with no obvious hedonic component. Work by Schultz and colleagues has demonstrated that the mesolimbic dopaminergic neurones may in fact serve as a learning or 'error' signal, and thereby play a role in learning about the motivational significance of a stimulus (see Schultz 1998, 2000). In monkeys, these neurones fire in response to an unanticipated reward. However, as the monkey learns to associate a particular cue with that reward, the dopaminergic neurones begin to fire in response to the cue, while the response to the reward itself habituates. If a predicted reward is omitted, however, the baseline firing of these neurones is suppressed. These neurones therefore may act as a reward 'error signal', indicating how the perceived rewarding effect of a stimulus compares to that expected. The general arousing effect of increased dopamine efflux in the forebrain, combined with more detailed information about the context and nature of the stimulus from areas such as the hippocampus and amygdala, is therefore believed to drive the processes involved with learning about the value of a stimulus. Since the strength and
persistence with which drugs of abuse activate this pathway is probably greater than that observed with natural reinforcers, it is postulated that they stimulate an exaggerated learning signal, which may drive the formation of changes in brain structure and function underpinning the development of addiction (Berke & Hyman 2000).

The NAcc has been intensively studied as a locus in which molecular, cellular and synaptic changes occur in response to drug administration. Changes in gene expression are postulated to be central to the development of addiction, by altering the molecular composition of neurones that are affected by drug administration and shaping future responses (Terwilliger et al. 1991). For example, chronic exposure to opiates, cocaine or alcohol reduces the levels of inhibitory G-proteins (McLeman et al. 2000; Nestler et al. 1990; Striplin & Kalivas 1993), and inactivation of G, and G, proteins with intra-accumbens infusions of pertussis toxin reduces the rewarding effects of intravenous cocaine and heroin (Self et al. 1994). Similarly, chronic morphine, cocaine, amphetamine or alcohol administration increases the levels and activity of CREB in a number of brain regions including the NAcc and VTA (Cole et al. 1995; Turgeon et al. 1997). CREB activates transcription when phosphorylated and has been shown to be involved in tolerance, physical dependence and possibly the conditioned aspects of addiction. Indeed, overexpression of CREB in the NAcc decreases the rewarding effects of opiates and cocaine, while a dominant-negative mutant form of CREB has the opposite effect (Carlezon et al. 1998). Changes in the expression of the immediate early genes (IEGs) c-fos and c-jun occur with drug administration (see Chapter 5), and the expression of related proteins, such as ΔFosB is believed to be of importance in mediating the long-term behavioural changes characteristic of the switch to an addicted state (Hope et al. 1994; Kelz et al. 1999; Moratalla et al. 1996). Changes in synaptic strength also occur in response to drug administration. Both long-term potentiation (LTP) and long-term depression (LTD) have been observed in the NAcc and VTA, and may be related to the effects of repeated drug exposure on brain function (Jones et al. 2000; Thomas et al. 2000). Indeed, LTD in the VTA is disrupted by amphetamine administration and in the CPu, whose cytoarchitecture is similar to that of the NAcc, LTP and LTD are known to occur in a dopamine receptor-dependent manner (Jones et al. 2000; Thomas et al. 2000). LTP between glutamatergic terminals and the dopaminergic cells of the VTA has also been observed in response to a single administration of cocaine (Ungless et al. 2001).
Furthermore, long lasting changes in the size and number of dendritic spines have been observed on dopaminergic neurones within the NAcc and PFC following chronic cocaine or amphetamine administration, whereas chronic opiate administration decreases their size (Robinson & Kolb 1997; Robinson & Kolb 1999; Sklair-Tavron et al. 1996). Similar changes to these have been observed in animal models of learning and memory, highlighting the similarity in the neural processes underlying these two forms of behavioural change, and in their persistence (Deisseroth et al. 1995; Lüscher et al. 2000; Martin & Kandel 1996; Silva & Murphy 1999; Yin & Tully 1996).

Whilst the NAcc is known to be important in mediating the reward of psychomotor stimulant drugs via dopamine-dependent mechanisms, the role of this nucleus in opiate reward is less clear. Although rats will self-administer opiates into the VTA (Bozarth & Wise 1981; Devine & Wise 1994; Phillips & Lepiane 1980), and this brings about increases in accumbal dopamine transmission (Devine et al. 1993; Hemby et al. 1995; Kiyatkin et al. 1993; Leone et al. 1991; Wise et al. 1995), dopaminergic lesions of the NAcc do not affect opiate self-administration behaviour, at least in non-dependent animals (Dworkin et al. 1988; Gerrits & Van Ree 1996; Pettit et al. 1984). Additionally, opiates infused directly into the NAcc are rewarding: microinjections of morphine into the nucleus support place preferences and rats will self-administer morphine and heroin into the NAcc (David & Cazala 2000; McBride et al. 1999; Nestler et al. 1990; Olds 1982; Schildein et al. 1998; Striplin & Kalivas 1993; van der Kooy et al. 1982). Since heroin self-administration and CPP are blocked by intra-NAcc injections of opiate receptor antagonists (Corrigall & Vaccarino 1988; Vaccarino et al. 1985), this effect is believed to be via μ-opiate receptors, which are abundant in this brain region. The mediation of opiate reward-related behaviours is therefore dependent not only on dopaminergic transmission in this pathway, but also by a more direct dopamine-independent action within this nucleus.

1.3.4.2. The amygdala

Although most of the studies examining the neural basis of drug reward and addiction have focused on the mesolimbic dopaminergic system, the influence of other areas of the brain, such as the amygdala, is becoming clearer. An important role for the amygdala in the recognition of unpleasant emotions and associating aversive sensory inputs with environmental stimuli is widely accepted (see Phillips & LeDoux 1992), but recent work
has illustrated its additional importance in processing positive emotions (see Baxter & Murray 2002). In particular, the basolateral nucleus of the amygdala is known to be critical in controlling reward-seeking behaviour, possibly through its interactions with the NAcc core, thereby providing information about the motivational and emotional significance of stimuli (Everitt et al. 1991). Although they do not affect cocaine self-administration, excitotoxic lesions of this nucleus prevent rats from developing responding for cocaine-associated cues (Whitelaw et al. 1996) and cue-induced reinstatement of cocaine-seeking behaviour (Meil & See 1997). They also impair responding in a second-order schedule of sexual or food reinforcement (Everitt et al. 1989; Hatfield et al. 1996) and prevent the acquisition of CPP to food (Everitt et al. 1991). Excitotoxic or electrolytic lesions of the lateral nucleus of the amygdala have been shown to disrupt the expression of a CPP to amphetamine in rats (Hiroi & White 1991), as well as impair acquisition of a conditioned cue preference task, in which a light cue is paired repeatedly with a food reward before a choice procedure (McDonald & White 1993). Furthermore, when human addicts are presented with cues previously associated with cocaine, activity in the amygdala, along with other areas of the brain is increased (Breiter et al. 1997; Grant et al. 1996). It is therefore postulated that the amygdala is of particular importance in mediating the conditioned aspects of reward, in a similar way to its accepted role in the conditioned aspects of fear and anxiety. As such, it probably plays an important role in cue-induced relapse, craving, and the persistence of addictive behaviour.

1.3.4.3. The hippocampus

The hippocampus sends strong projections to the striatum, including the NAcc, forming glutamatergic synapses on the same neurones that receive dopaminergic input from the VTA (Sesack & Pickel 1990). Direct evidence for a role in drug reward processes includes the observation that opioids are self-administered into the CA3 region of the hippocampus in a μ-opioid receptor-dependent manner (Self & Stein 1993; Stevens et al. 1991). The hippocampus may provide detailed information about drug-associated stimuli, which is combined with the motivational information encoded by dopamine release in the NAcc and translated into behavioural output. The hippocampus is known to be crucial for declarative and spatial learning and therefore may be of importance in cue-conditioned reinstatement of drug-seeking behaviour and relapse.
1.3.4.4. The locus coeruleus

The locus coeruleus has been studied as a model system for the upregulation of the cAMP pathway in response to repeated opiate administration (Nestler & Aghajanian 1997). Dramatic upregulation of CREB is observed in the noradrenergic neurones of this region in response to chronic opiate administration, which is believed to act as a compensatory or homeostatic mechanism to the inhibition mediated by persistent agonism of the μ-opioid receptor (Widnell et al. 1994). The subsequent increase in activity of the neurones brought about upon cessation of opiate administration is hypothesised to be an important mechanism underlying the development of physical opiate dependence and withdrawal (Lane-Ladd et al. 1997). This is supported by the observation that mice with mutations in the gene for CREB exhibit reduced opiate withdrawal symptoms (Maldonado et al. 1996).

1.3.4.5. The prefrontal cortex

The PFC is one of the terminal sites of the mesolimbic dopamine system, and sends glutamatergic projections to the NAcc. Rats will self-administer cocaine and phencyclidine into this region, in a dopamine receptor-dependent manner (Carlezon & Wise 1996; Goeders et al. 1986; Goeders & Smith 1986). It is believed to be of importance in the inhibition of behaviour (Knight et al. 1999), and may be involved in preventing initial over-indulgence of drugs. Indeed, dopaminergic lesions of the medial prefrontal cortex bring about a supersensitivity to the rewarding aspects of self-administered cocaine and amphetamine (Dalley et al. 1999; Schenk et al. 1991). Furthermore, human addicts exhibit behaviours similar to those observed in patients with damage to this region, including increased impulsivity and risk-taking, and poor decision making ability, which may correlate with drug-induced damage to this region (Rogers et al. 1999).

1.3.4.6. Other brain regions

Various other brain regions have been implicated in the motivational aspects of drugs, including their rewarding and addictive properties. These areas include the periaqueductal grey, which is involved in the mediation of withdrawal behaviours, and other areas of the cortex, the thalamus and the hypothalamus (see Nestler 2001). Since drug seeking is a behavioural phenomenon that can develop into the primary motivational goal of an addict, it is likely to involve a large number of different regions of the brain. While the systems
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-described above are probably some of the most important in drug reward and the
development of addiction, the nature of the contribution of other neural areas and systems
remains an area of immense research potential.

1.4. Summary and aims of this thesis

SP and its receptor, the NK1 receptor, are widely expressed throughout the nervous
system. Although previous work has concentrated on this system's role in pain
transmission and inflammation, more recent work, aided by the development of specific
brain-penetrant NK1 receptor antagonists and NK1<sup>−/−</sup> mice, has refined our understanding
of SP's participation in nociception. Furthermore, they have highlighted its role in
emotional behaviours, particularly in orchestrating the response to stress, as well as a
crucial role in the rewarding aspects of morphine. The nature of the NK1 receptor's
participation in reward processes is, however, unclear. Despite a large literature on the
neurochemical basis of drug reward and addiction, only a few previous studies have shown
a link between the motivational effects of drugs of abuse and the NK1 receptor. However,
the analysis of the neurobiology of drug reward and addiction indicates that many of the
areas known to play an important role in these processes, such as the NAcc, the VTA and
the amygdala, are areas of NK1 receptor expression, suggesting that they may be of
importance in the receptor's drug-related behavioural effects.

The primary aims of this thesis are to confirm and extend Murtra et al.'s (2000b) findings in
the mouse. To this end, the distribution of the NK1 receptor throughout the mouse brain
is described, followed by a confirmation of Murtra et al.'s (2000b) findings, and their
extension using the locomotor sensitisation model. Subsequently, the location of the NK1
receptor's effects in mediating morphine reward-related behaviours is sought using two
complementary techniques: the histological comparison of the expression of the immediate
early gene c-Fos between wild type and NK1<sup>−/−</sup> mouse brains, and the behavioural
examination of mice with region-specific ablation of NK1 receptor-expressing neurones.
Finally, the behaviour of NK1<sup>−/−</sup> mice is assessed in a range of learning and memory tasks,
in order to assess the contribution of the loss of NK1 receptor-mediating signalling to
mnemonic processes.
CHAPTER TWO

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1. Introduction

The techniques that are used in more than one results chapter are described in this chapter. Since much of the work in this thesis used mice in which the neurokinin-1 (NK1) receptor had been genetically disrupted, the generation, breeding and background of these animals are described. This is followed by descriptions of the histological and behavioural techniques that form a major part of the work presented. Methods that are unique to one chapter are described therein.

2.2. Materials

2.2.1. General laboratory solutions

**Phosphate buffer** (PB; 0.1 M; pH 7.4):

- 190 mM NaH$_2$PO$_4$ (BDH, Poole, UK)
- 810 mM Na$_2$HPO$_4$ (BDH)

**Tris buffer** (0.15 M; pH 7.6):

- 127 mM Tris HCl (Sigma, Poole, UK)
- 23 mM Tris base (Sigma)

**Heparinised phosphate-buffered saline (PBS):**

- Saline (0.9 % NaCl; Baxter, Lessines, Belgium)
- 5 u.ml$^{-1}$ heparin (Monoparin; CP Pharmaceuticals, Wrexham, UK)
- ~5 mM PB

**Normal Goat Serum (NGS) solution:**

- 0.1 M PB
- 3 % NGS (Vector Laboratories, Burlingame, USA)
- 0.3 % Triton X-100 (BDH)
- 0.02 % NaN$_3$ (Sigma)

*Store at 4 °C.*
2.2.2. Drugs

Morphine sulphate and cocaine HCl were purchased from Sigma. They were dissolved in sterile saline (Steri-Amp; Steripak, Runcorn, UK) and administered intraperitoneally (IP) using 0.5 ml Micro-Fine U-100 insulin syringes (Becton Dickinson, Le Pont de Claix, France).

2.3. Mice

All the experiments described in this thesis used mice. The mice were housed in Biological Services, UCL, London, in cages containing between 1 and 5 animals. Environmental conditions were maintained at 21 °C and 50 % humidity, with tap water and food (Harlan Teklad TRM Rat/Mouse Diet; Harlan, Bicester, UK) ad libitum. Lights were programmed on a 12:12 h cycle (lights on at 8.00 am).

2.3.1. Neurokinin-1 receptor knockout mice

The NK1 receptor knockout (NK1−/−) mice used were derived from those described by De Felipe et al. (1998). The knockout line was created by targeted disruption of the gene encoding the NK1 receptor, as illustrated in Figure 2.1. Using homologous recombination in embryonic stem cells, a 129/sv × C57BL/6 mouse line was generated, in which exon 1 of the gene was disrupted by insertion of a cassette at a unique StuI site. The cassette consisted of an internal ribosome entry site (IRES), the lacZ coding sequence and a neomycin resistance gene expressed from its own promoter.

Except where indicated, the NK1−/− mice used in the experiments described in this thesis were derived from homozygous mutant 129/sv × C57BL/6 mice crossed once onto the MF1 background (Harlan; Migaud et al. 1998). This crossing procedure allowed a rapid dilution of the 129/sv component of the mutant mice, since substrains of this background perform poorly in a number of behavioural tests, including conditioned place preference (CPP) and the Morris water maze, possibly due to their high anxiety levels (Balogh et al. 1999; Crawley et al. 1997; Dockstader & van der Kooy 2001; Gerlai 1996; Homanics et al. 1999; Miner 1997; Owen et al. 1997; Vöikar et al. 2001).

Mice were bred from homozygous or heterozygous breeding pairs. The mice within a homozygous breeding pair were always progeny of heterozygous mating, to ensure that
mutant and control mice were of similar genetic background. Mice were weaned at approximately three weeks of age.

2.3.1.1. Verification of genotype

The genotype of all mice used in experiments involving NK1−/− mice and wild type controls was determined or verified by performing the polymerase chain reaction (PCR) on DNA extracted from tail tip samples, as described in protocol 2.3.1.1 (see Appendix). This procedure uses three PCR primers: NK1-F and NK1-R amplify a 350-base section of exon 1 of the gene encoding the NK1 receptor, and NeoF and NK1-R amplify a 260-base section of the NK1 receptor gene including the inserted cassette. Resolution of these PCR products on a 2% agarose gel gives a single band at 260 bases for NK1−/− mice, bands at 260 and 350 bases for heterozygous mice, and a single 350-base band for wild type mice.

2.3.2. Culling

Mice were culled using CO₂ asphyxiation, with the exception of those animals undergoing perfusion (see below). Mice were placed in a CO₂ chamber into which a slow, steady flow of gas into the chamber was released, allowing CO₂ concentrations to rise slowly. When the mice had stopped breathing, death was verified by physical breaking of the neck, exsanguination or removal of the brain.
Figure 2.1 NK1 receptor gene disruption by homologous recombination. Diagram showing the targeting (replacement) vector, the region of the wild type NK1 locus containing exon 1, and the predicted structure of the NK1 gene following homologous recombination. The 5’ external probe fragment for Southern blot analysis is also shown, along with the sizes of restriction fragments following Xbal digestion. BGal: lacZ coding region; IRES: internal ribosome entry site; MC1: MC1 promoter; Neo: neomycin resistance gene; TK: Herpes simplex virus thymidine kinase gene. Small arrows indicate restriction sites. Adapted from De Felipe et al. (1998).

2.4. Immunohistochemistry

The major histological technique used in the experiments described in this thesis is immunohistochemistry (IHC). This process allows the identification of a tissue constituent by virtue of a specific antigen-antibody binding reaction, which is labelled with a visible marker.
To label antigens in fixed tissue, unlabelled primary antibodies were applied to free-floating tissue sections. This was followed by a biotinylated secondary antibody, which binds to antibodies produced by the species in which the primary antibody was raised. Visible labels bound to avidin were then applied, resulting in the formation of a visible macromolecular complex around antigen within the tissue. A general schema for IHC is given in Figure 2.2.
Chapter two  
Materials and methods

Figure 2.2 General schema for IHC used in this thesis.
2.4.1. Tissue preparation and fixation

Mice were terminally anaesthetised by IP injection of 0.2 ml pentobarbitone sodium (200 mg.ml⁻¹; Euthatal; Rhône Mérieux, Harlow, UK) and perfused intracardially with 50 ml heparinised PBS, followed by 100 ml ice-cold 4% paraformaldehyde (PFA; BDH) solution in 0.15 M PB. Brains were removed and post-fixed for 4 - 5 h in 4% PFA at 4 °C. They were then cryoprotected in 30% sucrose in 0.1 M PB containing 0.02% NaN₃ and stored at 4 °C for at least 12 h until ready for sectioning.

Brains were mounted onto the chuck of a sledge microtome (SM 2000 R; Leica Microsystems, Milton Keynes, UK) and frozen with dry ice. Sections were cut coronally at 40 μm, collected in 5% sucrose in 0.1 M PB containing 0.02% NaN₃ and stored at 4 °C.

2.4.2. Primary antibodies

The primary antibodies used in the experiments described in this thesis are listed in Table 2.1, showing their antigen, source, the species in which they were made, and the optimum dilution for each detection method (see below). Before experimental use, all primary antibodies were tested at a range of dilutions and with a variety of detection methods, in order to optimise their staining.
Table 2.1 Primary antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Animal in which raised</th>
<th>Monoclonal or polyclonal (M/P)</th>
<th>Detection method</th>
<th>Optimal dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK1 receptor. Raised against amino acid residues 393 to 407 of the carboxy-terminus of the rat receptor (KTMTESSSFYSNMLA; Vigna et al. 1994).</td>
<td>Chemicon International, Temecula, USA</td>
<td>Rabbit</td>
<td>P</td>
<td>DAB</td>
<td>1:10 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FITC</td>
<td>1:10 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TSA-FITC</td>
<td>1:100 000</td>
</tr>
<tr>
<td>NK1 receptor. Raised against amino acid residues 393 to 407 of the carboxy-terminus of the mouse receptor (KTMTESSSFYNSIL; Cook et al. 1994).</td>
<td>Eurogentec, Seraing, Belgium</td>
<td>Rabbit</td>
<td>P</td>
<td>DAB</td>
<td>1:10 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FITC</td>
<td>1:10 000</td>
</tr>
<tr>
<td>C-Fos. Raised against amino acid residues 4 to 17 of human c-fos (SGFNADYEASSSRC; De Togni et al. 1988).</td>
<td>Oncogene Research Products, Cambridge, USA</td>
<td>Rabbit</td>
<td>P</td>
<td>DAB (with Ni)</td>
<td>1:20 000</td>
</tr>
<tr>
<td>Choline acetyltransferase (ChAT). Raised against human placental enzyme.</td>
<td>Chemicon International</td>
<td>Goat</td>
<td>P</td>
<td>FITC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP). Raised against GFAP isolated from cow spinal cord.</td>
<td>Dako, Glostrup, Denmark</td>
<td>Rabbit</td>
<td>P</td>
<td>FITC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Neuronal Nuclei (NeuN). Raised against purified cell nuclei from mouse brain.</td>
<td>Chemicon International</td>
<td>Mouse</td>
<td>M</td>
<td>Texas Red</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

2.4.3. Labelling primary antibodies

Most of the IHC presented in this thesis utilised the avidin-biotin complex method. This process exploits the very high affinity of the egg white glycoprotein avidin \((K_a = 10^{15} \text{ M}^{-1})\) for the small molecular weight vitamin, biotin. This high affinity, which is over a million

---

1 Directly conjugated to secondary antibody.
times higher than most antigen-antibody reactions, is essentially irreversible. Since biotin and avidin can both be conjugated to most proteins and each avidin molecule can bind four biotin molecules, this reaction can be used to link biotinylated secondary antibodies to avidin-bound label molecules, thereby allowing the formation of visible macromolecular complexes around the antigen of interest.

The labels used to localise primary antibodies in this thesis are as follows:

- **Horseradish peroxidase (ABC method).** Horseradish peroxidase (HRP) is an enzyme (molecular weight 40 kDa), which forms a complex with hydrogen peroxide, causing it to decompose to water and atomic oxygen. In the presence of an electron donor, this peroxidase activity results in the oxidation of the donor. Several of these donors form an insoluble coloured product on oxidation and are termed chromagens. The chromagen used in this thesis is 3,3'-diaminobenzidine tetrahydrochloride (DAB), whose oxidised end product is a brown polymer which is insoluble in water or alcohol. When oxidised in the presence of nickel ions, a grey-black precipitate is formed, which enhances the intensity of the staining procedure. Since DAB is a potential carcinogen, it must be used with care. HRP is bound to avidin for use in IHC. In some experiments, further signal amplification was achieved using tyramide signal amplification (TSA). This process uses horseradish peroxidase to catalyse the deposition of biotinylated tyramide onto the complex bound to the primary antibody. The numerous biotin molecules deposited by this reaction are then visualised indirectly using an avidin-bound label step. Although chromogenic detection can be used following TSA, in this thesis fluorescent detection was carried out using avidin-FITC (see below). Use of TSA allows lower concentrations of primary antibody to be used, thereby increasing the signal:background ratio on tissue sections.

- **Fluorescein isothiocyanate (FITC).** This molecule fluoresces green (515 nm) when excited at a wavelength of 495 nm. It is bound to avidin for use in IHC.

In cases where staining does not require the amplification afforded by the avidin-biotin method, antibodies can be detected using labels bound directly to the secondary antibody. This method is used with primary antibodies raised against choline acetyltransferase (ChAT), neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP), which are
visualised by secondary antibodies directly conjugated to the fluorescent molecule Texas Red or FITC. Texas Red fluoresces red at 615 nm when excited at a wavelength of 595 nm.

The method for IHC with chromogenic detection using DAB is given in protocol 2.4.1 (see Appendix). TSA with fluorescent detection is given in protocol 2.4.2 (see Appendix), and conventional fluorescent detection is detailed in protocol 2.4.3 (see Appendix).

2.4.4. Microscopy and photography

Sections were viewed using either a Leica DMR (Leica Microsystems) or a Nikon Eclipse E800 microscope (Nikon, Kingston upon Thames, UK). Antigens labelled chromogenically were visualised under bright-field conditions, whilst fluorescent dyes were observed under appropriate excitation from a 50 W mercury lamp (HBO 50; Osram, Munich, Germany). Bright-field digital photography was carried out using a JVC Color Video camera (KY-F50; JVC, London, UK), and fluorescent photography using a Hamamatsu Chilled CCD camera (C5985; Hamamatsu Photonics, Welwyn Garden City, UK) attached to a Macintosh PowerPC G3 computer running Vision Explorer VA 1.11 (Graftek Imaging, Austin, USA). High-resolution and large images were created by fusing multiple smaller images using Adobe® Photoshop® 5.5 (Adobe Systems, San Jose, USA).

2.5. Behavioural techniques

The experiments presented in this thesis use a wide range of different behavioural techniques in the assessment of the relationship of the NK1 receptor to reward and learning and memory processes. However, the only techniques that are used in more than one chapter of this thesis are CPP and locomotor assessment, which are explained below.

2.5.1. General principles

The analysis of animal behaviour is a useful way in which links between neural processes and higher functions, such as reward and learning can be assessed. Due to the complex nature of the control of behaviour, care has to be taken to ensure that conclusions drawn from behavioural work are not affected by confounding variables, such as stress and anxiety. Since elevated anxiety levels can impede an animal's behaviour, habituation to handling and the test apparatus before the start of the experiment is often necessary to
minimise the effects of anxiety. The presence of the experimenter in the testing room can also increase anxiety levels. To avoid this, behavioural experiments were carried out in a dedicated room, with as little external disturbance as possible. Where the nature of the experiment allowed, experiments were videotaped or monitored using a PC, while the experimenter waited outside the behaviour room.

In most cases, each mouse was only used in one type of behavioural experiment. Where it was necessary to use a mouse on more than one occasion, a time period of at least a week was included between two experiments, and mice were never used for a new experiment after one involving drug administration. Because of the adverse effects of singular housing on behaviour (Bardo et al. 1995), only mice housed in groups were used for behavioural studies, and albino animals were avoided due to their poor visual acuity (Crawley et al. 1997; Owen et al. 1997; Rhoades & Henry 1977). Except where indicated, behavioural experiments were carried out under fluorescent strip lighting, at an intensity of 40 lx. Mice were transported into the behavioural room at least 24 h before the start of behavioural testing.

2.5.2. EthoVision

The behavioural monitoring software EthoVision 2.3 (Noldus Information Technology, Wageningen, The Netherlands) was used in many of the experiments described in this thesis. It converts analogue visual information from a video camera (High Resolution B/W CCD Camera Model VCB-3372P; Sanyo Electric Company, Osaka, Japan) to a digital signal, which is used to monitor the position of animals in up to 8 experimental arenas simultaneously. In the 'subtraction' mode used in the experiments presented here, it compares the image received from the camera to a previously collected reference image of the experimental apparatus, considering the largest area of discrepancy between these two images in each arena to be the animal under observation. In all the experiments described in this thesis, images were collected at a frequency of 5 Hz. Following recording, a wide variety of parameters can be calculated for each animal being observed. These include the time spent in user-defined zones within each experimental arena, the speed of movement and number of rearings (characterised by large reductions in the animal's surface area).
2.5.3. Conditioned place preference

CPP is a behavioural technique for assessing the rewarding properties of a drug or other stimulus (see Bardo & Bevins 2000). A typical CPP experiment consists of the differential pairing of one of two sets of contextual or environmental cues with the stimulus of interest. The contextual cues tend to differ in terms of wall colour or pattern, floor texture, smell, size or shape, and the animal is exposed to repeated pairings of the stimulus with one context, interspersed with exposures to the other context but without the conditioned stimulus. Following conditioning, the animal is given a choice test, in which it is given free access to both environments, and the amount of time spent in each is monitored. An increase in the amount of time spent in the environment paired with the stimulus of interest is taken as evidence that the stimulus was rewarding, whereas a decrease indicates that it was aversive. In such cases, the paradigm is often referred to as conditioned place aversion (CPA).

CPP has been used to demonstrate the rewarding properties of both natural and drug stimuli. Naturally occurring stimuli that can bring about an increase in preference in rodents include food, water, sweet fluids, interaction with conspecífics, ejaculation, copulation and novel stimuli, although these tend to be subject to changes in motivational state, such as hunger for food reward. A wide variety of drugs can also induce preference. The abused drugs which have been shown to induce CPP include psychostimulants (e.g. cocaine and amphetamine), opiates (e.g. morphine and heroin), nicotine, ethanol, caffeine, lysergic acid diethylamide (LSD), phencyclidine (PCP) and Δ⁹-tetrahydrocannabinol (THC; see Schechter & Calcagnetti 1993; Tzschentke 1998).

CPP is a relatively simple procedure to carry out, and has a number of methodological advantages that have made it an extremely popular tool for the analysis of reward. Its major advantage is that it tests the animal under study in a drug-free state, thereby avoiding the potential effects of the drug (or other stimulus) under test on perception or motivation. It can be used in a wide range of species, and does not require surgery before starting the experiment, unlike self-administration paradigms. It is sensitive to low doses of drugs, and a preference can often be produced following a single pairing of the stimulus and context. However, the task does have a number of conceptual drawbacks. The major criticism of CPP as a measure of reward is based on the fact that the animal under study is required to
form an association between the subjective effects of the stimulus and the context in which it is administered: the reward-associated context then takes on a secondary reinforcing effect in its own right, causing the animal to make more operant approaches towards the context, or alter its behaviour within it, such that the time spent in the context is increased. Drugs that interfere with learning and memory processes may prevent such associations being made, thereby masking a potential rewarding effect. Similarly, an effect of the drug under test on the ability of an animal to become familiar with a context may render the drug-paired compartment more ‘novel’ than the alternate compartment, to which it was exposed without the drug. In this case, the animal under test may move towards the drug-associated compartment during the test phase because of this novelty effect rather than a primary rewarding effect of the drug under study. A final caveat is that state-dependent learning may occur, such that associations made between the rewarding stimulus and the context are expressed only when the animal is under the influence of the drug stimulus under study. Since testing is usually carried out in a drug-free state, such an effect could mask a stimulus’s rewarding effect during this task. Nevertheless, the drawbacks of the CPP procedure are few in comparison to its simplicity and robustness. Despite the potential confounds of the task, CPP does provide information about the affective value of many stimuli, and unique information about the rewarding effect of contextual cues associated with such stimuli (see Bardo & Bevins 2000).

In the CPP experiments presented in this thesis, the rewarding properties of morphine or cocaine were assessed in a counterbalanced, unbiased design based on that of Maldonado et al. (1997). Preference scores were expressed as the within-subject difference in time spent in the drug-associated compartment after conditioning relative to that before conditioning. The scores of animals treated with drug in one of the two compartments were compared to those achieved by vehicle (saline) exposure in both compartments through the conditioning period, to control for the effects of stress and exposure to handling, injection per se and the novelty of the apparatus.

2.5.3.1. Apparatus

The CPP apparatus used in the experiments presented in this thesis consisted of two compartments (146 mm [l] × 138 mm [w] × 141 mm [h]) separated by a central neutral zone (50 mm [l] × 138 mm [w] × 141 mm [h]; Figure 2.3). All apparatus was constructed
from white Plexiglas. The two compartments differed in wall pattern and floor texture: one compartment had black horizontal stripes painted on the walls and floor, whilst the other had black spots painted on the walls and floor, and a metal grid on the floor. A removable guillotine door whose inner surface was painted in the same pattern as its compartment could be used to enclose each end compartment. Clear Plexiglas lids were in place to prevent escape.

Figure 2.3 CPP apparatus. View of apparatus from above showing spotted and striped compartments, position of the metal grid, and the central neutral zone. Small arrows indicate the positions of the removable guillotine doors (not shown), which are painted in the same pattern as the compartment walls.

2.5.3.2. Procedure

The procedure for CPP is illustrated in Figure 2.4.
Figure 2.4 CPP procedure. Following an exposure to both boxes during the preconditioning phase, mice receive drug injections when enclosed in one compartment and vehicle injections in the other compartment on alternate days for 6 d. During the test session, mice tend to spend more time in the compartment paired with a rewarding stimulus. Note that the arrangement of the compartments is altered in this figure for clarity.
2.5.3.2.1. Preconditioning

Prior to conditioning, the behaviour of the mice in the apparatus was monitored, in order to habituate them to the CPP apparatus, and to assess their innate preference for the two compartments. Although exposure to the conditioning compartments before pairing with drug or vehicle injections can reduce CPP scores due to latent inhibition (Bardo et al. 1995; Bardo & Bevins 2000), preconditioning trials are often used in order to isolate animals with unusually strong preferences for one compartment and to allow a completely unbiased design to be used.

Each mouse was placed the central neutral zone of the CPP apparatus, facing one of the sidewalls, with the guillotine doors removed. Its position was monitored for 18 min. This was achieved in one of three ways:

- Real time monitoring of each mouse’s behaviour by the experimenter, recording the amount of time spent in each compartment with two stopwatches. A mouse was considered to be within a compartment when its entire body, with the exception of the tail, had crossed the threshold of the compartment. This method was used before the acquisition of a camcorder, but was extremely time-consuming as only one mouse could be tested at any one time.

- Videotaping the mice’s behaviour using a video camera (Handycam Vision CCD-TRV36E; Sony Corporation, Tokyo, Japan) and measuring the time spent in each compartment by playing the videotape back at the end of the behavioural session. This method had the advantage of allowing up to four mice to be tested at once, in four sets of identical apparatus, although each mouse’s behaviour still had to be scored individually after recording. The experimenter did not need to be present in the behavioural testing room with this method, thereby minimising the effects of human presence on the animals’ behaviour.

- Following the acquisition of EthoVision (see above), the efficiency of monitoring was increased dramatically. In CPP experiments, the software was used to calculate the amount of time spent in each of the two compartments by each mouse. Four mice were tested simultaneously in identical sets of apparatus.

Since it is unclear whether the mouse is expressing a preference for either compartment when it is in the neutral central zone, the time spent in this area was not included in the
calculation of preference following preconditioning. As such, the time spent in each compartment was expressed as a proportion of the time spent in either of the two compartments excluding the central zone. This was then multiplied by the total time of the preconditioning session (1080 s), giving a measure of the time spent in each compartment in seconds. Animals spending more than 75 % (810 s) or less than 25 % (270 s) of the time in one compartment were excluded from further testing, as their innate bias for one of the two environmental contexts may prevent CPP from being demonstrated clearly (Maldonado et al. 1997)

During conditioning, mice were given drug injections (morphine or cocaine) in one of the two compartments, interspersed with vehicle (saline) injections in the alternate compartment. The assignment of mice to receive drug injections in either the striped or spotted compartment was carried out in a counterbalanced manner. In order to achieve this, care was taken to ensure that the total time spent by all mice of one experimental group during the preconditioning phase in the drug-associated compartments was approximately equal for mice receiving drug in the spotted and striped compartments. This ensured that there was an equal bias for the two compartments within the mice from each experimental group, thereby avoiding the problems that may be associated with a disproportionate number of animals receiving drug injections in the preferred or non-preferred compartment.

2.5.3.2.2. Conditioning

Conditioning took place on days 1 to 6, beginning 24 h after the preconditioning phase. On days 1, 3 and 5, mice received an injection of the test drug, whilst on days 2, 4 and 6, they were injected with an equivalent volume of vehicle. Mice were injected immediately before being placed in the appropriate compartment with the guillotine door in place for 20 min. Following exposure, the mice were returned to their home cage and the apparatus was thoroughly cleaned with water.

2.5.3.2.3. Testing

Following 6 d of conditioning, CPP was tested on day 7. Mice were exposed to the CPP apparatus with guillotine doors removed, and their behaviour monitored as in the preconditioning phase (see above). The time spent in each compartment was calculated as
for preconditioning and the preference score expressed as the time spent in the drug-associated compartment before conditioning subtracted from that after conditioning. Positive scores are indicative of a rewarding stimulus, although comparison with the behavioural effect of vehicle injection in both compartments is necessary to ensure that the observed phenomenon is not due to procedural aspects of the task, such as the order of stimulus presentations or the stress of handling and injection.

2.5.4. Locomotor assessment

At certain doses, all addictive drugs cause increases in locomotor behaviour in animals, and there is evidence to suggest that this hyperlocomotor response is mediated by the same neural mechanisms as reward (Wise 1987; Wise & Bozarth 1987). The analysis of locomotor stimulation by morphine and cocaine, as well as the sensitisation of locomotor stimulation caused by repeated drug administration, was used in many of the experiments of this thesis. Furthermore, differences in basal locomotor activity between groups of animals may be confounded with other behavioural measures. In CPP for example, elevated basal locomotion may lead to animals continually moving between the two boxes during the test phase, thereby masking a rewarding effect of the stimulus under study. Locomotor assessment of mice was achieved in one of two ways.

2.5.4.1. Open field

In the open field test, mice were placed in a square black arena (410 mm × 410 mm; Columbia Instruments) with 14 parallel infrared beams (27 mm apart and 28 mm above the floor) projecting across to detector cells on the opposite wall. The total number of times the mouse broke a beam by crossing it was counted automatically. The apparatus also counted the number of times a mouse crossed successive beams, i.e. discounting repeated crossings of the same beam. This measure of 'ambulatory' beam crosses was used as a measure of locomotor activity in mice, since it avoided the repeated beam crossings associated with movements such as grooming and scratching. The open field arena was cleaned thoroughly with water after use.
2.5.4.2. Small activity boxes

The activity of mice was also monitored using EthoVision (see above). Mice were placed individually in small black Plexiglas boxes (internal dimensions: 200 mm [l] × 90 mm [w] × 110 mm [h]), with up to 8 mice being tested at one time. Locomotor activity was expressed as mean speed of movement in mm.s\(^{-1}\) over the recording session. Activity boxes were cleaned thoroughly with water after use.

2.6. Statistical analysis

Except where indicated otherwise, all of the statistical analysis presented in this thesis was carried out using Minitab Release 12.1 (Minitab, State College, USA). The major technique used for the comparison of two or more groups within a data set was analysis of variance (ANOVA), using the General Linear Model (GLM) command. The principle behind this method of analysis is to partition the total variability of a set of data into components due to different sources of variation. The variability is divided into that which is due to systematic differences between groups (e.g. between wild type and knockout mice) and that due to individual differences within groups, i.e. the noise or 'error'. The test calculates the amount of between-group variability relative to the noise in the data set to assess whether the variable under test (e.g. genotype) has a significant influence on the measured parameter, with the null hypothesis that there is no difference between the groups.

ANOVAs can be used to assess the influence of a single independent variable on a data set, in which case it is referred to as a 1-way ANOVA: an example is in the analysis of the effect of mouse genotype on a behavioural parameter. Two- or more-way ANOVAs can be used to analyse the effects of more than one independent variable on a data set simultaneously: an example of a 2-way ANOVA is the analysis of the effects of mouse genotype and drug type on a behavioural task. In such cases, the ANOVA procedure also allows analysis of the interaction between the independent parameters (i.e. genotype and drug) in the model. A significant interaction indicates that the levels of the parameters under study influence the dependent variable in a complex manner, and that each parameter's influence depends on the others. For example, a significant interaction between genotype and drug in the 2-way ANOVA described above would suggest that the drugs under study have different effects on the behavioural parameter in each genotype. In contrast, significant main effects of genotype and drug without a significant interaction
term would suggest that although both factors affect the outcome on the behavioural task, the drugs have similar effects in all the genotypes under test, i.e. the effect of drug is independent of that of genotype.

Following significant main effects of such parameters or interactions, statistical differences between individual groups can be assessed using post hoc tests. In this thesis, Tukey comparisons were used for multiple pairwise comparisons, whilst Dunnett’s tests were used for comparisons with a control level. The use of such post hoc tests is desirable since the likelihood of detecting a false positive result when there is no real difference (a Type I error) increases during multiple pairwise comparisons of the same groups within a data set. Post hoc tests have built-in controls to keep the overall Type I error rate low, thus reducing the chance of false positive results, although they can be relatively conservative.

The ANOVA procedure is based on the assumption that the samples in a data set are independent, and they are from Normally distributed populations with the same variance. Non-independence of data points cannot be corrected mathematically, but in the special case where multiple measurements are made from one experimental subject, repeated-measures ANOVA can be used. In such cases, the use of this procedure ensures that comparisons between groups are made on the basis of within-subject comparisons, rather than between-subject differences, which can often be relatively large. For example, a comparison of the growth rate of a group of mice could be assessed by measuring the size of the mice every day for a period of time. A 1-way repeated-measures ANOVA could be used to analyse this data set, with day as a within-group factor. Flagging day as a within-group factor is necessary to ensure that the test does not assume that the values collected on separate days are independent, i.e. from different mice. Rather, the influence of day on the mice’s size is assessed by analysis of the change in size within each subject over the time period of the experiment. Repeated-measures can also be incorporated into 2- or more-way ANOVAs, for example in the comparison of growth rate between two mouse genotypes.

In the ANOVAs described in this thesis, Normality was assessed by constructing a Normal plot of the residuals (the difference of each value from its group mean), and testing whether they deviated from Normality using the Ryan-Joiner test. The assumption that the variance of the samples’ populations was homogeneous was tested using an F-test (for two
groups) or Bartlett's test (for three or more groups). Where these assumptions were not upheld, square root ($\sqrt{x}$, where $x$ is each value in the data set), logarithmic ($\ln[x]$), reciprocal ($-1/x$) or Box-Cox transformations ($x^y$, where $y$ produces a distribution of residuals as close to Normality as possible) were attempted. Where none of these transformations was successful in correcting violation of these assumptions, 1-way ANOVAs were substituted with Mann-Whitney tests (for two groups) or Kruskal-Wallis tests (for three or more groups). Repeated-measures ANOVAs and 2- or more-way ANOVAs cannot easily be substituted with nonparametric tests: in such cases, the transformed data set which brought about the smallest deviation from the test's assumptions was used.

In all statistical procedures used, $P \leq 0.05$ was considered statistically significant. Except where indicated, all data are presented as mean ± standard error (SEM).
CHAPTER THREE

IMMUNOHISTOCHEMICAL DETECTION OF THE NEUROKININ-1 RECEPTOR IN THE MOUSE BRAIN
3. IMMUNOHISTOCHEMICAL DETECTION OF THE NEUROKININ-1 RECEPTOR IN THE MOUSE BRAIN

3.1. Introduction

Knowledge of the distribution of the neurokinin-1 (NK1) receptor within the brain is vital for an analysis of its involvement in the control of behaviour. Although a number of studies have described the distribution of the receptor in the brains of a range of mammalian species, there are no published systematic descriptions of the receptor’s distribution in the mouse brain. A full analysis of the receptor’s expression was therefore carried out before experiments examining the receptor’s role in reward and learning and memory processes were begun.

To this end, this brief chapter describes an immunohistochemical study analysing the distribution of the NK1 receptor in the mouse brain. The findings are compared to previous anatomical studies in other species. The results of this experiment were used in the design and interpretation of subsequent studies of the involvement of the NK1 receptor in the control of behaviour.

3.2. Background

3.2.1. Distribution of the neurokinin-1 receptor: radioactively labelled agonists

Early studies of the distribution of the NK1 receptor in brain sections examined the binding pattern of radioactively labelled substance P (SP). These studies revealed that the highest levels of SP binding, and presumably NK1 receptor distribution in the rat brain were found in the olfactory bulbs, the dentate gyrus, the amygdala (especially the medial nucleus and amygdalo-hippocampal area), the superior colliculus, the parabrachial nucleus and the locus coeruleus, with more moderate staining in the striatum, including the nucleus accumbens (NAcc), the periaqueductal grey and the subiculum (Buck et al. 1986; Mantyh et al. 1984; Quirion et al. 1983; Rothman et al. 1984; Shults et al. 1982). The indirect nature of this approach calls the specificity of these findings into question, since, although the NK1 receptor is the preferred receptor for SP, it can also bind with low affinity to NK2 and NK3 receptors, whose distributions differ from that of the NK1 receptor (Ingi et al. 1991; Mantyh et al. 1989; Regoli et al. 1987; Saffroy et al. 1988). However, use of a radioactively...
labelled ligand with more selective binding properties for the NK1 receptor confirmed the earlier findings with labelled SP (Dam et al. 1990).

3.2.2. Distribution of the neurokinin-1 receptor: in situ hybridisation

In situ hybridisation histochemistry has also been used in a range of species to examine the distribution of cells expressing the NK1 receptor. In the rat brain, high levels of the mRNA encoding the receptor have been observed in the olfactory bulbs, the caudate putamen (CPu), the NAcc, the ventral pallidum, the olfactory tubercle, the medial nucleus of the amygdala, the amygdalo-hippocampal area, the dorsal raphe nucleus, the locus coeruleus, the dorsal tegmental nuclei and the parabrachial nucleus, with weak to moderate levels in the hippocampus, hypothalamus (especially the suprachiasmatic nucleus), the dorsal part of the periaqueductal grey and the superior colliculus. A small number of NK1 receptor mRNA-positive cells have also been observed in cortical areas. There were very low numbers of such cells in the thalamus, except for a group of positive cells in the habenular nucleus, the pretectum and the intergeniculate leaflet (Elde et al. 1990; Maeno et al. 1993; Mick et al. 1994). Neurones expressing mRNA for the NK1 receptor also express that for choline acetyltransferase (ChAT), particularly in the striatum, including the NAcc, as well as in the medial septal nucleus (Aubry et al. 1993; Gerfen 1991). In man, coexpression of ChAT and the NK1 receptor has been confirmed in the striatum using this technique (Aubry et al. 1994), whilst in the cat, in situ hybridisation has been used to demonstrate the presence of NK1 receptor mRNA in the visual cortex, the hypothalamus and the basolateral nucleus, but not the medial, lateral or cortical nuclei of the amygdala (Matute et al. 1993; Yao et al. 1999).

3.2.3. Distribution of the neurokinin-1 receptor: immunohistochemistry

The clearest demonstrations of the distribution of the NK1 receptor protein have been made using immunohistochemistry (IHC) on fixed brain sections. Such studies have generally produced similar results to those using radioactively labelled SP and in situ hybridisation. A systematic analysis of NK1 receptor immunoreactivity in the central nervous system of the rat revealed that the highest levels of staining in the brain occurred in the cortical amygdaloid nucleus and amygdalo-hippocampal area, the hilus of the dentate gyrus, the locus coeruleus and the nucleus ambiguus. Strong staining was also observed in
Chapter three Immunohistochemical detection of the neurokinin-1 receptor

the olfactory bulbs, the CPu, the NAcc shell and core subregions, the olfactory tubercles, the medial nucleus of the amygdala and the median, pontine and magnus raphe nuclei, as well as in various nuclei in the medulla oblongata and the brainstem. Weaker staining was seen in cortical and hypothalamic areas, septal nuclei, the nucleus basalis magnocellularis, the CA1 and CA3 regions of the hippocampus, the central, lateral and basolateral nuclei of the amygdala, the periaqueductal grey and the superior colliculus, but there was little staining in the thalamus (except for weak staining in the midline nuclei), the substantia nigra or the ventral tegmental area (VTA; Nakaya et al. 1994). Other studies in the rat have confirmed these findings, and have indicated the presence of NK1 receptor immunoreactivity specifically on the γ-aminobutyric acid (GABA)-ergic interneurones of the CA1 and CA3 regions of the hippocampus and on the somata of noradrenergic neurones in the locus coeruleus (Acsády et al. 1997; Barbaresi 1998; Chen et al. 2000; Commons & Valentino 2002; Jakab & Goldman-Rakic 1996; Kaneko et al. 1994; Li et al. 2001; Ogawa-Meguro et al. 1994; Pickel et al. 2000; Sloviter et al. 2001). In the guinea pig, similar patterns of immunoreactivity have been observed (Yip & Chahl 2000, 2001), whilst high levels of expression of the NK1 receptor in the anterior, amygdalo-hippocampal and medial nuclei of the amygdala has been confirmed in the gerbil, with lower levels of immunoreactivity in the basolateral nucleus (Smith et al. 1999). In man, NK1 receptor immunoreactivity has been reported in the prefrontal and cingulate cortices, the striatum, the globus pallidus, the hippocampus and the ventral pallidum (Burnet & Harrison 2000; Kowall et al. 1993; Mounir & Parent 2002; Parent et al. 1995; Tooney et al. 2000). Although there are no published systematic analyses of the distribution of the NK1 receptor in the mouse brain, the presence of neurones expressing the receptor in the paraventricular and supraoptic nuclei of the mouse hypothalamus has been demonstrated (Liu et al. 2002). Additionally a recent study has attempted to analyse differences between species in the distribution of NK1 receptor immunoreactivity, finding that although present in the suprachiasmatic nucleus of the hypothalamus of the rat, it is absent in this region in the mouse and hamster, whilst all three species possess NK1 receptor immunoreactivity in the intergeniculate leaflet (Piggins et al. 2001).

The expression of ChAT in NK1 receptor-expressing neurones observed with in situ hybridisation has also been observed using double-labelling IHC. In particular, the coexpression of these molecules in the large aspiny neurones of the striatum has been seen
in a number of studies examining a range of species (Jakab & Goldman-Rakic 1996; Kowall et al. 1993; Li et al. 2000; Pickel et al. 2000). However, an additional subpopulation of the NK1 receptor-expressing neurones in this region do not express ChAT but are somatostatin-positive, at least in the rat, macaque and human (Aubry et al. 1994; Jakab et al. 1996; Kaneko et al. 1993). Coexpression of the NK1 receptor and ChAT has also been seen in the ventral pallidum, the substantia innominata, the medial septal nucleus, the nucleus of the diagonal band of Broca, and the magnocellular preoptic nucleus (Chen et al. 2001b).

3.3. Materials and methods

Male wild type mice from homozygous breeding (see section 2.3.1) of approximately 8 weeks of age (n = 3) were perfused, and their brains removed and sectioned (see section 2.4.1). Immunohistochemistry (IHC) for the NK1 receptor was carried out on every 6th section through the brain from the caudal limit of the olfactory bulbs (approximately 3 mm rostral to Bregma) to the brainstem (approximately 6 mm caudal to Bregma), using an antibody raised against the NK1 receptor (Chemicon International; see section 2.4.2), visualised using tyramide signal amplification (TSA) and fluorescein isothiocyanate (FITC; see section 2.4.3). Sections were observed and photographed under fluorescent conditions (see section 2.4.4).

3.4. Results

The distribution of NK1 receptor immunoreactivity was identical in the three brains used in this experiment. Representative photomicrographs of sections taken from the mice are shown in Figure 3.1, along with diagrammatic representations adapted from a mouse brain atlas (Franklin & Paxinos 1997). Brain regions are labelled according to the scheme used in this atlas.
Chapter three
Immunohistochemical detection of the neurokinin-1 receptor

-0.3 mm

-0.6 mm

-0.7 mm

-0.9 mm
Chapter three

Immunohistochemical detection of the neurokinin-1 receptor

-4.0 mm

-4.2 mm

-4.6 mm

-4.8 mm
Figure 3.1 The NK1 receptor in the mouse brain. Sections from the brain of a mouse stained immunohistochemically for the NK1 receptor, and visualised with TSA-FITC. Numbers are approximate distances of sections from Bregma. Abbreviations given overleaf. Scale bars = 2 mm.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
<th>Abbreviation</th>
<th>Term</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>anterior amygdaloid area</td>
<td>IP</td>
<td>interpeduncular nucleus</td>
</tr>
<tr>
<td>AAD</td>
<td>anterior amygdaloid area, dorsal part</td>
<td>IPAC</td>
<td>interstitial nucleus of the posterior limb of the anterior commissure</td>
</tr>
<tr>
<td>aca</td>
<td>anterior commissure, anterior part</td>
<td>IRt</td>
<td>intermediate reticular nucleus</td>
</tr>
<tr>
<td>Acb</td>
<td>accumbens nucleus</td>
<td>LA</td>
<td>lateral amygdaloid nucleus</td>
</tr>
<tr>
<td>AcbC</td>
<td>accumbens nucleus, core</td>
<td>LDTg</td>
<td>laterodorsal tegmental nucleus</td>
</tr>
<tr>
<td>AcbSh</td>
<td>accumbens nucleus, shell</td>
<td>LGP</td>
<td>lateral globus pallidus</td>
</tr>
<tr>
<td>Aco</td>
<td>anterior cortical amygdaloid nucleus</td>
<td>LH</td>
<td>lateral hypothalamic area</td>
</tr>
<tr>
<td>AHC</td>
<td>anterior hypothalamic area, central part</td>
<td>LOT</td>
<td>nucleus of the lateral olfactory tract</td>
</tr>
<tr>
<td>AHiAL</td>
<td>amygdalohippocampal area, anterolateral part</td>
<td>LP</td>
<td>lateral posterior thalamic nucleus</td>
</tr>
<tr>
<td>AHP</td>
<td>anterior hypothalamic area, posterior part</td>
<td>LPO</td>
<td>lateral preoptic area</td>
</tr>
<tr>
<td>APT</td>
<td>anterior pretectal nucleus</td>
<td>MeA</td>
<td>medial amygdaloid nucleus, anterior part</td>
</tr>
<tr>
<td>APTD</td>
<td>anterior pretectal nucleus, dorsal part</td>
<td>MG</td>
<td>medial geniculate nucleus</td>
</tr>
<tr>
<td>APTV</td>
<td>anterior pretectal nucleus, ventral part</td>
<td>MHB</td>
<td>medial habenular nucleus</td>
</tr>
<tr>
<td>Arc</td>
<td>arcuate hypothalamic nucleus</td>
<td>ml</td>
<td>medial lemniscus</td>
</tr>
<tr>
<td>B</td>
<td>basal nucleus of Meynert (nucleus basalis magnocellularis)</td>
<td>MnR</td>
<td>median raphe nucleus</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdaloid nucleus</td>
<td>MS</td>
<td>medial preoptic nucleus</td>
</tr>
<tr>
<td>BMA</td>
<td>basomedial amygdaloid nucleus, anterior part</td>
<td>MTu</td>
<td>medial tuberal nucleus</td>
</tr>
<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>BSTL</td>
<td>bed nucleus of the stria terminalis, lateral division</td>
<td>PB</td>
<td>parabrachial nucleus</td>
</tr>
<tr>
<td>BSTM</td>
<td>bed nucleus of the stria terminalis, medial division</td>
<td>PCrTa</td>
<td>parvicellular reticular nucleus, alpha part</td>
</tr>
<tr>
<td>BSTMPL</td>
<td>bed nucleus of the stria terminalis, medial posterolateral part</td>
<td>PMCo</td>
<td>posteromedial cortical amygdaloid nucleus</td>
</tr>
<tr>
<td>Ce</td>
<td>central amygdaloid nucleus</td>
<td>PnC</td>
<td>pontine reticular nucleus caudal</td>
</tr>
<tr>
<td>CeC</td>
<td>central amygdaloid nucleus, capsular division</td>
<td>PnO</td>
<td>pontine reticular nucleus oral</td>
</tr>
<tr>
<td>CeL</td>
<td>central amygdaloid nucleus, lateral division</td>
<td>Po</td>
<td>posterior thalamic nuclear group</td>
</tr>
<tr>
<td>CIC</td>
<td>central nucleus inferior colliculus</td>
<td>PP</td>
<td>peripeduncular nucleus</td>
</tr>
<tr>
<td>CPu</td>
<td>caudate putamen</td>
<td>PPtG</td>
<td>pedunculopontine tegmental nucleus</td>
</tr>
<tr>
<td>CxA</td>
<td>cortex-amygdala transition zone</td>
<td>PV</td>
<td>paraventricular thalamic nucleus</td>
</tr>
<tr>
<td>DLG</td>
<td>dorsal lateral geniculate nucleus</td>
<td>RMC</td>
<td>red nucleus magnocellular</td>
</tr>
<tr>
<td>DM</td>
<td>dorsomedial hypothalamic nucleus</td>
<td>SCO</td>
<td>subcommissural organ</td>
</tr>
<tr>
<td>DpG</td>
<td>deep grey layer of the superior colliculus</td>
<td>SI</td>
<td>substantia innominata</td>
</tr>
<tr>
<td>DpMe</td>
<td>deep mesencephalic nucleus</td>
<td>SLEA</td>
<td>sublentic extended amygdala</td>
</tr>
<tr>
<td>DR</td>
<td>dorsal raphe nucleus</td>
<td>SuG</td>
<td>superficial grey, superior colliculus</td>
</tr>
<tr>
<td>En</td>
<td>endopiriform nucleus</td>
<td>TC</td>
<td>tuber cinereum area</td>
</tr>
<tr>
<td>f</td>
<td>fornix</td>
<td>Th</td>
<td>thalamus</td>
</tr>
<tr>
<td>fmi</td>
<td>forceps minor of the corpus callossum</td>
<td>Tu</td>
<td>olfactory tubercle</td>
</tr>
<tr>
<td>fr</td>
<td>fasciculus retroflexus</td>
<td>VDB</td>
<td>nucleus of the vertical limb of the diagonal band</td>
</tr>
<tr>
<td>Hb</td>
<td>habenular nucleus</td>
<td>VEn</td>
<td>ventral endopiriform nucleus</td>
</tr>
<tr>
<td>HDB</td>
<td>nucleus of the horizontal limb of the diagonal band</td>
<td>VLG</td>
<td>ventrolateral geniculate nucleus</td>
</tr>
<tr>
<td>Hp</td>
<td>hippocampus</td>
<td>VMH</td>
<td>ventromedial hypothalamic nucleus</td>
</tr>
<tr>
<td>ic</td>
<td>internal capsule</td>
<td>VOLT</td>
<td>vascular organ of the lamina terminalis</td>
</tr>
<tr>
<td>IcJ</td>
<td>islands of Calleja</td>
<td>VP</td>
<td>ventral pallidum</td>
</tr>
<tr>
<td>IcJM</td>
<td>islands of Calleja, major island</td>
<td>VPO</td>
<td>ventral periolivary nucleus</td>
</tr>
<tr>
<td>IGL</td>
<td>intergeniculate leaflet</td>
<td>xscp</td>
<td>decussation of the superior cerebellar peduncle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZI</td>
<td>zona incerta</td>
</tr>
</tbody>
</table>
3.4.1. Telencephalon

NK1 receptor immunoreactivity was modest throughout the cortex. In the prefrontal cortical areas, diffuse dendritic staining was observed, which was maintained caudally through the parietal, temporal and striate cortices, although sections in Figure 3.1 do not include cortical areas caudal to the temporal cortex. There was no clear lamination of NK1 receptor immunoreactivity in any cortical region.

The CPu exhibited strong immunoreactivity throughout. NK1 receptor immunoreactivity was seen on perikarya of a subset of neurones in this brain region, with a dense network of NK1 receptor immunoreactive dendrites extending throughout the nucleus. A similar pattern of immunoreactivity was observed in the NAcc, with no clear distinction between the shell and core subregions. Cells in the ventral pallidum, islands of Calleja and basal nucleus of Meynert (nucleus basalis magnocellularis) also exhibited clear NK1 receptor immunoreactivity of similar nature to that in the CPu, although with a more patchy distribution.

The hippocampus possessed low levels of NK1 receptor immunoreactivity in the dorsorostral regions shown in Figure 3.1. In more ventrocaudal portions of the hippocampus, NK1 receptor-immunoreactive neurones with pyramidal cell-like morphology were occasionally observed, with clearly labelled perikarya and dendrites (data not shown).

The amygdala possessed clear NK1 receptor immunoreactivity. The central, basomedial and medial nuclei of the amygdala displayed strong staining, as did some of the amygdala’s accessory regions, including the cortical amygdaloid nucleus, the cortex-amygdala transition zone, the anterior amygdaloid area and the amygdalo-hippocampal area. In the central nucleus of the amygdala, immunoreactivity, although strong, was not observed on perikarya or dendrites but there was a more diffuse staining of the neuropil. Conversely, in the basomedial and medial nuclei, NK1 receptor IHC revealed a network of clearly stained dendrites. Within the basolateral and lateral nuclei of the amygdala, immunoreactivity was generally of low intensity, although one or two immunoreactive neurones were often observed in each section, with clearly stained perikarya and dendrites.
In the septum, the medial nucleus exhibited slightly stronger immunoreactivity than surrounding areas, although this staining is diffuse without visible cell bodies or dendrites. A similar pattern of diffuse staining is observed in the bed nucleus of the stria terminalis.

3.4.2. Diencephalon

The habenular nucleus, particularly its medial division, possessed some of the strongest NK1 receptor immunoreactivity of the mouse brain, with a dense network of stained dendrites visible at higher magnifications. In general, however, the thalamus was devoid of NK1 receptor immunoreactivity, with the exception of the lateral geniculate nucleus and the intergeniculate leaflet, which are clearly visible with strong staining for NK1 receptors.

The hypothalamus exhibited diffuse NK1 receptor immunoreactivity throughout, although it was less strong in the ventromedial nucleus than other nuclei.

3.4.3. Mes- and metencephalon

The periaqueductal grey possessed modest NK1 receptor immunoreactivity, especially in the dorsomedial column. Immunoreactivity was also seen in the interpeduncular nucleus, although it was almost absent from the VTA and substantia nigra. The superior colliculus also possessed diffuse neuropil staining.

The tegmental pedunculopontine nucleus and medial and dorsal raphe nuclei both possessed modest NK1 receptor immunoreactivity and were clearly visible in stained sections. The NK1 receptor immunoreactivity in the parabrachial nucleus and the locus coeruleus were more intense, being two of the areas of the strongest immunoreactivity in the brain, with a dense network of immunoreactive perikarya and dendritic processes.

3.5. Discussion

3.5.1. Distribution of neurokinin-1 receptor immunoreactivity in the mouse brain

Immunoreactivity for the NK1 receptor in the mouse brain was strongest in the CPu, the NAcc core and shell subregions, the habenular nucleus, the lateral geniculate nucleus, the intergeniculate leaflet, the parabrachial nucleus and the locus coeruleus, with weaker staining observed in cortical areas, the hypothalamus, the central, basomedial and medial
nuclei of the amygdala, the amygdala’s accessory regions, the dorsal periaqueductal grey, the tegmental pendunculopontine nucleus and dorsal and medial raphe nuclei. NK1 immunoreactivity was generally absent from the hippocampus, thalamic regions, the substantia nigra, the VTA and the cerebellum. However, sections caudal to the level of the locus coeruleus (approximately 6 mm caudal to Bregma) were not analysed, comprising the medulla oblongata and brainstem nuclei. Similarly, sections rostral to the prefrontal cortex (approximately 2 mm rostral to Bregma) were not examined, thereby omitting the olfactory bulbs from analysis.

3.5.2. Comparison of mouse and rat neurokinin-1 receptor distributions

The distribution of immunoreactivity for the NK1 receptor in the mouse brain is very similar to that in the rat (Nakaya et al. 1994), and generally agrees with findings in this and other species using IHC, and using labelled NK1 receptor agonists or in situ hybridisation. This similarity is also reflected in the expression of ChAT in the NK1 receptor-expressing neurones of the mouse CPu and NAcc, as confirmed with dual-labelling IHC (Murtra et al., unpublished observations). However, in the hippocampus, the pattern of immunoreactivity differs between the present study and previous studies in the rat. Here, NK1 immunoreactivity was not seen in the dorsorostral hippocampus in any of the brains analysed, whilst at more ventrocaudal levels of the hippocampus and the subiculum, NK1 receptor-positive cells with pyramidal cell-like morphology were occasionally seen (data not shown). In the rat, NK1 receptor immunoreactivity is reported to be of high intensity and density in the hilus of the dentate gyrus, as well as on GABAergic interneurones of the CA1 and CA3 subregions (Acsády et al. 1997; Nakaya et al. 1994; Sloviter et al. 2001). Further minor differences between this and previous studies were observed in the septal nuclei, the amygdala and the thalamus. Although NK1 receptor immunoreactivity was observed in the septum of the mouse, especially the medial septal nucleus, it was of lower relative intensity than in the rat. Similarly, the density of NK1 receptor-immunoreactive neurones in the lateral and basolateral nuclei of the amygdala was noticeably lower in the mouse than in the rat, although the pattern of NK1 receptor distribution across the amygdala’s subnuclei was comparable. Finally, the low levels of NK1 receptor immunoreactivity in the midline nuclei of the rat thalamus, such as the parafascicular nucleus, were not observed in the present study. Elsewhere in the brain there were few
qualitative differences in the distribution of immunoreactivity between species, although differences may exist in the areas of the brain not examined in this study, such as the olfactory bulbs and the medulla oblongata.

3.5.3. Mismatch between substance P and the neurokinin-1 receptor

The expression of the NK1 receptor in the rat follows that of SP relatively closely. However, there are a few areas of mismatch of the receptor and its preferred ligand, most noticeably in the substantia nigra pars reticulata. This brain region possesses high levels of SP but seems to be devoid of NK1 receptors, at least in the majority of published studies (Gerfen 1991; Maeno et al. 1993; Mantyh et al. 1984; Nakaya et al. 1994; Rothman et al. 1984; Shults et al. 1982). Conversely, the hilus of the dentate gyrus, which is rich in NK1 receptor-positive dendrites in the rat, expresses low levels of SP (Nakaya et al. 1994). In the absence of a systematic analysis of the expression pattern of SP in the mouse, a comparison of the expression patterns of the receptor and ligand in this species is not possible. However, in a preliminary study, the expression pattern of SP immunoreactivity in the mouse forebrain was found to be similar to that of the NK1 receptor (data not shown).

3.5.3. Methodological considerations

Although the present results are similar to those observed in other species, the correspondence of the distribution of NK1 receptor immunoreactivity to the distribution of the receptor itself is dependent upon the specificity of the primary antibody. The antibody used was purchased from Chemicon International, who claim that it is specific for the NK1 receptor, reacting with that of the guinea pig, mouse or rat (see section 2.4.2). The observed staining pattern should therefore correspond exactly to the receptor's distribution. Furthermore, the present findings have been confirmed in the mouse using two other primary antibodies raised against the NK1 receptor (including an antibody raised against the amino acid sequence of the mouse NK1 receptor; see section 2.4.2), adding weight to the conclusion that the observed differences between this study and previous rat studies are due to a real interspecies difference rather than being a methodological artefact.
3.6. **Conclusions**

The distribution of NK1 receptor immunoreactivity in the mouse brain is similar to that in the rat and other species. However, differences between the rat and mouse do exist, primarily in the hippocampus. In the mouse, NK1 receptor immunoreactivity is almost absent from this region, whilst it is present at high levels in the hilus of the dentate gyrus and in the GABAergic interneurones of the CA1 and CA3 regions of the rat. Elsewhere in the brain, the distribution of NK1 receptor immunoreactivity is similar in the two species.
CHAPTER FOUR

REWARD AND ADDICTION BEHAVIOURS IN
NEUROKININ-1 RECEPTOR KNOCKOUT MICE
4. REWARD AND ADDICTION BEHAVIOURS IN NEUROKININ-1 RECEPTOR KNOCKOUT MICE

4.1. Introduction

In this chapter, results are presented from a range of experiments examining the behaviour of neurokinin-1 (NK1) receptor knockout (NK1<sup>-/-</sup>) mice in paradigms that assess the rewarding and addictive properties of drugs of abuse. These are discussed in relation to the work of Murtra et al. (2000b) and other published studies. Part of these findings has been published in a peer-reviewed journal (Ripley et al. 2002; see Appendix).

4.2. Background

4.2.1. Use of knockout mice in behavioural studies

The analysis of the behaviour of gene knockout mice is a useful way in which to assess the contribution of a molecule or system to the function of the brain. Indeed, behavioural analysis of NK1<sup>-/-</sup> mice led to the unexpected discovery of the NK1 receptor's crucial role in modulating opiate reward behaviours (Murtra et al. 2000b). However, there are a number of potential confounds which can complicate the interpretation of findings from such studies. Importantly, the induced mutation may bring about a number of compensatory processes in a mutant mouse, such as up- or downregulation of other genes, which may lead to secondary phenotypic changes, including changes in behaviour. Such changes will have different effects depending on the background in which the mutation is introduced, since mice of different genetic backgrounds possess different alleles of many genes.

In common with most gene targeting studies, the NK1<sup>-/-</sup> mice used by De Felipe et al. (1998) and Murtra et al. (2000b), and in the experiments described in this thesis, were produced by introducing the mutation into cultured embryonic stem cells derived from mice of the 129/sv strain. Successfully transformed cells were then introduced into blastocysts of the C57BL/6 strain, which went on to develop into chimaeric mice. Those with germline transmission of the mutant allele were then mated with C57BL/6 mice, giving heterozygous F<sub>1</sub> offspring. Such mice have one set of chromosomes from the 129/sv strain and the other from the C57BL/6 strain, and subsequent mating of these mice
with one another gives rise to homozygous mutant, heterozygous and homozygous wild type F₂ offspring, such as those used by De Felipe et al. (1998) and Murtra et al. (2000b). Since sibling mice of this generation have different patterns of recombination of 129/sv and C57BL/6 chromosomes, differences between mutant and wild type mice may therefore be caused not only by the targeted mutation but also by heterozygosity at other loci. Furthermore, the transmission of genes at loci closely linked to the targeted mutation will occur preferentially with the mutant allele; these linked background genes may therefore bring about changes in behaviour that are not due to the gene of interest (Gerlai 1996; Lariviere et al. 2001). Since the 129/sv strain exhibits a number of unusual behavioural phenotypes in relation to morphine administration, including deficits in CPP (Dockstader & van der Kooy 2001), such traits may be disproportionally expressed in the mutant mice.

4.2.2. Confirmation of Murtra et al.'s (2000b) findings

The NK1⁻/⁻ mice used in the experiments described in this thesis were derived from the original C57BL/6 × 129/sv line crossed onto the outbred MF1 background (see section 2.3.1). Since much of the work presented is based on Murtra et al.'s (2000b) findings, it was of interest to ensure that mice of this strain exhibit similar behaviours to the original strain before using them to further investigate the relationship between the NK1 receptor and morphine reward processes. Conditioned place preference (CPP) to morphine and the locomotor response to acute morphine were therefore assessed first in these mice. Additionally, the analysis of these behaviours was of use in verifying the specificity of Murtra et al.'s (2000b) findings on deletion of the NK1 receptor. Although the MF1 strain is an outbred, and hence genetically heterogeneous strain, the confirmation of Murtra et al.'s (2000b) behavioural findings in these mice would suggest that the background influences described above are less likely to be the cause of the observed behavioural differences. This approach cannot completely exclude the possibility that loci linked to the mutant gene disproportionately affect the NK1⁻/⁻ mice's behaviour, but testing an additional mouse background strain in this way is a rapid and simple way in which to increase certainty of the original conclusions. Furthermore, the examination of morphine-induced behaviours following genetic dilution of the 129/sv component is desirable, given their unusual behaviour in such tasks (Dockstader & van der Kooy 2001).
4.2.3. Locomotor sensitisation

In addition to confirming the findings of Murtra et al. (2000b), NK1^{-/-} mice of the MF1 background were used to analyse the contribution of NK1 receptors to locomotor sensitisation in response to chronic morphine and cocaine. The repeated administration of drugs of abuse causes a number of adaptive responses within the brain, which may eventually contribute to the development of addiction (see Chapter 1). Locomotor sensitisation is a typical behavioural response to chronic treatment with opiates and psychostimulants, consisting of a progressive and persistent enhancement of a drug's psychomotor properties with repeated, intermittent exposure, which persists even after long periods of withdrawal (Stewart & Badiani 1993).

Sensitisation depends on enhancement of glutamatergic and dopaminergic transmission within the basal forebrain, particularly in the interconnections between the ventral tegmental area (VTA), nucleus accumbens (NAcc), prefrontal cortex (PFC) and amygdala (see Kalivas 1995; Vanderschuren & Kalivas 2000; Wolf 1998). The pathway most often associated with sensitisation is the mesolimbic dopaminergic projection from the VTA to the NAcc, although the projections to the amygdala and PFC are also of importance: both these areas send glutamatergic projections to the NAcc, from which γ-aminobutyric acid (GABA)-ergic projections are sent to the motor circuitry, as well as back to the VTA (Figure 4.1). Indeed, the induction of sensitisation to psychostimulants and opioids is critically dependent upon the enhancement of N-methyl-D-aspartate (NMDA) receptor-mediated glutamate transmission within the VTA, since the co-administration of NMDA receptor antagonists, either systemically or into the VTA itself, blocks the development of sensitisation to amphetamine, cocaine and opioids (e.g. Cador et al. 1999; Jeziorski et al. 1994; Kalivas & Alesdatter 1993; Karler et al. 1989; Li et al. 1999). Similarly, repeated cocaine injections led to increases in NMDA receptor subunit 1 and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor GluR1 subunit levels in the VTA in rats which develop behavioural sensitisation (Churchill et al. 1999), whilst introduction of the GluR1 subunit into the rat VTA using viral-mediated gene transfer brings about an intensification of morphine's stimulant and rewarding properties (Carlezon et al. 1997). In contrast, the expression of a sensitised locomotor response to a drug challenge is dependent upon enhanced dopaminergic transmission within the NAcc, coupled with a hypersensitivity of the mesolimbic dopamine pathway. Supersensitivity of dopamine D1
receptors in the NAcc seems to be of particular importance in this process for both psychostimulants and opioids (e.g. Vanderschuren et al. 1999). Differences in the neurochemical mechanisms underlying sensitisation to different drugs of abuse do exist, including the additional requirement for enhancements in dopaminergic transmission in the induction of sensitisation to amphetamine, but not cocaine or opioids, and a crucial role for glutamate transmission during the expression of sensitisation to cocaine (see Vanderschuren & Kalivas 2000).

Sensitisation is believed to be of importance in the motivational aspects of drug addiction. The incentive-sensitisation theory of drug addiction posits that sensitisation not only occurs to the locomotor effects of drugs of abuse but also in the neural systems which are responsible for the attribution of motivational significance to the act of drug taking – such sensitisation thereby transforms ‘wanting’ the drug to the craving and compulsive drug-seeking behaviour characteristic of drug addiction (Robinson & Berridge 1993). In animal studies, links between locomotor sensitisation and drug-seeking behaviour have been demonstrated. For example, it has been shown in a number of studies that only those drugs that can elicit the expression of locomotor sensitisation in animals pre-exposed to a drug can cause re-instatement of drug-seeking behaviour following a period of abstinence.

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Figure 4.1 Circuitry involved in locomotor sensitisation. Model illustrating the dopaminergic, glutamatergic and GABAergic pathways involved in the induction and expression of behavioural sensitisation. BLA: basolateral nucleus of the amygdala; c: core; d: dorsal; GABA: \( \gamma \)-aminobutyric acid; NAcc: nucleus accumbens; PFC: prefrontal cortex; s: shell; VTA: ventral tegmental area. Adapted from Vanderschuren & Kalivas (2000).
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(De Vries et al. 1998, 1999; Vanderschuren et al. 1999). Furthermore, following longer periods of cocaine self-administration, rats are more sensitive to the motivational effects of cocaine as assessed by cocaine-induced re-instatement of drug-seeking behaviour and cocaine-induced runway behaviour (Deroche et al. 1999). Taken together, these findings support the hypothesis that hyperresponsiveness to drugs plays an important part in drug-seeking behaviour and possibly drug craving. Locomotor sensitisation can therefore be regarded as a simple measure of the adaptive changes which occur during chronic drug exposure, and which are correlated with some of the characteristics of addiction. Since the involvement of NK1 receptors in these processes has not been addressed directly, the effects of chronic morphine and chronic cocaine on locomotor activity in NK1−/− mice were assessed.

4.2.4. Morphine metabolism

Murtra et al. (2000b) found that the expression, distribution, agonist binding properties and activity of the μ-opioid receptor were unaffected by the genetic disruption of the NK1 receptor. However, it remains possible that mice of the two genotypes metabolise morphine differentially and that different circulating levels of the drug or its metabolites following morphine administration may contribute to the observed behavioural differences. In order to address this possibility, the metabolism of morphine was assessed in NK1−/− and wild type mice by measuring plasma levels of the drug and its major metabolites morphine-3β-D-glucuronide (M3G) and morphine-6β-D-glucuronide (M6G) at various times after systemic morphine administration. M6G is itself active, being a more potent agonist of the μ-opioid receptor with a longer duration of action than morphine (Frances et al. 1990; Osborne et al. 2000; Pasternak et al. 1987; Paul et al. 1989). Its analgesic effect is 4–5 times greater than that of morphine when given subcutaneously, and 45–200 times greater when injected into the cerebral ventricles (Abbott & Palmour 1988; Frances et al. 1990; Frances et al. 1992; Shimomura et al. 1971). Similar relative potencies have been observed for morphine-induced CPP (Abbott & Franklin 1991) and hyperlocomotion (Grung et al. 1998; Uchihashi et al. 1996). M3G, however, does not have a high affinity for the μ-opioid receptor (Chen et al. 1991), and does not have analgesic or locomotor stimulant activity in animals (Lipkowski et al. 1994; Morland et al. 1994; Shimomura et al. 1971). It has been suggested that it acts as an antagonist at the μ-opioid receptor and may be important in the
development of tolerance and dependence to morphine (Morland et al. 1994), although a more recent study has suggested that its role in dependence is minor (Salem & Hope 1997). Although a number of studies have failed to observe M6G following morphine administration in mice and rats (Coughtrie et al. 1989; Hasselström et al. 1996; Kuo et al. 1991; Nagano et al. 2000; Salem & Hope 1997), any differences in the production of these metabolites may account for the behavioural observations, at least in part.

4.3. Materials and methods

4.3.1. Morphine conditioned place preference

Adult (6 - 8 weeks) male NK1\(^{-/-}\) (n = 64) and wild type (n = 76) mice from homozygous breeding (see section 2.3.1) were tested for reward to morphine using the CPP paradigm (see section 2.5.3). Fifteen NK1\(^{-/-}\) and 18 wild type mice were excluded from the experiment after exhibiting strong preconditioning preferences for one compartment. The following doses of morphine were used: 0.0 mg.kg\(^{-1}\) (saline; NK1\(^{-/-}\): n = 14; wild type: n = 18); 3.0 mg.kg\(^{-1}\) (NK1\(^{-/-}\): n = 9; wild type: n = 11); 5.0 mg.kg\(^{-1}\) (NK1\(^{-/-}\): n = 7; wild type: n = 11); 7.5 mg.kg\(^{-1}\) (NK1\(^{-/-}\): n = 9; wild type: n = 8); 10.0 mg.kg\(^{-1}\) (NK1\(^{-/-}\): n = 10; wild type: n = 10). Injections were given in a volume of 10.0 ml.kg\(^{-1}\). Mice were tested in groups of 12.

CPP scores were analysed using a 2-way ANOVA with genotype and drug dose as between-subject factors followed by post hoc Tukey comparisons (see section 2.6). Due to considerable variability in the groups treated with saline and 10.0 mg.kg\(^{-1}\) morphine, mice whose CPP scores fell over 2 standard deviations (SDs) from the group mean were excluded from analysis. This involved the exclusion of 1 NK1\(^{-/-}\) mouse (saline group) and 4 wild type mice (three from the saline group and one from the 10.0 mg.kg\(^{-1}\) morphine group).

4.3.2. Morphine-induced locomotion

The locomotor response to acute morphine injection was assessed in adult (6 - 8 weeks) male NK1\(^{-/-}\) (n = 60) and wild type (n = 60) mice from homozygous breeding (see section 2.3.1). The following doses of morphine were used (n = 10 per dose per genotype): 0.0 mg.kg\(^{-1}\) (saline); 3.0 mg.kg\(^{-1}\); 5.0 mg.kg\(^{-1}\); 7.5 mg.kg\(^{-1}\); 10.0 mg.kg\(^{-1}\); 15.0 mg.kg\(^{-1}\). Injections were given in a volume of 6.67 ml.kg\(^{-1}\), 30 min before locomotor activity was monitored for
10 min in small activity boxes using EthoVision (see section 2.5.4.2). Mice were not habituated to the apparatus before testing. All locomotor assessment was carried out between 10.00 am and 1.00 pm. Mice were tested in groups of eight.

Mean speeds were analysed using a 2-way ANOVA with genotype and drug dose as between-subject factors, followed by post hoc Tukey comparisons (see section 2.6). Mice whose mean speeds fell over 2 SDs from the group mean were excluded from analysis. This involved the exclusion of 6 NK1−/− mice (three from the 5.0 mg.kg−1 group, two from the 10.0 mg.kg−1 group and one from the 15.0 mg.kg−1 group) and 2 wild type mice (one from the 3.0 mg.kg−1 group and one from the 10.0 mg.kg−1 group).

4.3.3. Locomotor sensitisation

The sensitisation of locomotor stimulation by morphine and cocaine were assessed in adult (> 7 weeks) male NK1−/− (n = 48) and wild type (n = 48) mice from homozygous breeding (see section 2.3.1). The sensitisation procedure used was modified from that of Martin et al. (2000). Mice were tested for behavioural sensitisation to repeated injections of morphine (n = 19 per genotype), cocaine (n = 10 per genotype) or saline (n = 19 per genotype). Locomotor activity was assessed in small activity boxes (see section 2.5.4.2) between 9.30 and 11.00 am. The protocol for the assessment of sensitisation is illustrated in Figure 4.2.

![Figure 4.2 Sensitisation protocol](image.png)

**Figure 4.2 Sensitisation protocol.** Mice were habituated to the apparatus for 3 d before chronic drug treatment was begun on day 4 for 15 d, during which locomotor activity was measured every 3 d. Following a 7-d drug-free period, maintenance of sensitisation was tested after a single injection on day 26.

The animals were habituated to the test environment on days 1, 2 and 3, in order to obtain a stable baseline. On day 4, chronic treatment with morphine (15.0 mg.kg−1), cocaine (10.0
mg.kg$^{-1}$) or saline was begun for a period of 15 d. The mice received two injections per day: the morning injections were performed between 9.15 am and 10.30 am, and the evening injections between 6.30 pm and 7.30 pm. Injections were given in a volume of 6.67 ml.kg$^{-1}$.

The acute locomotor effects of morphine or cocaine were measured on day 4, 10 minutes after the first injection of morphine, cocaine or saline. Thereafter, during chronic drug treatment, locomotor activity was measured every 3 d (on days 7, 10, 13, 16 and 19), 10 min after the morning injection.

At the end of the chronic drug administration on day 19, the animals did not receive any treatment from days 20 to 25. On day 26, they received a challenge dose of morphine (15.0 mg.kg$^{-1}$), cocaine (10.0 mg.kg$^{-1}$) or saline, and locomotor activity was measured again.

Due to power failure, no recordings of locomotor activity were made from 8 morphine-treated and 8 saline-treated wild type mice on day 7, or any of the morphine-treated wild type mice on day 16. Three morphine-treated wild type mice were removed from the experiment on day 9 following erroneous injection with cocaine. Nine mice of each genotype from the morphine- and saline-treated groups were culled for use in another experiment after locomotor assessment on day 13.

Mean speeds were analysed separately for morphine / saline and cocaine / saline using 3-way repeated-measures ANOVAs with drug and genotype as between-group factors, and day as a within-group factor. Subsequent 1-way ANOVAs with genotype as the between-group factor were calculated for the 3 habituation days, and 2-way ANOVAs were calculated for days 4 to 26 with treatment and genotype as between-group factors, followed by post hoc Tukey comparisons (see section 2.6).

4.3.4. Morphine metabolism

Morphine metabolism was assessed in adult (6 – 8 weeks) male NK1$^{-/-}$ and wild type mice ($n = 20$ per genotype) from homozygous breeding (see section 2.3.1). The plasma levels of morphine and its major metabolites M3G and M6G were measured at various time points after morphine injection using high performance liquid chromatography (HPLC).
Sixteen mice of each genotype were injected with 10.0 mg.kg$^{-1}$ morphine in a volume of 6.67 ml.kg$^{-1}$. After 20, 40, 90 or 150 min ($n = 4$ per genotype per time point), they were terminally anaesthetised in CO$_2$ and exsanguinated. Four mice of each genotype were exsanguinated without morphine injection, in order to assess baseline levels of morphine and its metabolites.

Mice were placed into CO$_2$ chamber and the concentration of CO$_2$ slowly increased until breathing had stopped. They were exsanguinated by cardiac stab, inserting a 21G needle (Monoject®; Kendall, Gosport, UK) attached to a 1 ml syringe (Plastipak™; Becton Dickinson), previously flushed with heparin solution (5000 u.ml$^{-1}$; Monoparin, CP Pharmaceuticals), directly into the heart via the abdominal wall and diaphragm. The heparin flush prevented the blood from clotting. Gentle negative pressure was applied until approximately 1 ml of blood had been drawn.

The blood was immediately transferred into lithium heparin tubes (Teklab, Durham, UK) and centrifuged at 3000 rpm for 12 min, causing separation of plasma from blood cells. The plasma supernatant was carefully transferred to 1.5 ml microfuge tubes (Sarstedt, Nürmbrecht, Germany) and frozen at −80 °C until ready for analysis. Each mouse gave between 400 and 600 µl plasma.

Analysis of morphine and its metabolites was performed by Ines Kastner at the Children Nationwide Research Centre, based at the Institute of Child Health, London. The concentrations of morphine, M3G and M6G were measured simultaneously by solid phase extraction (SPE) followed by reverse-phase ion-paired chromatography with electrochemical and fluorescence detection (Joel et al. 1988). The process consisted of three phases:

- **Extraction.** Plasma samples were purified using a SPEC® disc (C18AR; Ansys Technologies, London, UK) and extraction was carried out using an ASPEC XLI automated sample injector (Gilson, Middleton, USA). SPE disc columns containing 15.0 mg C18AR were conditioned with 500 µl acetonitrile (Sigma) followed by 250 µl water, activating the C18 chains of the bonded phase to enable them to interact with the sample matrix. Plasma samples were diluted 1:1 in 500 mM ammonium sulphate (pH 9.30; Sigma), and 1.00 ml was loaded onto the SPE column. Interfering matrix compounds were removed from the sorbent with
250 μl 5.00 mM ammonium sulphate (pH 9.30; Sigma) followed by 100 μl water. The analyte produced was then desorbed with 220 μl 50.0 mM NaH₂PO₄ (BDH) in 10.0 % methanol (Sigma). 250 μl of this eluate was filtered through a 0.45 μm filter (Millipore, Watford, UK) and injected onto the HPLC column.

- **Chromatography.** Separation of the analytes was achieved using a 250-mm silica-based column (Reverse Phase-Select [5 μm] LiChrospher® 60; BDH/Merck, Leicester, UK) at a temperature of 35.0 °C and a flow rate of 1.00 ml.min⁻¹. The composition of the mobile phase was 50.0 mM NaH₂PO₄ (BDH) + 750 μM sodium dodecyl sulphate (SDS; Sigma) in 20.0 % acetonitrile (pH 2.10; Sigma).

- **Detection.** Morphine and M6G were measured using electrochemical detection with a Coulochem® 5200A detector (ESA, Chelmsford, USA). M3G is not electrochemically active, so was measured using an ultraviolet fluorescence detector (122 UV/VIS Detector; Gilson). Recordings were presented as chromatograms and peak heights calculated using UniPoint™ (Gilson).

Morphine concentrations were analysed separately for NK1⁻/⁻ and wild type mice by fitting a 1-tailed exponential decay curve of concentration against time using GraphPad Prism® Version 3.00 (GraphPad Software, San Diego, USA). Fitted curves were constrained to plateau at a y-value of zero. The rate constants (K) for each genotype were compared using an unpaired Student's t-test. M3G concentrations were compared using a 2-way ANOVA with genotype and time point as between-subject factors (see section 2.6). M6G levels were below the level of detection (< 10 ng.ml⁻¹) in all samples, so were not analysed.

4.4. **Results**

4.4.1. **Morphine conditioned place preference**

Reward to a range of morphine doses from 0.0 to 10.0 mg.kg⁻¹ was assessed in NK1⁻/⁻ and wild type mice using the CPP paradigm. The scores of wild type mice were generally higher than those of NK1⁻/⁻ mice, although at the highest dose tested, the scores obtained by NK1⁻/⁻ mice approached those of wild type mice (see Figure 4.3). A 2-way ANOVA of the CPP scores revealed significant main effects of genotype (F₁,₉₂ = 7.32; P = 0.008) and drug dose (F₄,₉₂ = 4.52; P = 0.002), but the interaction term did not reach statistical significance (F₄,₉₂ = 1.33; P = 0.265). This suggests that the dose of morphine increased
CPP scores in both genotypes, but that wild type mice had persistently greater scores than NK1⁻/⁻ mice across the range of doses tested. *Post hoc* Tukey comparisons failed to reveal any differences between genotypes at any morphine dose (P > 0.4).

Figure 4.3 Morphine CPP. Mean ± SEM CPP scores obtained by NK1⁻/⁻ and wild type mice tested with a range of morphine doses.

### 4.4.2. Morphine-induced locomotion

The locomotor response to acute morphine administration was tested in NK1⁻/⁻ and wild type mice in small activity boxes for 10 min, beginning 30 min after injection. Both groups of mouse exhibited similar levels of locomotor activity at low doses of morphine (0.0 – 10.0 mg.kg⁻¹), but their behaviour became distinct at the highest dose used (15.0 mg.kg⁻¹; Figure 4.4). A 2-way ANOVA revealed significant main effects of genotype (F₁,₁₀₀ = 9.83; P = 0.002) and drug dose (F₅,₁₀₀ = 8.69; P < 0.001), and a significant drug by genotype interaction (F₅,₁₀₀ = 4.90; P < 0.001), suggesting that the two genotypes responded in different ways to increasing morphine dose. *Post hoc* Tukey comparisons revealed that NK1⁻/⁻ mice displayed a significant reduction in locomotor activity relative to vehicle at 10.0 mg.kg⁻¹ (P = 0.013) and 15.0 mg.kg⁻¹ (P < 0.001), and at this highest dose, wild type mice moved at a higher speed than NK1⁻/⁻ mice (P < 0.001). Using these doses and
conditions, a hyperlocomotor effect of acute morphine administration was not detected in either genotype.

![Graph showing morphine-induced locomotion](image)

**Figure 4.4 Morphine-induced locomotion.** Mean ± SEM speeds moved by NK1/− and wild type mice over 10 min, beginning 30 min after injection. **|**<sup>***</sup>P < 0.001 vs. NK1/−; **|**<sup>#</sup>P < 0.05, **|**<sup>###</sup>P < 0.001 vs. vehicle (post hoc Tukey comparisons).

### 4.4.3. Locomotor sensitisation

#### 4.4.3.1. Morphine

On days 1, 2 and 3, mice were exposed to the locomotor activity boxes for 15 min in order to habituate them to the test environment. The locomotor activity of NK1/− and wild type mice did not differ on any of the three days (1-way ANOVAs: P > 0.4). Subsequent chronic administration of morphine (15.0 mg.kg<sup>−1</sup>) caused an increase in locomotor activity in wild type mice, with NK1/− mice moving at similar speeds to those receiving chronic injections of vehicle (Figure 4.5). A 3-way repeated-measures ANOVA with genotype (G), drug treatment (T) and day (D) as factors revealed significant main effects of G and D, and significant G × T, G × D, T × D, and G × T × D interactions (Table 4.1).
Figure 4.5 Locomotor sensitisation to morphine. Mean ± SEM speeds of movement of NK1-/- and wild type mice across habituation, sensitisation and challenge phases of the sensitisation procedure, with 15.0 mg.kg⁻¹ morphine. *P < 0.05, ***P < 0.001 vs. NK1-/-; **P < 0.01; ###P < 0.001 vs. saline (post hoc Tukey comparisons).

Table 4.1 Results of 3-way repeated-measures ANOVA of morphine sensitisation data. Significant P values are underlined.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Error degrees of freedom</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (G)</td>
<td>1</td>
<td>72</td>
<td>18.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drug treatment (T)</td>
<td>1</td>
<td>72</td>
<td>0.83</td>
<td>0.365</td>
</tr>
<tr>
<td>Day (D)</td>
<td>8</td>
<td>478</td>
<td>19.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>G × T</td>
<td>1</td>
<td>73</td>
<td>9.37</td>
<td>0.003</td>
</tr>
<tr>
<td>G × D</td>
<td>8</td>
<td>476</td>
<td>8.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T × D</td>
<td>8</td>
<td>476</td>
<td>10.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>G × T × D</td>
<td>8</td>
<td>476</td>
<td>5.87</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.2 shows the results of 2-way ANOVAs on the speeds of mice recorded on separate days during the experiment. On day 4, acute morphine administration caused a slight
decrease in the locomotor activity of NK1^−/− mice, and a slight increase in the locomotion of wild type mice (Figure 4.5). There was a significant main effect of genotype and a significant genotype by drug interaction. Post hoc Tukey comparisons indicated that wild type mice treated with morphine moved significantly more quickly than morphine-treated NK1^−/− mice (P < 0.001), but not than wild type mice treated with saline (P = 0.878). The difference between the speeds of morphine- and saline-treated NK1^−/− mice narrowly missed statistical significance (P = 0.068), implying that they showed a mild but non-significant inhibition of locomotor activity following acute morphine at 15.0 mg.kg⁻¹.

On days 7, 10 and 13 there was a significant interaction between genotype and drug, indicating a differential effect of morphine on locomotor activity in the two genotypes on these three days. On day 19, there were significant main effects of treatment and genotype, but the interaction term did not reach statistical significance. Post hoc Tukey comparisons revealed significant differences between the speeds moved by morphine-treated NK1^−/− and wild type mice on all 4 days (P < 0.04; Figure 4.5). There were significant differences between the speeds of wild type mice treated with morphine and those treated with saline on days 10 and 13 (P < 0.002), but no significant differences in the locomotor activity induced by saline and morphine in NK1^−/− mice on days 7, 10, 13 or 19 (P > 0.1; Figure 4.5). Taken together, these data indicate that there was a significant locomotor sensitisation to morphine in wild type mice, but that this did not occur in NK1^−/− mice.

One week after finishing chronic morphine treatment, the mice were given a challenge dose of morphine (15.0 mg.kg⁻¹) or saline before locomotor activity was measured, in order to assess the maintenance of sensitisation to morphine’s locomotor effects. Locomotor sensitisation was maintained in morphine-treated wild type mice, but morphine- and saline-treated NK1^−/− mice demonstrated similar speeds of locomotion (Figure 4.5). A 2-way ANOVA demonstrated significant main effects of treatment and genotype on day 26, and a significant genotype by treatment interaction (Table 4.2). Post hoc Tukey comparisons revealed that this interaction was due to significant differences between the locomotor activity of morphine-treated wild type mice and both morphine-treated NK1^−/− mice (P < 0.001) and saline-treated wild type mice (P < 0.001; Figure 4.5). The speeds of morphine- and saline-treated NK1^−/− mice did not differ significantly (P = 0.380).
Table 4.2 Results of 2-way ANOVAs of morphine sensitisation data on days 4 – 26. Significant P values are underlined.

<table>
<thead>
<tr>
<th>Day</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>$F_{1,72} = 11.9$</td>
<td>$F_{1,72} = 1.54$</td>
<td>$F_{1,72} = 5.28$</td>
</tr>
<tr>
<td>7</td>
<td>$F_{1,56} = 12.8$</td>
<td>$F_{1,56} = 0.37$</td>
<td>$F_{1,56} = 8.28$</td>
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<tr>
<td>10</td>
<td>$F_{1,69} = 14.7$</td>
<td>$F_{1,69} = 3.34$</td>
<td>$F_{1,69} = 11.5$</td>
</tr>
<tr>
<td>13</td>
<td>$F_{1,69} = 23.2$</td>
<td>$F_{1,69} = 7.49$</td>
<td>$F_{1,69} = 19.4$</td>
</tr>
<tr>
<td>19</td>
<td>$F_{1,33} = 5.69$</td>
<td>$F_{1,33} = 8.75$</td>
<td>$F_{1,33} = 2.05$</td>
</tr>
<tr>
<td>26</td>
<td>$F_{1,33} = 21.8$</td>
<td>$F_{1,33} = 11.5$</td>
<td>$F_{1,33} = 7.43$</td>
</tr>
</tbody>
</table>

4.4.3.2. Cocaine

On days 1, 2 and 3, mice were exposed to the locomotor activity boxes in order to habituate them to the test environment. The locomotor activity of NK1$^{-/-}$ and wild type mice did not differ on any of the three days (1-way ANOVAs: $P > 0.4$). Subsequent chronic administration of cocaine (10.0 mg.kg$^{-1}$) caused an increase in locomotor activity in both groups of mice after three days of drug treatment (Figure 4.6). A 3-way repeated-measures ANOVA with G, T and D as factors revealed significant main effects of T and D, and significant G $\times$ D and T $\times$ D interactions (Table 4.3).
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*Reward and addiction behaviours in neurokinin-1 receptor knockout mice*

Sensitisation Challenge

$40-1$

$3\,0-##$

Cocaine $+/+$

Cocaine $-/-$

$20$

Cocaine / saline injections

$0$

$10$

$20$

$30$

$40$

Day

**Figure 4.6 Locomotor sensitisation to cocaine.** Mean ± SEM speeds of movement of NK1$^-$ mice and wild type mice across habituation, sensitisation and challenge phases of the sensitisation procedure, with 10.0 mg.kg$^{-1}$ cocaine. $^*P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$ vs. saline (*post hoc* Tukey comparisons).

**Table 4.3 Results of 3-way repeated-measures ANOVA of cocaine sensitisation data.** Significant P values are underlined.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Error degrees of freedom</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (G)</td>
<td>1</td>
<td>54</td>
<td>0.02</td>
<td>0.882</td>
</tr>
<tr>
<td>Drug treatment (T)</td>
<td>1</td>
<td>54</td>
<td>14.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Day (D)</td>
<td>9</td>
<td>425</td>
<td>13.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>G × T</td>
<td>1</td>
<td>54</td>
<td>2.97</td>
<td>0.091</td>
</tr>
<tr>
<td>G × D</td>
<td>9</td>
<td>423</td>
<td>1.99</td>
<td>0.039</td>
</tr>
<tr>
<td>T × D</td>
<td>9</td>
<td>423</td>
<td>20.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>G × T × D</td>
<td>9</td>
<td>423</td>
<td>1.69</td>
<td>0.089</td>
</tr>
</tbody>
</table>

**Table 4.4 shows the results of 2-way ANOVAs on the speeds of mice recorded on separate days during the experiment.** On day 4, acute cocaine administration did not cause any
significant changes in locomotor activity in NK1−/− or wild type mice. However, on days 7, 10, 13, 16 and 19, there were significant main effects of treatment, but no significant main effects of genotype or interactions between treatment and genotype. This suggests that on these recording days, cocaine induced an increase in locomotor activity, which was independent of genotype. Chronic cocaine therefore elicits locomotor sensitisation in both NK1−/− and wild type mice.

One week after finishing chronic cocaine treatment, the mice were given a challenge dose of cocaine (10.0 mg.kg⁻¹) or saline before locomotor activity was measured, in order to assess the maintenance of sensitisation to cocaine's locomotor effects. Both wild type and NK1−/− mice maintained their speeds of day 19, with NK1−/− mice demonstrating a slight increase in locomotor activity. A 2-way ANOVA revealed a significant main effect of treatment, and a significant interaction between treatment and genotype (Table 4.4). Post hoc Tukey comparisons revealed a highly significant difference in locomotor activity between cocaine- and saline-treated NK1−/− mice (P < 0.001; Figure 4.6), but no significant differences between cocaine-treated NK1−/− and wild type mice (P = 0.187), or between cocaine- and saline-treated wild type mice (P = 0.226).

Table 4.4 Results of 2-way ANOVAs of cocaine sensitisation data on days 4 – 26. Significant P values are underlined.

<table>
<thead>
<tr>
<th>Day</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>F₁,₅₄ = 1.01</td>
<td>P = 0.319</td>
<td>F₁,₅₄ = 0.41</td>
</tr>
<tr>
<td>7</td>
<td>F₁,₄₆ = 0.03</td>
<td>P = 0.870</td>
<td>F₁,₄₆ = 17.3</td>
</tr>
<tr>
<td>10</td>
<td>F₁,₅₄ = 0.67</td>
<td>P = 0.417</td>
<td>F₁,₅₄ = 14.8</td>
</tr>
<tr>
<td>13</td>
<td>F₁,₄₄ = 0.14</td>
<td>P = 0.713</td>
<td>F₁,₄₄ = 20.4</td>
</tr>
<tr>
<td>16</td>
<td>F₁,₃₆ = 0.19</td>
<td>P = 0.663</td>
<td>F₁,₃₆ = 24.1</td>
</tr>
<tr>
<td>19</td>
<td>F₁,₃₆ = 0.43</td>
<td>P = 0.518</td>
<td>F₁,₃₆ = 25.7</td>
</tr>
<tr>
<td>26</td>
<td>F₁,₃₆ = 0.63</td>
<td>P = 0.433</td>
<td>F₁,₃₆ = 23.7</td>
</tr>
</tbody>
</table>
4.4.4. Morphine metabolism

Concentrations of morphine and its major metabolites M3G and M6G were measured using HPLC. Baseline plasma concentrations of all three compounds from mice of both genotypes were below the level of detection (morphine: < 5 ng.ml\(^{-1}\); M3G: < 60 ng.ml\(^{-1}\); M6G: < 10 ng.ml\(^{-1}\)). In mice treated with morphine, M6G levels were below the level of detection in all samples, implying that, unlike in man, M6G is not a major metabolite in the mouse.

Injection of morphine caused plasma morphine levels to increase in mice of both genotypes. For both genotypes, the reduction in plasma morphine concentrations over time approximately fitted a one-tailed exponential decay curve (NK1\(^{-/-}\): \(R^2 = 0.642\); wild type: \(R^2 = 0.622\); Figure 4.7A). The rate constants (K) for the two genotypes did not differ significantly (NK1\(^{-/-}\): 0.0308 ± 0.0126 min\(^{-1}\); wild type: 0.0361 ± 0.0119 min\(^{-1}\); unpaired Student’s \(t\)-test: \(T_{30} = 0.304\); \(P = 0.763\)), suggesting that the rate of morphine metabolism was similar in the two genotypes.

Morphine administration also increased plasma concentrations of M3G in both genotypes, to levels approximately 13-fold higher than those of morphine. The concentration of M3G decreased in both genotypes over time (Figure 4.7B). A 2-way ANOVA revealed a significant main effect of time on M3G concentration (\(F_{3,24} = 23.3\); \(P < 0.001\)) but no significant effects of genotype (\(F_{1,24} = 0.27\); \(P = 0.608\)) or time by genotype interaction (\(F_{3,24} = 2.63\); \(P = 0.073\)). Both genotypes therefore rapidly metabolised morphine to M3G, which itself was metabolised at similar rates in the two groups of mice.
Figure 4.7 Morphine metabolism. A: Mean ± SEM morphine concentrations in plasma of NK1⁻/⁻ and wild type mice taken at various time points after injection of morphine, and best-fit one-tailed exponential decay curves. B: Mean ± SEM M3G concentrations in plasma of NK1⁻/⁻ and wild type mice taken at various time points after injection of morphine. Note that y-axis scales are different in the two plots.

4.5. Discussion

The behavioural and biochemical studies presented in this chapter examined the responses of NK1⁻/⁻ mice to morphine and cocaine. The analysis of CPP and locomotor in response to morphine were carried out in order to confirm the findings of Murtra et al. (2000b) in mice of a different background strain, whilst the examination of locomotor sensitisation and morphine metabolism were novel investigations.

4.5.1. Morphine conditioned place preference

CPP to morphine was tested in a variety of doses from 3.0 to 10.0 mg.kg⁻¹, as well as a vehicle (saline) control. The CPP scores achieved by the mice increased with morphine dose in both genotypes, but the dose-response curve of the NK1⁻/⁻ mice was shifted to the right, suggesting that they are less sensitive to the rewarding properties of morphine than wild type mice at the doses tested. This finding is slightly different to that of Murtra et al. (2000b), who claimed to see a complete absence of morphine reward in NK1⁻/⁻ mice. This conclusion was drawn from the CPP scores obtained at a morphine dose of 3 mg.kg⁻¹: higher doses of morphine were not tested. In the present experiment, 3.0 mg.kg⁻¹ morphine induced a small separation of the two genotypes’ CPP scores, but this difference was more pronounced at higher doses, implying that the mice used in this experiment (original C57BL/6 × 129/sv strain crossed onto the MF1 background) are less sensitive to the rewarding properties of morphine than the original strain. Despite this difference in
sensitivity of the background strains, the present results support Murtra et al.'s (2000b) conclusion that substance P (SP) and the NK1 receptor play an important role in the reward to morphine, and possibly other opiates. However, they also suggest that the genetic deletion of the NK1 receptor does not bring about a complete insensitivity to morphine reward, but rather a reduction in sensitivity. At higher doses of morphine, SP and the NK1 receptor may not be crucial for morphine reward behaviours, but alternative neural mechanisms may come into play in mediating reward-related behaviours in these mice. Identifying the nature of such processes would require further study.

4.5.2. Morphine-induced locomotion

In the analysis of locomotor behaviour induced by acute morphine injections, an inhibition of locomotor activity was observed in NK1\(^{-/-}\) mice at doses of 10.0 and 15.0 mg.kg\(^{-1}\), with the locomotor behaviour of the two genotypes differing significantly at the higher dose. No stimulant effect of morphine was observed in either genotype at any of the doses tested. At similar doses to these, Murtra et al. (2000b) did see a stimulant effect of morphine in wild type mice, along with a similar inhibition of locomotor activity in NK1\(^{-/-}\) mice. They also observed that the locomotor activity of NK1\(^{-/-}\) mice recovered to the level of saline controls at the higher dose of 20.0 mg.kg\(^{-1}\). The reasons for the discrepancy between the present findings and those of Murtra et al. (2000b) could lie either in the experimental procedure, or in the genetic background of the mice used. In the present study, the mice were observed in small boxes, but Murtra et al. (2000b) tested their mice in an open field: the small activity boxes used here may not have favoured larger, ambulatory movements, thereby reducing the measured locomotor scores. More likely, however, the difference in genetic background of the mice used in the two experiments may explain the difference. As in the CPP experiments discussed above, the strain used here seem to display a lower sensitivity to the effects of morphine than those used by Murtra et al. (2000b), with a rightward shift in the dose-response curves for both behavioural paradigms. It is postulated that use of a higher dose of morphine may indeed have brought about locomotor stimulation in wild type mice of the MF1 strain, thereby reflecting the observations seen by Murtra et al. (2000b) at the doses of 10.0 and 20.0 mg.kg\(^{-1}\). The present data do support a role for SP and the NK1 receptor in morphine's locomotor effects, thereby supporting Murtra et al.'s (2000b) findings: importantly, the confirmation of
these findings in mice of a different genetic background renders the potential confounding influence of genetic elements other than the gene for the NK1 receptor in mediating the behavioural differences between NK1$^{-/-}$ and wild type mice less likely (Gerlai 1996).

Inter-strain differences in the effects of morphine and other drugs such as those observed here are well documented. Although there is no published data on the MF1 strain per se, other mouse strains have been shown to exhibit different degrees of morphine-induced behaviours, including CPP (Cunningham et al. 1992; Dockstader & van der Kooy 2001; Semenova et al. 1995), locomotor activity (Belknap et al. 1989, 1998; Brase et al. 1977; Castellano & Oliverio 1975; Moskowitz et al. 1985; Muraki et al. 1982, 1984; Muraki & Kato 1985; Shuster et al. 1975), self-administration (Semenova et al. 1995), dependence (Brase et al. 1977), oral morphine preference (Belknap 1990; Belknap et al. 1993; Berrettini et al. 1994; Horowitz et al. 1977), and analgesic tolerance (Kest et al. 2002), as well as analgesia, hypothermia, respiratory depression, antidiuresis, muscular rigidity, constipation and mortality (Belknap et al. 1989; Brase et al. 1977; Castellano & Oliverio 1975; Elmer et al. 1998; Gebhart & Mitchell 1973; Moskowitz et al. 1985; Muraki et al. 1984; Muraki & Kato 1986, 1987; Semenova et al. 1995; Shuster et al. 1975). The genetic basis of these differences is probably complex and multifactorial, and is beyond the scope of this discussion.

The lack of an observable difference in locomotor activity following morphine administration between genotypes at doses of between 0.0 and 10.0 mg.kg$^{-1}$ adds weight to the reliability of the CPP findings. Although the measurement of locomotor activity was made at 30 min after injection and the conditioning sessions of the CPP paradigm takes place over the 20 min immediately following drug injection, it is unlikely that the two genotypes exhibited gross differences in locomotion during the conditioning phase of the CPP paradigm. Differences in drug-induced locomotor activity between genotypes are unlikely to have interfered with conditioning.

4.5.3. Locomotor sensitisation

In the test of locomotor sensitisation, the involvement of the NK1 receptor in the adaptive responses to repeated exposure to morphine and cocaine were assessed. The chronic administration of opiates or psychostimulant drugs tends to cause a gradual enhancement
of both the stimulant and rewarding properties of that drug. This response is believed to be due to an enhancement of dopaminergic and glutamatergic transmission within the forebrain, especially in the connections between the VTA, NAcc, prefrontal cortex and amygdala (see Figure 4.1; Kalivas 1995; Vanderschuren & Kalivas 2000; Wolf 1998). These changes are analogous to the increases in motivational aspects of drugs of abuse that occur during the development of addiction, such as reinstatement of compulsive drug-seeking behaviour and craving (De Vries et al. 1998, 1999; Deroche et al. 1999; Vanderschuren et al. 1999).

In this study, acute administration of morphine caused a slight reduction in the locomotor activity of NK1−/− mice: their speed of movement differed significantly from that of morphine-treated wild type mice, but not NK1−/− saline controls. This finding is similar to that observed in mice which were not habituated to the test apparatus, at the same dose (see above). However, chronic morphine treatment caused a dramatic increase in the speed of locomotion of wild type mice, while the locomotor activity of NK1−/− mice remained around the level of saline-treated animals. Following a week's abstinence, the maintenance of sensitisation was tested with a challenge dose of morphine. Sensitisation was maintained in wild type mice, and NK1−/− mice continued to behave in a similar manner to vehicle controls. These data strongly indicate that the activation of NK1 receptors is necessary for the normal development of locomotor sensitisation to morphine. Cocaine, on the other hand, had similar effects in both genotypes. Although acute administration of cocaine did not affect locomotor activity in either genotype, subsequent chronic administration of cocaine caused a similar locomotor sensitisation in both genotypes, which was maintained over a one-week drug-free period.

These results extend those of Murtra et al. (2000b), strongly suggesting that the receptor for SP is involved not only in the acute rewarding and stimulant effects of morphine, but also in the long-term alterations in behaviour accompanying chronic morphine exposure. In addition to emphasising the differences in the neural mechanisms underlying opiate- and psychostimulant-induced motivational responses, they suggest that the NK1 receptor is critical for the alterations in motivational aspects of opiates contributing towards the development of addiction. However, in the light of the CPP observations presented here, it may be the case that NK1−/− mice are less sensitive to morphine, and that utilisation of a higher dose of morphine could actually bring about some form of behavioural sensitisation.
Although this possibility remains to be tested explicitly, it does not detract from the conclusion that the NK1 receptor plays an important role in the behavioural response to morphine reward and addiction, but that alternative mechanisms may be in place that can mediate these behaviours at higher doses. This phenomenon has already been described in the NK1⁻⁄ mouse, since their reduced analgesic response to morphine in the tail-flick test is overcome at higher morphine doses (De Felipe et al. 1998).

Whether NK1 receptors are necessary for the development of sensitisation to locomotor sensitisation to morphine, or for the expression of a sensitised response, is not clear from this study. One way in which this question could be addressed is by examining the response of NK1⁻⁄ mice in a cross-sensitisation paradigm. Cross-sensitisation is the phenomenon whereby the repeated administration of one drug can induce an enhanced locomotor response to another, due to the shared neurochemical processes occurring in response to chronic administration of different classes of drug (Bonate et al. 1997; Cunningham et al. 1997; Hirabayashi & Tadokoro 1992; Kalivas & Weber 1988; Schenk et al. 1991; Shuster et al. 1977; Vanderschuren et al. 1997; Vezina et al. 1989). If repeated morphine administration brings about a cross-sensitisation to a challenge dose of cocaine in NK1⁻⁄ mice it is likely that the expression of morphine sensitisation is disrupted by the absence of NK1 receptors. However, if these mice fail to cross-sensitise to cocaine, it is more likely that the acquisition of sensitisation is disrupted, and dependent upon NK1 receptor activation.

4.5.4. Morphine metabolism

The metabolism of morphine in NK1⁻⁄ mice was assessed by examining the concentrations of morphine and its metabolites M3G and M6G in plasma at various times after injection. There was no detectable difference in the rate of morphine metabolism between genotypes, and both groups of mice seemed to metabolise the drug via M3G, with no detectable M6G in any sample. This is in agreement with published observations of morphine metabolism in mice and rats, in which M6G is only produced at low levels (Coughtrie et al. 1989; Hasselström et al. 1996; Kuo et al. 1991; Nagano et al. 2000; Salem & Hope 1997). The observed differences in morphine-mediated behaviour cannot therefore be attributed to differences in circulating morphine concentrations, or that of its metabolites, thereby
extending the conclusions of Murtra et al. (2000b), who found no differences in the
distribution, number, affinity or activity of the μ-opioid receptor.

4.5.5. Comparison with cannabinoid-1 receptor knockout mice

The results from the NK1<sup>−/−</sup> mouse are strikingly similar to those found in mice lacking
cannabinoid-1 (CB1) receptors. Like NK1<sup>−/−</sup> mice, these mice do not show CPP to
morphine, but the response to cocaine is normal (Martin et al. 2000). CB1 knockout mice
do not self-administer morphine, as has also been observed in NK1<sup>−/−</sup> mice (Ripley et al.
2002; see Appendix), and they do not experience many of the somatic responses to
morphine withdrawal (Ledent et al. 1999). Additionally, although CB1 knockout mice
display normal acute hyperlocomotor responses to morphine, locomotor sensitisation to
morphine is absent, whereas cocaine sensitisation is normal (Martin et al. 2000). The
interaction between the opioid and cannabinoid systems is hypothesised to be due to
interaction or competition between opioid and cannabinoid receptors at the level of signal
transduction cascades. Neurones coexpressing these receptors are found within a number
of brain areas associated with motivational behaviours, within the limbic system and
periaqueductal grey (Martin et al. 2000).

4.5.6. Possible mechanisms for the neurokinin-1 receptor's action

The exact mechanisms by which NK1 receptors participate in the affective aspects of
opiates remain unclear. NK1 receptors are expressed in many areas of the brain associated
with opiate reward and addiction processes, such as the NAcc and amygdala subnuclei (see
Chapter 3). One area in which the expression of the NK1 receptor and the μ-opioid
receptor overlap is the locus coeruleus, the major supplier of noradrenergic input to the
forebrain, which has been implicated in the development of opiate dependence, as well as
stress and arousal (Nestler et al. 1993, 1994, 1999; Nestler & Aghajanian 1997). Since both
μ-opioid and NK1 receptors are localised on these cells' somata (Chen et al. 2000; Moyse et
al. 1997; Santarelli et al. 2001), it is possible that the alterations in noradrenaline release
within the forebrain in NK1<sup>−/−</sup> mice could affect the motivational responses to opiate
administration: noradrenaline has been implicated in the motivational properties of opiates,
including sensitisation to their locomotor effects (Airio & Ahtee 1997).
Although the noradrenergic system is one plausible candidate for the behavioural changes observed in NK1 knockout mice, other systems could be involved. The dopaminergic input from the VTA to the NAcc is critical for the expression of sensitisation to both psychostimulants and opioids (Vanderschuren & Kalivas 2000), and administration of the SP amino terminal fragment, SP_{1-7}, which acts at NK1 receptors, into the VTA modulates turnover of dopamine in the NAcc during morphine withdrawal (Zhou & Nyberg 2002). A lack of NK1 receptors in the VTA or on the cholinergic interneurones of the NAcc in the NK1^{-/-} mice could perturb dopaminergic transmission between these nuclei and bring about changes in behaviour as observed here. Although it has been reported that microinjections of SP into the ventral pallidum increase locomotor activity (Kalivas et al. 1993), and may support place preference conditioning (Hasenohrl et al. 1998a; Holzhäuer-Oitzl et al. 1988), and elevate dopamine levels in the NAcc (Boix et al. 1995), it is unlikely that this system represents a site for a direct interaction between the NK1 receptor and the µ-opioid receptor, since intracranial injections into the ventral pallidum of cocaine (Gong et al. 1996), but not of morphine (Olmstead & Franklin 1997), support place preference conditioning.

An additional candidate is the 5-hydroxytryptamine (5HT) system. 5HT transmission is known to be enhanced in the dorsal raphe nucleus following pharmacological or genetic disruption of the NK1 receptor through 5HT_{1A} receptor desensitisation (Froger et al. 2001; Haddjeri & Blier 2001; Santarelli et al. 2001). Although this is known to have no effects on the basal concentrations of extracellular 5HT in the forebrain, sensitisation to morphine can be blocked by pre-treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Sills & Fletcher 1997), suggesting a possible link between NK1 receptor disruption and the loss of the adaptive response to morphine.

Whereas morphine reward, withdrawal and addiction behaviours are disrupted in NK1^{-/-} mice, all cocaine-related behaviours studied are unaffected. It is therefore difficult to reconcile these behavioural differences solely with global alterations in monoamine function. Although, in contrast to psychostimulants, opiates additionally achieve reinforcing effects through actions in dorsal hippocampus, periaqueductal grey and lateral hypothalamus (Corrigall & Linseman 1988; David & Cazala 1994; McBride et al. 1999; Olmstead & Franklin 1997; van der Kooy et al. 1982), the observation that CPP to amphetamine is also impaired in mice lacking NK1 receptors (Murtra et al. 2000a), suggests
that the dissociation of the NK1 receptor's effects does not fall simply between psychostimulants and opiates. Only a few studies have reported similar dissociations between the behavioural effects of morphine and amphetamine and those of cocaine. Firstly, ibotenic acid lesions of the rat tegmental pedunculopontine nucleus abolish CPP to both morphine and amphetamine but leave cocaine CPP intact, at least in non-dependent animals (Bechara & van der Kooy 1989; Parker & van der Kooy 1995). Conversely, quinolinic acid lesions of the prelimbic area of the medial prefrontal cortex abolish CPP to cocaine, leaving responses to morphine and amphetamine intact (Tzschentke & Schmidt 1998). Although both of these areas have only weak NK1 receptor immunoreactivity (see Chapter 3), these findings confirm that separate neural pathways mediate the motivational properties of these groups of drugs. The behavioural observations in NK1⁻/⁻ mice suggest that substance P and the NK1 receptor may represent a further aspect of this separation, although further work is required to identify the nature of their participation in reward and addiction behaviours.

4.6. Conclusions

The results presented here confirm the behavioural findings of Murtra et al. (2000b) in a different background strain of mouse. However, they suggest that, although the NK1 receptor is important in the rewarding and stimulant aspects of morphine, it may not be crucial for them at higher doses of the drug. NK1⁻/⁻ mice also fail to sensitise to the locomotor stimulant effects of morphine, but not cocaine, suggesting that SP and the NK1 receptor may be important in the development of opiate addiction. These observations are not confounded by alterations in morphine metabolism in NK1⁻/⁻ mice. NK1 receptor antagonists may therefore offer a powerful new approach to the management of opiate abuse and addiction, in addition to their known effectiveness in alleviating stress, anxiety and depression.
CHAPTER FIVE

ANALYSIS OF C-FOS EXPRESSION IN THE BRAINS OF NEUROKININ-1 RECEPTOR KNOCKOUT MICE FOLLOWING MORPHINE TREATMENT AND CONDITIONED PLACE PREFERENCE
5. ANALYSIS OF C-FOS EXPRESSION IN THE BRAINS OF NEUROKININ-1 RECEPTOR KNOCKOUT MICE FOLLOWING MORPHINE TREATMENT AND CONDITIONED PLACE PREFERENCE

5.1. Introduction

In an attempt to identify some of the brain regions in which substance P (SP) and the neurokinin-1 (NK1) receptor exert their actions in the mediation of morphine reward behaviours, a set of experiments was carried out in which the expression of the immediate early gene (IEG) c-fos in the brains of NK1 knockout (NK1−/−) mice was analysed. The effects of acute or chronic morphine administration and the morphine conditioned place preference (CPP) procedure on the pattern of c-Fos protein expression were compared between wild type and NK1−/− mice, giving some clues as to the brain regions in which perturbations in neuronal activity occur in mice lacking NK1 receptors. These findings are discussed in relation to previous work.

5.2. Background

The behavioural observations of a lack of morphine reward- and addiction-related behaviours in NK1−/− mice provide clear evidence for a role of SP and its receptor in morphine, and possibly opiate abuse and addiction (Murtra et al. 2000b; Ripley et al. 2002; Chapter 4). However, the analysis of these mice's behaviour gives little evidence as to the location of this effect, since they lack NK1 receptors throughout the body, and throughout life. It is a reasonable assumption that these effects are occurring within the brain, since the behaviours studied (CPP, self-administration and locomotor sensitisation) depend upon the integrity of brain structures, and the NK1 receptor is expressed in many of the same brain regions which are involved.

5.2.1. C-Fos

One way in which the differences in activation of brain regions can be assessed is by the analysis of the expression of IEGs. These genes' products are transcription factors, which can couple short-term environmental signals received at the cell surface to longer-term alterations in a cell's phenotype by regulating the expression of specific target genes. IEGs
Chapter five

Analysis of c-Fos expression in the brains of neurokinin-1 receptor knockout mice

such as c-fos and c-jun tend to be activated within minutes of trans-synaptic stimulation (Greenberg et al. 1985; Greenberg & Ziff 1984). C-Fos, the protein product of the c-fos gene, forms a dimer with c-Jun, to produce an AP-1 binding complex, which, in neurones, can alter the transcription of genes such as those encoding tyrosine hydroxylase, the D1 dopamine receptor, subunit 1 of the N-methyl-D-aspartate (NMDA) receptor and protein kinase A (Nye & Nestler 1996). C-Fos expression can therefore mediate activity-dependent changes in neuronal structure and function (see Kovacs 1998; Morgan & Curran 1991; Pennypacker et al. 1994; Pennypacker 1998; Sheng & Greenberg 1990).

C-Fos expression is induced by a number of different intracellular signalling cascades, including the diacylglycerol-protein kinase C (Greenberg et al. 1986), cyclic adenosine monophosphate (cAMP; Greenberg et al. 1985) and calcium-calmodulin pathways (Morgan & Curran 1986). Since it acts as a site of convergence of these and possibly other pathways, c-Fos is expressed in the majority of neurones throughout the brain in response to trans-synaptic stimulation. C-Fos is expressed at low levels in most unstimulated neurones and has a short half-life of around two hours (Curran et al. 1984; Müller et al. 1984). A difference in the expression levels of c-Fos between two treatments can therefore be used as a marker of differential neuronal activity within a relatively specific timeframe, allowing the expression of c-Fos mRNA or protein to be used to ‘map’ regions of differential activity with cellular resolution (Hunt et al. 1987; Morgan et al. 1987; Sagar et al. 1988). C-Fos has been used in this way in a wide range of studies, including those analysing the effects of drug stimuli on specific brain regions (see Harlan & Garcia 1998).

5.2.2. Effects of morphine administration on c-Fos expression

In the rat, single systemic injections of morphine have been shown to bring about an upregulation of c-Fos in the neurones of a number of brain regions. These include the nucleus accumbens (NAcc), especially the shell subregion (Curran et al. 1996; Garcia et al. 1995; Liu et al. 1994; Nye & Nestler 1996), the lateral septal nucleus (Erdtmann-Vourliotis et al. 1998, 1999a,b; Liu et al. 1994), and the caudate putamen (CPu; Chang et al. 1988), notably in its dorsomedial portion (Bontempi & Sharp 1997; Garcia et al. 1995; Gutstein et al. 1998; Harlan et al. 2001; Liu et al. 1994; Nye & Nestler 1996). The upregulation in the NAcc and CPu is dependent upon D1 dopamine receptor and NMDA glutamate receptor activation, and is regulated by µ-opioid receptors located in the substantia nigra and the
ventral tegmental area (VTA). These findings have supported the suggestion that the morphine-induced upregulation of c-Fos in striatal neurones is mediated by a disinhibition of nigrostriatal and mesolimbic dopaminergic projections to the striatum following μ-opioid receptor-mediated inhibition of γ-aminobutyric acid (GABA)-ergic interneurones. This disinhibition leads to the release of dopamine in the striatum, activating D1 dopamine receptors, which, coupled with coactivation from glutamate, possibly from cortical or thalamic regions, induces the expression of c-Fos (Bontempi & Sharp 1997; Frankel et al. 1999; Liu et al. 1994).

More caudally in the brain, there have been reports of c-Fos upregulation in response to acute morphine administration in the thalamus (midline-intralaminar and paraventricular nuclei; Garcia et al. 1995; Gutstein et al. 1998; Harlan et al. 2001), the hypothalamus, especially ventromedial, paraventricular and supraoptic nuclei (Chang & Harlan 1990; Laorden et al. 2002), the periaqueductal grey, the inferior and superior colliculi, the lateral parabrachial nucleus (Gutstein et al. 1998), various brainstem nuclei (Gutstein et al. 1998; Laorden et al. 2002) and the parietal (somatosensory) and insular cortices (Garcia et al. 1995). Acute morphine injection has also been shown to bring about a decrease in the expression of c-Fos in the locus coeruleus (Hayward et al. 1990). In the guinea pig, intracerebroventricular infusion of morphine brought about an increase in the expression of c-Fos in a wide range of brain regions in a μ-opioid receptor-dependent manner. This included those observed following systemic injections in the rat, but with the addition of the bed nucleus of the stria terminalis, the medial nucleus of the amygdala, the CA1 region of the hippocampus, the dentate gyrus, the paraventricular and medial geniculate nuclei of the thalamus, the habenular nuclei and the piriform and entorhinal cortices (Bot & Chahl 1996).

The chronic administration of opiates brings about a different pattern of c-Fos expression to an acute dose. Reports have indicated that whilst c-Fos is expressed in the NAcc, dorsomedial CPu and lateral septal nucleus following repeated exposure to morphine (Erdtmann-Vourliotis et al. 1998, 1999a; Nye & Nestler 1996), it is also observed in anterior portions of the thalamus, the medial mammillary nuclei and cingulate and somatosensory cortices (Curran et al. 1996; Erdtmann-Vourliotis et al. 1998, 1999a). However, relative to the expression induced by single morphine injections, decreases have been reported in the
paraventricular and supraoptic nuclei of the hypothalamus (Chang et al. 1996; Laorden et al. 2002), whilst the reduction in expression observed with acute morphine relative to vehicle controls in the locus coeruleus is maintained following chronic morphine exposure (Hayward et al. 1990).

5.2.3. Effects of drug-conditioned stimuli on c-Fos expression

In addition to its regulation by non-contingent administration of drugs of abuse, the expression of c-Fos in response to exposure to an environment previously associated with drug administration has revealed some clues as to the regions of the brain which are activated by drug-conditioned stimuli, and which may be important in the reinstatement of drug-seeking behaviours and drug craving, as well as in performance in the CPP task. For example, rats given morphine once a day for ten days in a specific environment showed conditioned behavioural activation when re-exposed to that environment with only a mock injection of vehicle. This conditioned activity was paralleled by an upregulation of c-Fos relative to saline-treated control rats in a number of cortical regions and components of the limbic system, such as the prefrontal and cingulate cortices, the NAcc shell and the preoptic area of the hypothalamus. This upregulation was not due to the increase in locomotor activity per se, as its intensity was not correlated with locomotion; it was therefore due to the drug-associated conditioning itself. Trends for an upregulation were also observed in the NAcc core, the lateral septal nucleus and the basolateral nucleus of the amygdala, although these did not reach statistical significance (Schroeder et al. 2000). The upregulation of c-Fos in cortical areas and the amygdala has also been observed following exposure to an environment previously associated with amphetamine (Mead et al. 1999) and cocaine (Brown et al. 1992; Neisewander et al. 2000). These areas also are activated in human cocaine abusers when presented with drug-exposed stimuli which induce craving (Childress et al. 1999; Grant et al. 1996; Maas et al. 1998), suggesting that the prefrontal and cingulate cortices and the amygdala are involved in the emotional responses to drug-associated cues.

In a similar study examining the phenomenon of associative tolerance to morphine's analgesic effects, rats were given alternating daily injections of morphine and saline in distinct environments over twelve days. An upregulation of c-Fos was observed in the lateral and basolateral nuclei of the amygdala when these rats were given an injection of
morphine in the morphine-associated environment, but not when given saline in the saline-associated environment. In the CA1 region of the hippocampus an upregulation was observed after both treatments relative to rats given the same amount of morphine in a non-contingent manner (Mitchell et al. 2000). These brain regions, especially the amygdala, therefore participate in processing and assigning value to drug-associated environmental stimuli, and may contribute to associative tolerance. Given the similarity in the requirements of this task to those of CPP, it is possible that similar mechanisms are acting in the association of environmental cues with both the conditioned analgesic and conditioned rewarding properties of morphine, as well as in the conditioned locomotor properties described above. Although there are no published studies of the expression of c-Fos during CPP to morphine, it has been demonstrated that suppression of c-fos translation using an antisense oligonucleotide prevents morphine CPP when injected into the NAcc before the morphine injections in the conditioning phase of the task. Conversely, there was no effect of c-Fos suppression when the oligonucleotide was injected before the test session (Tolliver et al. 2000). C-Fos is therefore not only a useful marker of neuronal activity but at least in the NAcc, it also plays a role in the acquisition of the CPP procedure itself. This work also indicates that different neural mechanisms are at work during the acquisition and expression of drug-conditioned behaviours, with accumbal c-Fos expression being crucial for the plastic changes required to learn the CPP task, but activation in other areas, possibly including the amygdala and cortical regions, being important for the expression of conditioned responses, including the approach behaviour characteristic of the test session of the CPP task.

5.2.4. Links between the neurokinin-1 receptor and c-Fos expression

The aim of the experiments described in this chapter was to probe the brains of NK1−/− mice for alterations in c-Fos expression following morphine administration or the morphine CPP procedure, in order to identify regions of the brain that are activated differentially between wild type mice and those lacking NK1 receptors. A number of studies have examined the relationship between the NK1 receptor and the expression of c-Fos, although they are primarily concerned with the receptor's involvement in pain processing. NK1 receptor antagonists have been shown to bring about reductions in the expression of c-Fos in the rat spinal cord and trigeminal nucleus caudalis following noxious
peripheral stimulation (Bereiter et al. 1998; Chapman et al. 1996; Cutrer et al. 1995; Shepheard et al. 1995; Tao et al. 1997). Subcutaneous injection of formalin also induced c-Fos expression in a range of brain regions. This upregulation was blunted following intracerebroventricular pretreatment with the NK1 receptor antagonist RP 67 580 in the prefrontal cortex, the dorsomedial, ventromedial and paraventricular nuclei of the hypothalamus, the periaqueductal grey and the locus coeruleus, but not in the septohypothalamic nucleus, the medial thalamus, the central nucleus of the amygdala or the parabrachial nucleus (Baulmann et al. 2000). The same antagonist, as well as L 760 735, has also been shown to reduce the induction in c-Fos expression in the locus coeruleus brought about by restraint stress (Hahn & Bannon 1999), suggesting that NK1 receptor antagonists can reduce the activation of neurones in areas of the brain and spinal cord which are normally activated by pain and stressful stimuli.

In the present study, mice of both genotypes were treated with an acute injection of morphine, or a series of morphine injections of increasing dose over five days. A further group of mice underwent the morphine CPP paradigm. The number of immunohistochemically labelled c-Fos-positive neurones was analysed in a range of brain regions. While this study was not exhaustive, a number of candidate brain regions were analysed quantitatively: the NAcc (core and shell subregions); the dorsomedial CPu; the amygdala (central and basolateral nuclei); the motor and somatosensory cortices and the locus coeruleus. In the mice undergoing morphine CPP the hippocampus (CA1 and CA3 regions), the dentate gyrus, the VTA and the dorsal raphe nucleus were also analysed quantitatively. These regions were chosen on the basis of their known roles in drug reward (the NAcc, the dorsomedial CPu, the amygdala and the VTA; see Chapter 1), previous c-Fos studies in the rat and guinea pig indicating alterations in c-Fos expression by acute or chronic morphine or drug-associated stimuli (the NAcc, the dorsomedial CPu, the amygdala, the somatosensory cortex and the locus coeruleus), the results of a preliminary study examining the expression of c-Fos in response to acute and chronic morphine in the mouse (the motor and somatosensory cortices) and the effects of NK1 receptor antagonists on c-Fos expression in the brain (the locus coeruleus). The hippocampus, including the dentate gyrus, was analysed following morphine CPP on the basis of this regions’ known role in the formation of contextual associations (Anagnostaras et al. 2001; Fanselow 2000), which may be central to performance on the CPP task (Bardo & Bevins
Finally, the dorsal raphe nucleus was analysed because of the desensitisation of presynaptic 5HT_1A receptors observed in this region in NK1^{-/} mice (Froger et al. 2001; Santarelli et al. 2001), which may be of importance in mediating the behavioural differences between genotypes. Additionally, most of these areas are regions with moderate expression of NK1 receptors in the mouse (see Chapter 3), and may be important loci for the NK1 receptor's effects in morphine reward behaviours. Lastly, the relationship of the NK1 receptor to c-Fos was examined in a double-labelling immunohistochemical study, in order to assess the degree to which c-Fos is expressed in NK1 receptor-expressing neurones in response to morphine and the CPP paradigm.

5.3. Materials and methods

Some of the work described in this chapter was carried out by two students whom I supervised in the laboratory. Catherine Hall, a rotation student on the Wellcome Trust Four-year PhD in Neuroscience scheme carried out approximately 60% of the treatment, single-labelling immunohistochemistry (IHC) and c-Fos counting of the morphine CPP experiment described in section 5.3.2.3. Magdalen Gana, an Intercalated BSc student, carried out approximately 55% of the double-labelling IHC described in section 5.3.3.2.

5.3.1. Mice

Adult (4 - 6 weeks) male NK1^{-/} and wild type mice (n = 30 per genotype) from homozygous breeding were used in this experiment (see section 2.3.1).

5.3.2. Treatment protocols

5.3.2.1. Acute morphine

Mice (n = 4 per genotype) were given a single injection of 10.0 mg.kg^{-1} morphine in a volume of 6.67 ml.kg^{-1} (Figure 5.1). Control animals (n = 4 per genotype) received an equivalent volume of saline. Mice were returned to the home cage after injection. They were perfused 2 h after injection (see section 2.4.1).
5.3.2.2. Chronic morphine

Mice (n = 4 per genotype) received two injections of morphine per day, one between 10.00 and 11.00 am and the other between 6.30 and 7.30 pm, according to the following schedule, adapted from that of Murtra et al. (2000b): day 1: 10 mg.kg$^{-1}$, 20 mg.kg$^{-1}$; day 2: 40 mg.kg$^{-1}$ × 2; day 3: 60 mg.kg$^{-1}$ × 2; day 4: 80 mg.kg$^{-1}$ × 2; day 5: 100 mg.kg$^{-1}$ (Figure 5.2). Injections were given in a volume of 6.67 ml.kg$^{-1}$. Control mice (n = 4 per genotype) received equivalent volumes of saline. The mice were returned to the home cage after injections. They were perfused 2 h after the last injection (see section 2.4.1).

Figure 5.2 Protocol for c-Fos experiment: chronic morphine. Mice were injected twice daily with increasing doses of morphine or with saline for 4 d. On the fifth day, they were perfused 2 h after the last injection.

5.3.2.3. Morphine conditioned place preference

Mice (n = 4 per genotype) underwent the CPP procedure as described in section 2.5.3 with 7.5 mg.kg$^{-1}$ morphine in a volume of 10.0 ml.kg$^{-1}$. This dose was chosen on the basis of performance in the test session of the CPP procedure, bringing about the clearest separation of CPP scores between the two genotypes (see Chapter 4). They were perfused 2 h after the beginning of the test session (see section 2.4.1).
There were three groups of control mice:

- **Saline CPP**: In order to control for the effects of injection, handling and exposure to the CPP apparatus, mice \( n = 4 \) per genotype underwent the CPP procedure with an equivalent volume of saline replacing morphine during conditioning. They were perfused 2 h after the beginning of the test session (see section 2.4.1).

- **'Home cage' injections**: In order to control for the effects of morphine and saline administration that were independent of the CPP apparatus, mice \( n = 3 \) per genotype received equivalent morphine and saline injections to animals undergoing morphine CPP but without exposure to the CPP apparatus. Mice were returned to the home cage immediately after injection. They were perfused at an equivalent time to the morphine CPP group, i.e. 26 h after the last injection (see section 2.4.1).

- **No test session**: The final control group was included in order to assess the contribution of the final test session of the CPP procedure to the expression of c-Fos. Mice \( n = 3 \) per genotype underwent the morphine CPP procedure as described above, but did not undergo the test session on the last day. They were perfused 26 h after the last injection (see section 2.4.1).
Figure 5.3 Protocol for c-Fos experiment: morphine CPP. Mice underwent the morphine CPP procedure (7.5 mg kg⁻¹) and were perfused 2 h after the final test session. Control mice underwent saline CPP, injections of morphine (7.5 mg kg⁻¹) and saline in the home cage, or the morphine CPP procedure but without the final test session. ?: ‘choice’ session (pre- or post-conditioning).
5.3.3. Immunohistochemistry

Following perfusion, the mice's brains were removed, postfixed, cryoprotected and sectioned as described in section 2.4.1.

5.3.3.1. Single-labelling immunohistochemistry: c-Fos

IHC for c-Fos (see section 2.4.2) was carried out on every third section through the brain, visualised using the Ni-enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB) method (see section 2.4.3).

5.3.3.2. Double-labelling immunohistochemistry: c-Fos / neurokinin-1 receptor

Every 6th section through the brains of the wild type mice was processed for double-labelling IHC for c-Fos and the NK1 receptor. The method for IHC with DAB visualisation was used, without Ni enhancement (see section 2.4.3). The primary antibody step consisted of a 24-h incubation in the NK1 receptor antibody (1:5000; Chemicon International), immediately followed by incubation in the c-Fos antibody overnight (1:50 000), both at room temperature (see section 2.4.2).

5.3.4. Analysis

Single-labelled sections were visualised under bright field conditions (see section 2.4.4). The observer was blinded to genotype and drug treatment. The number of c-Fos positive nuclei was assessed in a range of brain regions, according to a mouse brain atlas (Franklin & Paxinos 1997). For each brain region under study, an objective was chosen (× 10, × 20 or × 40) which allowed as much of the region to be visible in a single field of view as possible. The number of dark grey / black nuclei was counted bilaterally in all sections through the brain in which the brain region under study was larger than the chosen field of view. The number of c-Fos-positive nuclei for each mouse was then expressed as the mean density of stained nuclei per mm$^3$. Comparisons between groups of mice were made separately for each brain region using 2-way ANOVAs with genotype and treatment as between-subject factors (see section 2.6). Post hoc Tukey comparisons were used following significant main effects or interaction terms.
Chapter five  Analysis of c-Fos expression in the brains of neurokinin-1 receptor knockout mice

The following brain regions were analysed (see Figure 5.4):

- NAcc core;
- NAcc shell;
- dorsomedial CPu;
- basolateral nucleus of the amygdala;
- central nucleus of the amygdala;
- motor cortex;
- somatosensory cortex;
- locus coeruleus;
- CA1 region of the hippocampus (CPP sections only);
- CA3 region of the hippocampus (CPP sections only);
- dentate gyrus (CPP sections only);
- VTA (CPP sections only);
- dorsal raphe nucleus (CPP sections only).

In double-labelled sections, the colocalisation of c-Fos-positive nuclei and the NK1 receptor was assessed qualitatively. Neurones co-expressing c-Fos and NK1 receptors were characterised by brown nuclei (c-Fos) surrounded by a pale brown cell membrane (NK1 receptor). Since this technique requires the presence of the NK1 receptor around the soma, it was only attempted in regions of clear somal NK1 immunoreactivity, i.e. the core and shell subregions of the NAcc and the dorsomedial CPu.
Figure 5.4 Brain regions for c-Fos counting. Diagrams of mouse brain sections indicating typical positions of areas in which the density of c-Fos-positive nuclei was measured. Numbers are approximate distances of sections from Bregma. BLA: basolateral nucleus of the amygdala; CA1: CA1 region of the hippocampus; CA3: CA3 region of the hippocampus; CeA: central nucleus of the amygdala; CPu: dorsomedial caudate putamen; DG: dentate gyrus; DR: dorsal raphe nucleus; LC: locus coeruleus; MCo: motor cortex; NAcC: nucleus accumbens core; NAcS: nucleus accumbens shell; SCo: somatosensory cortex; VTA: ventral tegmental area.

5.4. Results

5.4.1. Single-labelling immunohistochemistry: c-Fos

5.4.1.1. Acute morphine

The results of 2-way ANOVAs of the density of c-Fos-positive nuclei in various brain regions following acute morphine or saline treatment are shown in Table 5.1.
Table 5.1 Results of ANOVAs of c-Fos expression following acute morphine administration. The significant P-value is underlined.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAcc core</td>
<td>( F_{1,12} = 0.07 ) P = 0.801</td>
<td>( F_{1,12} = 0.18 ) P = 0.678</td>
<td>( F_{1,12} = 0.02 ) P = 0.891</td>
</tr>
<tr>
<td>NAcc shell</td>
<td>( F_{1,12} = 1.50 ) P = 0.244</td>
<td>( F_{1,12} = 0.01 ) P = 0.921</td>
<td>( F_{1,12} = 0.29 ) P = 0.599</td>
</tr>
<tr>
<td>Dorsomedial CPu</td>
<td>( F_{1,12} = 0.23 ) P = 0.641</td>
<td>( F_{1,12} = 0.55 ) P = 0.472</td>
<td>( F_{1,12} = 1.08 ) P = 0.320</td>
</tr>
<tr>
<td>Basolateral nucleus of the amygdala</td>
<td>( F_{1,12} = 2.36 ) P = 0.151</td>
<td>( F_{1,12} = 1.33 ) P = 0.272</td>
<td>( F_{1,12} = 1.62 ) P = 0.227</td>
</tr>
<tr>
<td>Central nucleus of the amygdala</td>
<td>( F_{1,12} = 0.21 ) P = 0.653</td>
<td>( F_{1,12} = 9.24 ) P = 0.010</td>
<td>( F_{1,12} = 0.42 ) P = 0.530</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>( F_{1,12} = 2.84 ) P = 0.118</td>
<td>( F_{1,12} = 4.16 ) P = 0.064</td>
<td>( F_{1,12} = 0.35 ) P = 0.565</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>( F_{1,12} = 1.35 ) P = 0.267</td>
<td>( F_{1,12} = 3.92 ) P = 0.071</td>
<td>( F_{1,12} = 0.23 ) P = 0.642</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>( F_{1,12} = 1.39 ) P = 0.262</td>
<td>( F_{1,12} = 0.04 ) P = 0.845</td>
<td>( F_{1,12} = 0.10 ) P = 0.752</td>
</tr>
</tbody>
</table>

In the central nucleus of the amygdala, there was a significant main effect of drug treatment, but there was no significant main effect of genotype and no significant genotype by treatment interaction. This suggests that acute morphine administration caused an increase in the density of nuclei expressing c-Fos in this brain region but that this increase was similar in both genotypes (Figure 5.5).
Figure 5.5 C-Fos expression following acute morphine administration: central nucleus of the amygdala. Mean ± SEM densities of c-Fos-positive nuclei in the central nucleus of the amygdala of wild type and NK1-/- mice 2 h after a single systemic injection of morphine (10.0 mg.kg⁻¹) or saline. Acute morphine caused an increase in the number of stained nuclei in both genotypes relative to saline controls. **p < 0.01.

Acute morphine at this dose (10.0 mg.kg⁻¹) did not affect the number of nuclei expressing c-Fos and there was no effect of genotype or interaction between drug administration and genotype in any of the other brain regions analysed quantitatively.

Qualitative assessment of the expression of c-Fos between treatment groups revealed trends for an increase in the number of c-Fos-positive nuclei following acute morphine administration in the bed nucleus of the stria terminalis, the lateral nucleus of the amygdala, the centromedial nucleus of the thalamus, and the paraventricular nucleus and lateral area of the hypothalamus. Decreases were observed in the lateral posterior nucleus of the thalamus and the medial preoptic nucleus of the hypothalamus. No trends for a difference between the two genotypes were noticed.

5.4.1.2. Chronic morphine

The results of 2-way ANOVAs of the density of c-Fos-positive nuclei in various brain regions following chronic morphine or saline treatment are shown in Table 5.2.
Table 5.2 Results of ANOVAs of c-Fos expression following chronic morphine administration. Significant P-values are underlined.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAcc core</td>
<td>$F_{1,12} = 0.16$</td>
<td>$P = 0.693$</td>
<td>$F_{1,12} = 0.29$</td>
</tr>
<tr>
<td>NAcc shell</td>
<td>$F_{1,12} = 1.45$</td>
<td>$P = 0.252$</td>
<td>$F_{1,12} = 0.05$</td>
</tr>
<tr>
<td>Dorsomedial CPu</td>
<td>$F_{1,12} = 0.61$</td>
<td>$P = 0.450$</td>
<td>$F_{1,12} = 1.10$</td>
</tr>
<tr>
<td>Basolateral nucleus of the amygdala</td>
<td>$F_{1,12} = 0.57$</td>
<td>$P = 0.465$</td>
<td>$F_{1,12} = 0.01$</td>
</tr>
<tr>
<td>Central nucleus of the amygdala</td>
<td>$F_{1,12} = 1.13$</td>
<td>$P = 0.309$</td>
<td>$F_{1,12} = 11.7$</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>$F_{1,12} = 0.02$</td>
<td>$P = 0.900$</td>
<td>$F_{1,12} = 5.36$</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>$F_{1,12} = 0.13$</td>
<td>$P = 0.721$</td>
<td>$F_{1,12} = 9.02$</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>$F_{1,12} = 3.72$</td>
<td>$P = 0.078$</td>
<td>$F_{1,12} = 7.56$</td>
</tr>
</tbody>
</table>

In the central nucleus of the amygdala (Figure 5.6), the motor (Figure 5.7) and somatosensory cortices (Figure 5.8) and the locus coeruleus (Figure 5.9) there were significant main effects of drug treatment, but no significant main effects of genotype or significant treatment by genotype interactions. Chronic morphine administration therefore caused increases in the density of c-Fos-positive nuclei relative to saline controls in these regions, but this effect was similar in both genotypes.
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Figure 5.6 C-Fos expression following chronic morphine administration: central nucleus of the amygdala. Mean ± SEM densities of c-Fos-positive nuclei in the central nucleus of the amygdala of wild type and NK1⁻/⁻ mice 2 h after the last of a series of systemic injections of morphine (10 - 100 mg.kg⁻¹) or saline. Chronic morphine caused an increase in the number of stained nuclei in both genotypes relative to saline controls. **P < 0.01.

Figure 5.7 C-Fos expression following chronic morphine administration: motor cortex. Mean ± SEM densities of c-Fos-positive nuclei in the motor cortex of wild type and NK1⁻/⁻ mice 2 h after the last of a series of systemic injections of morphine (10 - 100 mg.kg⁻¹) or saline. Chronic morphine caused an increase in the number of stained nuclei in both genotypes relative to saline controls. *P < 0.05.
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Figure 5.8 C-Fos expression following chronic morphine administration: somatosensory cortex. Mean ± SEM densities of c-Fos-positive nuclei in the somatosensory cortex of wild type and NK1⁻/⁻ mice 2 h after the last of a series of systemic injections of morphine (10 - 100 mg kg⁻¹) or saline. Chronic morphine caused an increase in the number of stained nuclei in both genotypes relative to saline controls. *P < 0.05.

Figure 5.9 C-Fos expression following chronic morphine administration: locus coeruleus. Mean ± SEM densities of c-Fos-positive nuclei in the locus coeruleus of wild type and NK1⁻/⁻ mice 2 h after the last of a series of systemic injections of morphine (10 - 100 mg kg⁻¹) or saline. Chronic morphine caused an increase in the number of stained nuclei in both genotypes relative to saline controls. *P < 0.05.

In all other brain regions analysed quantitatively, there were no significant main effects of drug treatment or genotype and no significant treatment by genotype interactions, suggesting that neither chronic morphine administration nor genotype affected the number of neurones expressing c-Fos in these regions.

Qualitative assessment of the expression of c-Fos between treatment groups revealed a trend for an increase in the number of c-Fos-positive nuclei following chronic morphine administration in the ventral pallidum, the bed nucleus of the stria terminalis, the CA1 region of the hippocampus, the anterior, centromedial and subparafascicular nuclei of the
thalamus, the lateral habenular nucleus, the arcuate and paraventricular nuclei and the lateral area of the hypothalamus, the cingulate, orbital, retrosplenial and agranular insular cortices and the dorsal, medial and pontine raphe nuclei. Decreases were observed in the medial preoptic nucleus of the hypothalamus. No trends for a difference between the two genotypes were noticed.

5.4.1.3. Morphine conditioned place preference

The results of 2-way ANOVAs of the density of c-Fos-positive nuclei in various brain regions following morphine CPP or control treatments are shown in Table 5.3.

Table 5.3 Results of ANOVAs of c-Fos expression following morphine CPP. Significant P-values are underlined.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAcc core</td>
<td>F_{1,20} = 3.42</td>
<td>P = 0.079</td>
<td>F_{3,20} = 8.37</td>
</tr>
<tr>
<td>NAcc shell</td>
<td>F_{1,20} = 3.35</td>
<td>P = 0.082</td>
<td>F_{3,20} = 11.2</td>
</tr>
<tr>
<td>Dorsomedial CPU</td>
<td>F_{1,20} = 2.33</td>
<td>P = 0.142</td>
<td>F_{3,20} = 14.6</td>
</tr>
<tr>
<td>Basolateral nucleus of the amygdala</td>
<td>F_{1,20} = 3.44</td>
<td>P = 0.078</td>
<td>F_{3,20} = 18.9</td>
</tr>
<tr>
<td>Central nucleus of the amygdala</td>
<td>F_{1,20} = 0.18</td>
<td>P = 0.676</td>
<td>F_{3,20} = 7.67</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>F_{1,20} = 13.3</td>
<td>P = 0.002</td>
<td>F_{3,20} = 18.7</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>F_{1,20} = 5.44</td>
<td>P = 0.030</td>
<td>F_{3,20} = 12.4</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>F_{1,17} = 2.45</td>
<td>P = 0.136</td>
<td>F_{3,17} = 27.8</td>
</tr>
<tr>
<td>Hippocampus: CA1</td>
<td>F_{1,20} = 5.36</td>
<td>P = 0.031</td>
<td>F_{3,20} = 8.75</td>
</tr>
<tr>
<td>Hippocampus: CA3</td>
<td>F_{1,20} = 5.47</td>
<td>P = 0.030</td>
<td>F_{3,20} = 105</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>F_{1,20} = 12.3</td>
<td>P = 0.002</td>
<td>F_{3,20} = 12.2</td>
</tr>
<tr>
<td>VTA</td>
<td>F_{1,20} = 0.76</td>
<td>P = 0.395</td>
<td>F_{3,20} = 5.59</td>
</tr>
<tr>
<td>Dorsal raphe nucleus</td>
<td>F_{1,19} = 0.54</td>
<td>P = 0.472</td>
<td>F_{3,19} = 17.0</td>
</tr>
</tbody>
</table>
In the NAcc core, there was a significant main effect of treatment, but not of genotype. There was also no significant genotype by treatment interaction. *Post hoc* Tukey comparisons within treatment revealed that mice undergoing morphine CPP had higher densities of c-Fos-positive nuclei in this brain region than those undergoing control home cage injections of morphine and saline ($P = 0.010$), but not those undergoing saline CPP ($P = 0.698$) or those undergoing morphine CPP without the final test session ($P = 0.505$; Figure 5.10). Exposure to the CPP procedure therefore caused an increase in the number of neurones expressing c-Fos in this brain region in both genotypes, although this was not affected by the omission of the final test session or the replacement of morphine injections with vehicle.

![Graph](Image)

**Figure 5.10 C-Fos expression following morphine CPP: NAcc core.** Mean ± SEM densities of c-Fos-positive nuclei in the NAcc core of wild type and NK1- mice 2 h after the test session of morphine CPP (7.5 mg.kg$^{-1}$), saline CPP, control injections of morphine (7.5 mg.kg$^{-1}$) and saline in the home cage or morphine CPP (7.5 mg.kg$^{-1}$) without exposure to the final test session. Morphine CPP caused increases in the number of stained nuclei relative to home cage controls in both genotypes. **$P < 0.01$.**

There was a similar pattern of c-Fos expression in the NAcc shell, with a significant main effect of treatment but no significant main effect of genotype and no significant genotype by treatment interaction. *Post hoc* Tukey comparisons within treatment revealed that mice undergoing morphine CPP had higher densities of c-Fos-positive nuclei in this brain region than mice receiving control home cage injections of morphine and saline ($P < 0.001$), but
not those undergoing saline CPP (P = 0.188) or undergoing morphine CPP but without the final test session (P = 0.883; Figure 5.11). As in the NAcc core, exposure to the CPP procedure therefore caused an increase in the number of neurones expressing c-Fos in the shell subregion of the NAcc in both genotypes, although this was not affected by the omission of the final test session or the replacement of morphine injections with vehicle.

![Graph showing c-Fos expression in the NAcc shell](image)

**Figure 5.11 C-Fos expression following morphine CPP: NAcc shell.** Mean ± SEM densities of c-Fos-positive nuclei in the NAcc shell of wild type and NK1- mice 2 h after the test session of morphine CPP (7.5 mg kg⁻¹), saline CPP, control injections of morphine (7.5 mg kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg kg⁻¹) without exposure to the final test session. Morphine CPP caused increases in the number of c-Fos-positive nuclei relative to home cage controls in both genotypes. ***P < 0.001.

In the dorsomedial portion of the CPu, there was also a significant main effect of treatment, but not genotype, and no significant genotype by treatment interaction. *Post hoc* Tukey comparisons revealed that mice undergoing morphine CPP exhibited higher densities of stained nuclei that those receiving control morphine and saline injections in the home cage (P < 0.001) but not those undergoing saline CPP (P = 0.931) or morphine CPP without the final test session (P = 0.996; Figure 5.12). The pattern of expression in this region was similar to that in the subregions of the NAcc and the central nucleus of the amygdala, since exposure to the CPP procedure caused an increase in the number of neurones expressing c-Fos in both genotypes, although this was not affected by the omission of the final test session or the replacement of morphine injections with vehicle.
In the basolateral nucleus of the amygdala, there was a significant main effect of treatment and a significant treatment by genotype interaction, suggesting that the pattern of c-Fos expression differed between the two genotypes following the various treatment protocols. Post hoc Tukey comparisons revealed that wild type mice undergoing morphine CPP exhibited higher densities of c-Fos-positive nuclei in this brain region than NK1<sup>-/-</sup> mice undergoing the same treatment (P = 0.032; Figure 5.13). They also exhibited higher densities of these nuclei than wild type mice undergoing saline CPP (P = 0.035) and those receiving control morphine and saline injections in the home cage (P < 0.001), but not those that did not undergo the final test session of the CPP procedure (P = 0.922). There were no differences between genotypes in the density of c-Fos-positive nuclei after any other treatment protocol (P = 1.0). There were also no differences in the density of stained nuclei in NK1<sup>-/-</sup> mice between the morphine CPP group and the three control treatment groups (P > 0.2). When the final test session was included in the procedure, morphine CPP therefore caused a significant increase in the number of neurones in this brain region expressing c-Fos relative to saline CPP and homecage controls, but this did not occur in NK1<sup>-/-</sup> mice.
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Figure 5.13 C-Fos expression following morphine CPP: basolateral nucleus of the amygdala. A: Representative sections from the basolateral nucleus of the amygdala (see Figure 5.4) of wild type and NK1⁻/⁻ mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session, immunohistochemically stained for c-Fos and visualised using nickel-enhanced DAB. Scale bar = 500 µm. B: Mean ± SEM densities of c-Fos-positive nuclei. *P < 0.05; ***P < 0.001. Morphine CPP caused an increase in the number of neurones expressing c-Fos in wild type, but not NK1⁻/⁻ mice, relative to saline CPP and home cage controls.
The pattern of expression of c-Fos in the central nucleus of the amygdala was similar to that in the striatum, with a significant main effect of treatment but no significant main effect of genotype and no significant genotype by treatment interaction. *Post hoc* Tukey comparisons within treatment revealed that mice undergoing morphine CPP had higher densities of c-Fos-positive nuclei in this brain region than mice receiving control home cage injections (*P* = 0.004) but not those undergoing saline CPP (*P* = 0.795) or morphine CPP without the final test session (*P* = 0.865; Figure 5.14). Exposure to the CPP procedure therefore caused an increase in the number of neurones expressing c-Fos in this region in both genotypes, although this was not affected by the omission of the final test session or the replacement of morphine injections with vehicle.

![Figure 5.14 C-Fos expression following morphine CPP: central nucleus of the amygdala.](image)

Mean ± SEM densities of c-Fos-positive nuclei in the central nucleus of the amygdala of wild type and NK1−/− mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session. Morphine CPP caused an increase in the number of neurones expressing c-Fos relative to home cage controls in both genotypes. **P < 0.01.

In the motor cortex there were significant main effects of genotype and treatment, and a significant genotype by treatment interaction. *Post hoc* Tukey comparisons revealed that wild type mice undergoing morphine CPP exhibited higher densities of c-Fos-positive nuclei in this brain region than NK1−/− mice undergoing the same treatment (*P* = 0.012; Figure 5.15). They also exhibited higher densities of these nuclei than wild type mice undergoing saline CPP (*P* = 0.003) and those receiving morphine and saline injections in the home cage (*P* < 0.001), but not those that did not undergo the final test session of the
morphine CPP procedure (P = 0.554). There were no differences between genotypes in the density of c-Fos-positive nuclei after any other treatment protocol (P > 0.4). There were also no differences in the density of stained nuclei in NK1$^{-/-}$ mice between the morphine CPP group and the three control groups (P > 0.2). The morphine CPP procedure therefore caused an increase in the number of neurones expressing c-Fos in this brain region in wild type mice, but this did not occur in NK1$^{-/-}$ mice.
Figure 5.15 C-Fos expression following morphine CPP: motor cortex. **A:** Representative sections from the motor cortex (see Figure 5.4) of wild type and NK1/- mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session, immunohistochemically stained for c-Fos and visualised using nickel-enhanced DAB. Scale bar = 500 μm. **B:** Mean ± SEM densities of c-Fos-positive nuclei. *P < 0.05; **P < 0.01; ***P < 0.001. Morphine CPP caused an increase in the number of neurones expressing c-Fos in wild type, but not NK1/- mice, relative to saline and home cage controls.
There was a similar pattern of c-Fos expression in the somatosensory cortex. Analysis of the density of c-Fos-positive nuclei revealed significant main effects of both genotype and treatment, and a significant genotype by treatment interaction. *Post hoc* Tukey comparisons revealed that wild type mice undergoing morphine CPP exhibited higher densities of c-Fos-positive nuclei in this brain region than NK1\(^{-/}\) mice undergoing the same treatment \((P = 0.013; \text{Figure 5.16})\). They also exhibited higher densities of these nuclei than wild type mice undergoing saline CPP \((P = 0.007)\) and those receiving morphine and saline injections in the home cage \((P = 0.001)\), but not those that did not undergo the final test session of the morphine CPP procedure \((P = 0.276)\). There were no differences between genotypes in the density of c-Fos-positive nuclei after any other treatment protocol \((P = 1.0)\). There were also no differences in the density of stained nuclei in NK1\(^{-/}\) mice between the morphine CPP group and the three control groups \((P > 0.3)\). In wild type mice the morphine CPP procedure therefore caused an increase in the number of neurones expressing c-Fos in this brain region, which was not affected by omission of the final test session: this increase did not occur in NK1\(^{-/}\) mice.
Figure 5.16 C-Fos expression following morphine CPP: somatosensory cortex. A: Representative sections from the somatosensory cortex (see Figure 5.4) of wild type and NK1⁻/⁻ mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session, immunohistochemically stained for c-Fos and visualised using nickel-enhanced DAB. Scale bar = 500 μm. B: Mean ± SEM densities of c-Fos-positive nuclei. *P < 0.05; **P < 0.01; ***P < 0.001. Morphine CPP caused an increase in the number of neurones expressing c-Fos in wild type, but not NK1⁻/⁻ mice, relative to saline CPP and home cage controls.
In the locus coeruleus, there was a significant main effect of treatment on the density of c-Fos-positive nuclei, but no significant main effect of genotype or treatment by genotype interaction. Post hoc Tukey comparisons within treatment revealed that mice undergoing morphine CPP exhibited higher densities of stained nuclei in this brain region than those undergoing saline CPP (P = 0.004), and those receiving control injections of morphine and saline in the home cage (P < 0.001), but not mice undergoing the conditioning sessions of the morphine CPP procedure but without the final test session (P = 0.354; Figure 5.17). The number of neurones expressing c-Fos in the locus coeruleus therefore increased following the morphine CPP procedure in both genotypes but it was not modulated by omission of the final test session of the CPP procedure.

Figure 5.17 C-Fos expression following morphine CPP: locus coeruleus. Mean ± SEM densities of c-Fos-positive nuclei in the locus coeruleus of wild type and NK1⁻/⁻ mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session. Morphine CPP caused an increase in the number of neurones expressing c-Fos relative to saline CPP and home cage controls in both genotypes. **P < 0.01; ***P < 0.001.

In the CA1 region of the hippocampus, there were significant main effects of both genotype and treatment on the density of nuclei expressing c-Fos, but no significant interaction term, suggesting that both factors affect the expression of c-Fos in this brain region independently. NK1⁻/⁻ mice tended to possess fewer stained nuclei than wild type mice in this brain region (Figure 5.18). Post hoc Tukey comparisons within treatment revealed that mice undergoing morphine CPP had greater densities of these nuclei than those receiving morphine and saline injections in the home cage (P < 0.001), but not those
undergoing saline CPP ($P = 0.912$) or those undergoing morphine CPP but without the final test session ($P = 0.559$). Therefore, despite an overall tendency for NK1/− mice to express c-Fos in fewer neuronal nuclei in this brain region, both genotypes demonstrated higher densities following exposure to the CPP procedure. However this was not modulated by replacement of morphine with vehicle, or omission of the final test session.

![Figure 5.18 C-Fos expression following morphine CPP: CA1](image)

**Figure 5.18 C-Fos expression following morphine CPP: CA1.** Mean ± SEM densities of c-Fos-positive nuclei in the CA1 region of the hippocampus of wild type and NK1/− mice 2 h after the test session of morphine CPP (7.5 mg.kg$^{-1}$), saline CPP, control injections of morphine (7.5 mg.kg$^{-1}$) and saline in the home cage or morphine CPP (7.5 mg.kg$^{-1}$) without exposure to the final test session. Morphine CPP caused an increase in the number of neurones expressing c-Fos relative to home cage controls but NK1/− mice generally exhibited lower densities of these neurones. ***$P < 0.001$.

In the CA3 region of the hippocampus, there were significant main effects of genotype and treatment, as well as a significant genotype by treatment interaction. However, *post hoc* Tukey comparisons did not reveal a significant difference between genotypes following any of the treatment protocols ($P > 0.1$). In a similar manner to that observed in the CA1 region, NK1/− mice tended to exhibit lower densities of c-Fos-positive nuclei than wild type mice (Figure 5.19). *Post hoc* Tukey comparisons within treatment revealed that mice undergoing morphine CPP exhibited greater densities of stained nuclei than those undergoing saline CPP ($P < 0.001$) and morphine and saline injections in the home cage ($P < 0.001$), but not those undergoing morphine CPP without the final test session ($P = 0.847$). The morphine CPP procedure therefore brought about an increase in the
number of neurones expressing c-Fos in this brain region in both genotypes, although this was unaffected by omission of the final test session.

![Graph showing C-Fos expression following morphine CPP: CA3.](image)

**Figure 5.19 C-Fos expression following morphine CPP: CA3.** Mean ± SEM densities of c-Fos-positive nuclei in the CA3 region of the hippocampus of wild type and NK1−/− mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session. Morphine CPP caused an increase in the number of neurones expressing c-Fos relative to saline CPP and home cage controls but NK1−/− mice generally exhibited lower densities of these neurones. ***P < 0.001.

In the dentate gyrus there were significant main effects of genotype and treatment, as well as a significant genotype by treatment interaction. *Post hoc* Tukey comparisons revealed that wild type mice undergoing morphine CPP exhibited higher densities of stained nuclei than NK1−/− mice undergoing the same treatment (P < 0.001; Figure 5.20). They also possessed a higher density of these nuclei than wild type mice of all three control treatment groups (saline CPP: P = 0.012; home cage injections: P < 0.001; no test session: P = 0.005). There were no significant differences between genotypes after any of the control treatment protocols (P > 0.9). There were also no differences in the density of stained nuclei in NK1−/− mice between the morphine CPP group and the three control groups (P > 0.2). Therefore in wild type mice, morphine CPP caused a significant increase in the number of neurones expressing c-Fos in this brain region, whilst this did not occur in NK1−/− mice. This increase was dependent on the performance of the final test session of the morphine CPP procedure.
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Figure 5.20 C-Fos expression following morphine CPP: dentate gyrus. A: Representative sections from the dentate gyrus (see Figure 5.4) of wild type and NK1−/− mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session, immunohistochemically stained for c-Fos and visualised using nickel-enhanced DAB. Scale bar = 500 μm. B: Mean ± SEM densities of c-Fos-positive nuclei. *P < 0.05; **P < 0.01; ***P < 0.001. Morphine CPP caused an increase in the number of neurones expressing c-Fos in wild type, but not NK1−/− mice, relative to all three controls.
In the VTA, there was a significant main effect of treatment on the density of stained nuclei, but no significant main effect of genotype and no significant genotype by treatment interaction. *Post hoc* Tukey comparisons revealed that mice undergoing morphine CPP exhibited higher densities of c-Fos-positive nuclei than those receiving control injections of morphine and saline in the home cage (P = 0.005), but not those undergoing saline CPP (P = 0.475) or those not undergoing the final test session (P = 0.981; Figure 5.21). C-Fos expression in this region was therefore similar to that observed in the NAcc subregions and in the central nucleus of the amygdala: exposure to the CPP procedure caused an increase in the number of neurones expressing c-Fos in both genotypes, although this was not affected by the omission of the final test session or the replacement of morphine injections with vehicle.

**Figure 5.21 C-Fos expression following morphine CPP: VTA.** Mean ± SEM densities of c-Fos-positive nuclei in the VTA of wild type and NK1-/- mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session. Morphine CPP caused an increase in the number of neurones expressing c-Fos relative to home cage controls in both genotypes. **P < 0.01.

Finally, in the dorsal raphe nucleus, although there was a significant main effect of treatment and a significant genotype by treatment interaction on the density of c-Fos-positive nuclei, *post hoc* Tukey comparisons failed to reveal significant differences between genotypes after any of the treatment protocols (P > 0.2). However, *post hoc* Tukey comparisons within treatment demonstrated that mice undergoing morphine CPP exhibited higher densities of c-Fos-positive nuclei in this brain region than those
undergoing saline CPP (P = 0.002), injections of morphine and saline in the home cage (P < 0.001) and those that did not undergo the final test session of the morphine CPP procedure (P = 0.005; Figure 5.22). Morphine CPP therefore caused an increase in the number of neurones expressing c-Fos in this brain region relative to all control groups, but without a consistent difference between genotypes.

![Figure 5.22 C-Fos expression following morphine CPP: dorsal raphe nucleus. Mean ± SEM densities of c-Fos-positive nuclei in the dorsal raphe nucleus of wild type and NK1−/− mice 2 h after the test session of morphine CPP (7.5 mg.kg⁻¹), saline CPP, control injections of morphine (7.5 mg.kg⁻¹) and saline in the home cage or morphine CPP (7.5 mg.kg⁻¹) without exposure to the final test session. Morphine CPP caused an increase in the number of neurones expressing c-Fos relative to all three control groups with no consistent effect of genotype across the treatment groups. **P < 0.01; ***P < 0.001.](image)

In summary, morphine CPP caused a significant increase in the number of neurones expressing c-Fos in all brain regions studied in wild type mice relative to those injected with morphine and saline in the same doses and volumes and at the same times as those undergoing the CPP procedure, but without exposure to the CPP apparatus. However, this increase was specific to morphine CPP, as opposed to saline CPP, only in the basolateral nucleus of the amygdala, the motor and somatosensory cortices, the locus coeruleus, the CA3 region of the hippocampus, the dentate gyrus and the dorsal raphe nucleus. Omission of the final test session of the morphine CPP procedure reduced the number of neurones expressing c-Fos only in the dentate gyrus and the dorsal raphe nucleus. Significant reductions in the number of neurones expressing c-Fos in response to morphine CPP in NK1−/− mice were observed in the basolateral nucleus of the amygdala,
the somatosensory and motor cortices and the dentate gyrus. In all other areas, the expression pattern was similar in the two genotypes.

5.4.2. Double-labelling immunohistochemistry: c-Fos / neurokinin-1 receptor

Double-labelled neurones were sought in the brain regions of wild type mice in which NK1 receptor immunoreactivity can be observed surrounding the soma, i.e. the NAcc subregions and the CPu. NK1 receptor immunoreactivity was similar across all treatment protocols, whilst the expression c-Fos followed a similar pattern to that observed following single-labelling IHC. However, c-Fos-positive nuclei were never observed in the same neurones with NK1 receptor immunoreactivity on the plasma membrane surrounding the soma (Figure 5.23).
Figure 5.23 Double-labelling IHC: c-Fos / NK1 receptor. Representative sections from the NAcc core of a wild type mouse following chronic morphine administration, immunohistochemically stained for c-Fos and the NK1 receptor visualised using DAB. A: low magnification. Scale bar = 500 μm. B: high magnification. Scale bar = 250 μm. c-Fos-positive nuclei (grey arrow) were never seen within the somata of NK1 receptor-expressing neurones (black arrow) in the NAcc subregions or the CPu.
5.5. **Discussion**

### 5.5.1. Effects of morphine on c-Fos expression

The acute or repeated administration of morphine brought about a number of changes in the expression pattern of the c-Fos protein in the brains of mice. Both acute and chronic injections of morphine caused increases in number of neurones expressing c-Fos in the central nucleus of the amygdala, whilst chronic injections brought about additional increases in the motor and somatosensory cortices and the locus coeruleus. There were no differences between genotypes in any of these areas, suggesting that the lack of NK1 receptors did not affect the expression of c-Fos, and possibly the activation of these neurones, in response to these morphine treatments. There were no changes in c-Fos expression in the NAcc core or shell subregions, the dorsomedial CPu or the basolateral nucleus of the amygdala.

The pattern of activation of c-Fos in the brain following acute and chronic morphine administration was generally less widespread than that observed in the rat. In the present study, acute morphine administration brought about an increase in c-Fos expression only in the central nucleus of the amygdala. This region has not been reported as a site of c-Fos upregulation in response to similar treatment protocols in the rat. However, it is connected to the core of the NAcc and receives dense reciprocal dopaminergic afferents from the VTA (Ben-Ari et al. 1975; Ungerstedt 1971; Wallace et al. 1992), whilst blockade of dopamine D2 receptors in this region reduces the acquisition and expression of CPP to morphine (Rezayof et al. 2002), suggesting an important role of this region in modulating reward-related behaviour. The majority of published studies cite the NAcc and dorsomedial CPu as regions with clear upregulation of c-Fos. The lack of a similar upregulation in this study may be due to the different species used: although an upregulation of c-fos mRNA in the NAcc shell and dorsomedial CPu of mice has been observed following acute morphine administration at a dose of 5 mg.kg\(^{-1}\) (Spielewoy et al. 2000), the strain used here may require a different dose of morphine in order to see an upregulation of the IEG in these regions.

Following chronic morphine injections, an additional upregulation in cortical areas and the locus coeruleus was observed. One study has reported an upregulation of c-Fos with chronic morphine administration in the somatosensory cortex of the rat, although this was
superimposed on a high background of c-Fos expression induced by control saline injections (Curran et al. 1996). The authors speculated that this region might be of importance in sensitisation to the locomotor effects of drugs of abuse, since it is also activated by cocaine and amphetamine, and may even be a locus for cross-sensitisation between drugs. Although the behavioural effects of the morphine administration protocol used here have not been examined, the lack of sensitisation to the locomotor effects of morphine in NK1 knockout mice (Ripley et al. 2002; Chapter 4) suggests that a simple association between c-Fos expression in this region and the expression of behavioural sensitisation cannot be made. The similar upregulation with chronic morphine in the motor cortex is a novel finding that has not been reported previously, in the mouse or rat. Again, given the difference in the motor effects of chronic morphine between the two genotypes, the similarity of their c-Fos expression in this area suggests that this upregulation is not linked directly to locomotor sensitisation. However, both regions are clearly activated by repeated morphine administration: this activation may be representative of the somatic effects of repeated morphine administration, although the nature of such effects remains to be determined.

Finally, the observation of an increase in c-Fos expression in the locus coeruleus is an opposite finding to that seen in the rat (Hayward et al. 1990). The chronic administration of opiates is known to bring about a compensatory increase in levels of guanine nucleotide binding proteins (G-proteins; Nestler et al. 1989), adenylate cyclase (Duman et al. 1988; Matsuoka et al. 1994), cAMP-dependent protein kinase (Nestler & Tallman 1988) and a number of morphine- and cAMP-regulated phosphoproteins in the neurones of the locus coeruleus (Guitart & Nestler 1989), which counteracts the acute μ-opioid receptor-mediated inhibition brought about by morphine (Aghajanian 1978; Andrade et al. 1983; Bird & Kuhar 1977; Korf et al. 1974; Nestler et al. 1994). Withdrawal from chronic opiate administration removes this inhibitory action and causes a dramatic increase in the activity of these cells (Rasmussen et al. 1990), coupled with an increase in c-Fos expression (Chahl et al. 1996; Frenois et al. 2002; Georges et al. 2000; Hayward et al. 1990; Stornetta et al. 1993).

In the present study, it is unlikely that the final injection of morphine was insufficient to counteract the compensatory adaptations in cells of this brain region, since the morphine dose was increased day by day, with a final dose of 100 mg kg⁻¹. The observed upregulation of c-Fos with chronic morphine is therefore difficult to reconcile with previous findings.
However, the present study used repeated bolus injections of morphine, whilst Hayward et al. (1990) used a subcutaneous morphine pellet to provide continuous infusion of the drug at a dose of 75 mg.kg$^{-1}$ per day. It remains possible, therefore, that the method of administration used in the present experiment has a different effect on the activity of the neurones of the locus coeruleus and on its cells' expression of c-Fos, possibly by bringing about an overcompensation of the intracellular signalling cascades resulting in a net upregulation of c-Fos and increased physiological activity.

These findings extend the results of Murtra et al. (2000b), who analysed the expression of a related immediate early gene in the NAcc of NK$1^{-/}$ mice in response to chronic morphine administration. Using an antibody raised against the Fos-related antigen FosB, they analysed the expression pattern of this protein, plus its truncated ΔFosB isoform, which is postulated to be of importance in the plastic changes occurring with chronic psychostimulant or opiate administration in the NAcc and CPu (Kelz et al. 1999; Nye & Nestler 1996). Murtra et al. (2000b) found no differences between wild type and NK$1^{-/}$ mice in the number of neurones expressing FosB isoforms following chronic morphine exposure. However they did report that the increased intensity of the immunofluorescent signal observed in the core and shell subregions of wild type mice did not occur in NK$1^{-/}$ mice in response to chronic morphine, suggesting a link between opiate reward and the expression of products of the fosb gene in the NAcc.

5.5.2. Effects of morphine conditioned place preference on c-Fos expression

The morphine CPP procedure brought about increases in the number of neurones expressing c-Fos in a range of areas, relative to the observed expression levels in mice undergoing the saline CPP procedure or those receiving the equivalent morphine and saline injections in a non-contingent manner without exposure to the CPP conditioning chambers. They were therefore brought about by the morphine CPP procedure itself, and not by the stress or pain of the CPP procedure or the drug injections per se. These increases were observed in similar areas to chronic injections of morphine, namely in the motor and somatosensory cortices and the locus coeruleus, but not in the central nucleus of the amygdala. Morphine CPP also resulted in upregulation of c-Fos expression in the basolateral nucleus of the amygdala and the dorsal raphe nucleus, as well as in the CA3 region of the hippocampus and the dentate gyrus. Of these regions, disruptions in the
expression pattern were observed in NK1\(^{-/+}\) mice in the motor and somatosensory cortices, the basolateral nucleus of the amygdala and the dentate gyrus. These regions are therefore potential sites for differences in activation between the brains of wild type and NK1\(^{-/-}\) mice in response to this procedure, and may hold clues as to the location of SP's and the NK1 receptor's effects in mediating morphine reward-related behaviours.

5.5.2.1. **Regions of differential c-Fos expression between genotypes**

The basolateral nucleus of the amygdala is a strong candidate as a locus for differences in NK1 receptor-mediated signalling in the control of morphine reward-related behaviours. The failure of morphine CPP to bring about an upregulation of c-Fos in this region in NK1\(^{-/-}\) mice suggests that this region is not activated in the same way in mice lacking NK1 receptors. Furthermore, the similar levels of expression of c-Fos in the central nucleus of the amygdala following acute and chronic morphine administration suggest that the basolateral nucleus may be more important in mediating the NK1 receptor’s effects in morphine-related behaviours, although this conclusion would benefit from analysis of the other subnuclei of the amygdala. The potential role of amygdalar NK1 receptors in mediating reward-related behaviours is discussed more thoroughly in Chapter 6.

The reductions in expression of c-Fos in cortical areas and the dentate gyrus also suggest that these brain regions are not activated to the same extent in NK1\(^{-/-}\) mice as they are in animals with intact NK1 receptors. While the present findings cannot distinguish between brain regions which are directly involved in the control of morphine reward behaviours and those which are affected by the execution of behaviour itself, the absence of NK1 receptors from these regions, at least in the mouse (see Chapter 3), suggests that they are less likely to be loci for the NK1 receptor’s crucial effects in mediating morphine CPP. Nevertheless, they do indicate that these areas contribute to the neural system that is differentially activated by morphine CPP in NK1\(^{-/-}\) mice. The upregulation of c-Fos in the dentate gyrus (and CA3 region of the hippocampus) of wild type mice may be related to the formation of contextual associations between the rewarding stimulus and the environment in which it was administered, a process that presumably is disrupted in NK1\(^{-/-}\) mice. Similarly, the activation of motor and somatosensory cortices could represent a conditioned activation of these areas in response to presentation of drug-associated cues in the CPP apparatus: the expression of c-Fos here may represent a cellular ‘memory’ for the
properties of repeated morphine exposure observed with non-contingent chronic morphine administration, which does not occur in mice lacking NK1 receptors.

5.5.2.2. Nature of the stimulus under test

Despite these observations, the exact nature of the stimulus under test with this protocol is unclear. The final test session of the CPP procedure involves the animal under study being faced with a choice situation, in which it is presented with two contexts, one of which was paired with a rewarding stimulus, and should have gained conditioned rewarding value, at least in wild type mice. While the upregulation of c-Fos observed may correspond to the conditioned rewarding nature of the drug-associated context, it is also possible that it reflects the choice procedure itself, the expectation of injection or the conditioned aspects of either of the two conditioning compartments, including the vehicle-associated context. Previous studies have reported consistent upregulations in c-Fos expression and activity in the amygdala and cortical regions, especially the cingulate and prefrontal cortices, in response to placement in a drug-associated context (Brown et al. 1992; Mead et al. 1999; Mitchell et al. 2000; Neisewander et al. 2000; Schroeder et al. 2000). In wild type mice in this experiment, the basolateral (but not the central) nucleus of the amygdala was found to exhibit increased expression of c-Fos, and a qualitative analysis of these cortical areas showed a similar trend, thereby supporting the hypothesis that these regions are activated in response to drug-conditioned stimuli, possibly along with hippocampal regions, the motor and somatosensory cortices and the dorsal raphe nucleus.

5.5.2.3. Expression of c-Fos following omission of the final test session

Omission of the final test session of the morphine CPP procedure resulted in similar levels of expression of c-Fos to those in mice undergoing the full CPP paradigm in all of the brain regions examined, except for the dentate gyrus, the dorsal raphe nucleus and the locus coeruleus. This treatment was intended to act as a control for the effects of the conditioning phase of the CPP procedure. Significant increases in the morphine CPP group over that of the 'no test session' group indicated that the observed upregulation of c-Fos was specifically due to the execution of the final test session in these three regions. However, the failure to observe such a difference in other regions is difficult to explain. It may reflect persistent synaptic activation of the neurones in these brain regions in response
to the conditioning phase of the morphine CPP procedure, resulting in c-Fos upregulation
despite these mice not having received a specific stimulus at two hours before perfusion.
In the case of the brain regions where a significant difference between genotypes was
observed following the morphine CPP test session, an inhibition of this persistent c-Fos
expression may occur in NK1⁻/⁻ mice during the test session.

5.5.3. Methodological considerations

Although differences in the expression of c-Fos in a particular brain region between
treatment groups may indicate that its neurones were differentially activated by the stimulus
under test, the lack of such a difference is difficult to interpret. In particular, the lack of an
increase in the density of neurones expressing c-Fos in the NAcc core and shell subregions
after all three treatment protocols was unexpected on the basis of previous findings,
despite evidence that it is not necessary for the expression of CPP to morphine (Tolliver et
al. 2000). However, c-Fos is not a definitive marker of all types of activation in all neurone
types, but simply a transcription factor that is upregulated in the majority of neurones
following stimulation at the cell surface. The lack of a difference in c-Fos expression
between two treatments, such as morphine and vehicle, does not necessarily imply that the
treatment did not activate the neurones of this region. The discrepancy between the
present findings and previously published work may therefore be due to a difference in the
requirements of these areas' cells for the induction of a detectable level of c-Fos, perhaps
due to the different species, drug dose or administration protocol used. Similarly, the lack
of an observed difference between genotypes in other regions does not preclude their
potential involvement in the NK1 receptor's behavioural effects. Conversely however, the
observation of a statistically significant difference in c-Fos expression between two
treatments is a clear indication that a difference in physiological activity took place in the
brain region under study. The failure of morphine CPP to bring about the upregulation of
c-Fos in the motor and somatosensory cortices, the basolateral nucleus of the amygdala
and the dentate gyrus therefore indicates that these regions form a part of a system which is
not activated by the morphine CPP procedure in NK1⁻/⁻ mice. However, firm conclusions
on the role of the other regions analysed await further study.
5.5.4. Double-labelling immunohistochemistry

In the double-labelling IHC study, the identity of the neurones expressing c-Fos was determined in the NAcc and CPu. After all treatment protocols, c-Fos-positive nuclei were observed in neurones other than those expressing the NK1 receptor on their perikarya. This suggests that the type of activation required to upregulate c-Fos did not occur in NK1 receptor-expressing neurones in these regions in response to single or chronic injections of morphine, or the morphine CPP procedure. While it is possible that this result reflects an inability for both antigens to be labelled in the same cells with this technique, the visualisation of the two proteins in the spinal cord in response to noxious stimulation has been shown in a number of studies in the rat using double-labelling IHC (Doyle & Hunt 1999; Lü et al. 1995; Todd et al. 2002). Therefore the observed lack of colocalisation in this study is probably due to a lack of expression of c-Fos in these cells. While it would have been of interest to examine the distribution of c-Fos-positive nuclei in other regions of the brain, especially those with significant increases in the density of such nuclei in response to morphine or the CPP procedure, it is limited by the presence of clear NK1 receptor immunoreactivity on the perikarya. Nevertheless, these observations suggest that the cells in which c-Fos-mediated plastic changes are occurring with chronic drug exposure, and which may be crucial for the acquisition of CPP behaviour (Tolliver et al. 2000), may be a separate population to those which express the NK1 receptor.

5.5.5. Opportunities for further study

While these findings are enlightening and provide evidence for the basolateral nucleus of the amygdala as a potential area for the NK1 receptor’s effects in mediating morphine reward behaviours, they would benefit from the quantitative analysis of further brain regions. Following acute and chronic morphine administration, qualitative analysis revealed some areas of c-Fos upregulation which were similar to that observed in the rat, such as thalamic, hypothalamic and cortical regions. The present findings would also benefit from the analysis of the effects of a range of morphine doses on the expression of c-Fos, since activation of areas such as the NAcc in wild type mice would enable more certain conclusions about the role of the NK1 receptor in these regions to be drawn. An interesting extension of this work would be the analysis of c-Fos expression following the locomotor sensitisation or self-administration procedures, or following the precipitation of
withdrawal from chronic morphine exposure in NK1\(^{-}\) mice. Finally, the comparison of these findings with cocaine, which induces normal reward- and addiction-related behaviours in NK1\(^{-}\) mice, would allow the specificity of these observations for the behavioural effects of morphine to be assessed.

5.6. Conclusions

The results presented in this chapter have demonstrated that NK1\(^{-}\) mice exhibit alterations in the expression pattern of the IEG *c-fos* in the brain in response to the morphine CPP procedure. While they do not necessarily indicate a causal role of these areas in bringing about the behavioural changes observed in this task, they do illustrate some components of the neural systems that are differentially activated in mice lacking NK1 receptors, including the basolateral nucleus of the amygdala, the dentate gyrus and cortical regions. Although they would benefit from the analysis of additional brain regions and drug doses, these data suggest that the amygdala is an important area in the NK1 receptor's effects on morphine reward behaviours.
CHAPTER SIX

BEHAVIOURAL EFFECTS OF ABLATION OF NEURONES EXPRESSING THE NEUROKININ-1 RECEPTOR
6. BEHAVIOURAL EFFECTS OF ABLATION OF NEURONES EXPRESSING THE NEUROKININ-1 RECEPTOR

6.1. Introduction

In this chapter, results are presented from a series of experiments attempting to identify the localisation of the population of neurones expressing the neurokinin-1 (NK1) receptor that are necessary for morphine reward and anxiety behaviours. The neurotoxin substance P-saporin (SP-SAP) was used to selectively ablate NK1 receptor-expressing neurones from discrete brain regions of the mouse brain, before the behaviour of these mice was assessed. The experiments described here include those involving optimisation of the stereotaxic injection technique, comparison of two brain regions in a preliminary experiment, and the controlled analysis of the effects of such ablation in the amygdala. The findings are discussed in relation to the published literature.

6.2. Background

6.2.1. Localisation of substance P and the neurokinin-1 receptor's effects

6.2.1.1. Local infusion of an antagonist

In order to gain more information about the location of substance P (SP) and the NK1 receptor's effects in these behaviours, it would be of use to functionally remove NK1 receptors from various brain regions and compare morphine reward-related behaviours to those of NK1 receptor knockout (NK1−/−) mice. A common technique used to approach this problem is to infuse a suitable antagonist into candidate brain regions using guide cannulae before or during a behavioural task. Whilst there are numerous NK1 receptor antagonists available, this approach would be complicated in studies involving rodents, as the majority of available NK1 receptor antagonists do not show high affinity for the rodent receptor (Appell et al. 1992; Beresford et al. 1991; Jensen et al. 1994; Pradier et al. 1995; Sachais et al. 1993). Furthermore, the behavioural analysis of morphine reward involves long-term experiments often lasting several days: repeated administration of an antagonist across the experimental procedure would therefore be required, as it is unclear which phase or phases of the tasks are dependent upon NK1 receptor activation. Whilst such repeated
administration of drugs is commonly undertaken in studies of reward behaviours (e.g. Packard et al. 1998; Popik & Kolasiewicz 1999; Ranaldi & Wise 2001), the effects of chronic NK1 receptor antagonism may bring about adaptive changes secondary to the acute blockade of the receptor – conversely, it may be true that it is these very changes which are necessary for the prevention of the expression of morphine reward behaviours. Indeed, NK1+/− mice show many of the neural adaptations characteristic of chronic treatment with antidepressant drugs, such as desensitisation of presynaptic 5HT1A receptors in the dorsal raphe nucleus (Froger et al. 2001; Santarelli et al. 2001), enhanced proliferation of new neurones in the dentate gyrus of the hippocampus, and elevated hippocampal levels of brain-derived neurotrophic factor (BDNF; Morcuende et al., submitted). In the absence of data on the effects of systemic NK1 receptor antagonism on morphine reward behaviours, it is not possible to assess the degree to which these adaptations are responsible for the observed behavioural effects of genetic disruption of the receptor. An analysis of the effects of NK1 receptor antagonists on morphine reward behaviours would therefore be necessary before attempting this in separate brain regions; however, due to the species specificity difficulties, re-design of the behavioural testing parameters would be necessary for use with species such as the gerbil, hamster or guinea pig.

6.2.1.2. Region-specific knockout technology

An alternative strategy would be to engineer lines of mice that lack NK1 receptor expression in specific regions of their brain, and to compare their behaviour in morphine reward-related paradigms to that of NK1+/− mice. This could theoretically be achieved using the bacteriophage P1-derived Cre/loxP recombination system (Tsien et al. 1996a). This process involves crossing transgenic mice that express Cre recombinase under the control of a promoter active within a specific brain region with a second transgenic mouse line in which one or more of the exons of the gene of interest has been flanked by two loxP sites. In the offspring of such a cross, the Cre recombinase catalyses recombination between the two flanking loxP sites, thereby excising the gene of interest, solely in those cells that express Cre in the parental mouse line. This procedure has already been used in mouse behavioural studies to examine the effects of deletion of the N-methyl-D-aspartate (NMDA) receptor subunit 1 from the CA1 (Tsien et al. 1996b) or CA3 (Nakazawa et al. 1996).
Chapter six Behavioural effects of ablation of neurones expressing the neurokinin-1 receptor

2002) regions of the hippocampus on learning and memory behaviours. However, the production of such lines of mice is extremely time-consuming and expensive, and therefore impractical for the comparison of numerous brain regions.

6.2.1.3. Ablation of neurokinin-1 receptor-expressing neurones

The approach taken in these experiments was to use the neurotoxin SP-SAP (Wiley & Lappi 1997). This is a conjugate of SP with the ribosome-inactivating protein saporin (SAP) from the seeds of the plant Saponaria officinalis (Lappi et al. 1985; Stirpe et al. 1983, 1992). Since SAP cannot cross the plasma membrane, the only way by which it can get into a cell and lead to its death is by 'piggybacking' on to SP. SP, when it binds to the NK1 receptor, is rapidly internalised along with the receptor, and enters the endosomal compartment. The fall in pH within the endosome causes the dissociation of the ligand from its receptor: SP is subsequently degraded in the lysosome whilst its receptor is recycled back to the membrane, where it can bind ligand once more (Figure 6.1A; Garland et al. 1994; Grady et al. 1995; Mantyh et al. 1995a,b; Southwell et al. 1996). Conjugation of SAP to SP allows the toxin to enter the cell, reach the ribosomes, and causing their inactivation, and eventual cell death (Figure 6.1B). Since SP-SAP is only internalised by those cells expressing NK1 receptors, the toxin is specific for this cell population, leaving cells that do not express the receptor intact (Mantyh et al. 1997; Wiley & Lappi 1997).
Figure 6.1 Internalisation of SP and SP-SAP. A: Upon binding to the NK1 receptor (1), SP is internalised via clathrin-coated pits (2) to the endosomal compartment (3), where the decrease in pH causes the receptor and ligand to dissociate. SP is degraded in the lysosome (4), while the NK1 receptor is recycled back to the synaptic membrane (5). B: Conjugation of SAP to SP allows the SAP toxin to 'piggyback' into the cell. SP-SAP follows the same route as SP into the cell (1-3) and travels, possibly via the lysosome (4), to inactivate ribosomes and eventually cause cell death (5). The NK1 receptor is recycled to the synaptic membrane as normal (6). NK1R: NK1 receptor; SP: substance P; SP-SAP: substance P-saporin.

The use of SP-SAP therefore allows the selective ablation of cells expressing the NK1 receptor within a specific area of tissue. This represents a different approach to the effects of genetic manipulation or pharmacological blockade of the receptor, since this method brings about destruction of entire cells – in the case of neurones, NK1 receptor-expressing
cells also express other receptors and neurotransmitters, and may have numerous connections with other neurones. Ablation of NK1 receptor-expressing neurones therefore brings about the additional removal of these connections and the receptor and neurotransmitter systems coexpressed with the NK1 receptor. Although the neurochemical effects of NK1 receptor gene knockout on these cells has not been thoroughly investigated, it is clear that NK1^+ mice still possess the cells which express the receptor in wild type mice (De Felip et al. 1998). The use of SP-SAP therefore may bring about more dramatic changes in local circuitry than genetic loss of the receptor, albeit lifelong, and certainly than its acute or chronic antagonism. Nevertheless, this approach does form a compromise between experimental simplicity and an accurate assessment of the role of the substance P / NK1 receptor system in the control of behaviour.

6.2.1.3.1. Previous studies using substance P-saporin

SP-SAP has already been used to demonstrate a role for NK1 receptor-expressing neurones in lamina I of the spinal cord in the transmission of hyperalgesia (Mantyh et al. 1997) and chronic pain signals (Nichols et al. 1999), as well as a role for superficial medullary and cervical dorsal horn cells in the oral irritation mediated by capsaicin (Simons et al. 2002). Additionally, destruction of NK1 receptor-expressing cells in laminae VII and X of lumbar segments 3 and 4 of the spinal cord using a similar compound brought about deficits in ejaculation (Truitt & Coolen 2002), whilst ablation of NK1 receptor-expressing neurones in the rostral ventrolateral medulla suggested that they play a role in the control of respiratory rhythm and blood pressure (Wang et al. 2002). Although there have been no published studies examining the behavioural effects of ablation of these cells in the mouse, the experiments described here were carried out in order to investigate the role of these cells in specific brain regions in morphine reward-related behaviours.

6.2.2. Experimental approach

Two brain regions were chosen for analysis in this study. Although there are numerous potential sites of action of the NK1 receptor in mediating morphine reward behaviours, the nucleus accumbens (NAcc) and amygdala were selected for a preliminary investigation. Both of these regions express NK1 receptors (see Chapter 3), and have been implicated in the mediation of reward behaviours (see Chapter 1). However, in this study no attempt
was made to bring about ablation selectively in the core or shell regions of the NAcc or in the various subnuclei of the amygdala. However, this more general approach was used as a first attempt at replicating the behaviours of NK1\(^{-/-}\) mice following NK1 receptor-expressing cell ablation, with the aim of isolating the site of action of the SP / NK1 receptor system to one of these two regions of the mouse brain. The effects of cell ablation on anxiety behaviours were also assessed in these mice.

6.3. General materials and methods

Much of the work presented in this chapter was carried out as a joint study with Patricia Murtra, a PhD student based at the Universitas Miguel Hernández, Alicante, Spain. The work described in the following sections was divided equally between Dr. Murtra and myself: 6.4.1; 6.4.2; 6.4.3; 6.4.5; and 6.5.1.2.

6.3.1. Stereotaxic surgery

Stereotaxic surgery was used to deliver compounds to specific regions within the brains of mice. This technique involves the stabilisation of the anaesthetised mouse’s head within a frame. Following exposure of the skull, the position of landmark joints in the skull (Bregma and / or Lambda; see Figure 6.2) are measured, from which the position of the targets within the brain can be calculated on the basis of a brain atlas (Franklin & Paxinos 1997). A syringe containing the substance to be injected is attached to a manoeuvrable holder, fixed to the stereotaxic frame, which can be moved separately in three dimensions by way of calibrated screws. This fine movement of the syringe allows the tip of the needle to be positioned at a precise spot (0.1 mm accuracy) within the brain relative to the skull joint.
Mice were anaesthetised with halothane (Concord Pharmaceuticals, Dunmow, UK) in oxygen (flow rate: 0.6 l.min⁻¹). Anaesthesia was maintained with inhaled halothane throughout surgery (1.5 to 3 h) at a level at which there was no motor response to a foot pinch, but at which breathing rate and depth were normal. Levels of inspired anaesthetic were altered as appropriate. Mice were placed on a heated pad throughout surgery.

Mice were placed in a stereotaxic frame (Model 900 Small Animal Stereotaxic Instrument; David Kopf Instruments, Tujunga, USA) fitted with an adjustable palate holder and tooth plate for the mouse (Mouse Adaptor; David Kopf Instruments). Rat ear bars (David Kopf Instruments) were used, but positioned against the skull of the mouse rather than in the ear canal, since pressure on the external auditory meatus can lead to breathing difficulties. The hair over the scalp was trimmed with scissors, and the skin was swabbed with 10% povidone-iodine solution (Betadine® Antiseptic Solution; Seton Healthcare Group, Oldham, UK). A midline incision was made using a sterile razor blade, exposing the skull. An empty 5 μl Hamilton Microliter™ Syringe (700 Series; Hamilton Bonaduz, Bonaduz, Switzerland) fitted with a 22G needle (RN Series; Hamilton Bonaduz) was placed in a holder attached to the stereotaxic frame (Universal Holder; David Kopf Instruments) and used to measure the vertical position of Bregma and Lambda, and points 2.0 mm lateral of Bregma in each direction. The position of the tooth and ear bars were adjusted until the vertical positions of Bregma and Lambda, and the positions to either side of Bregma did not differ by more than 0.1 mm.

The antero-posterior (AP) and medio-lateral (ML) position of Bregma was measured by placing the tip of the needle at Bregma. The AP and ML positions of the injection sites were calculated and marked on the skull. Holes of approximately 1 mm diameter were
drilled through the skull, and the dura mater was cleared using a needle, exposing the top of the brain.

For each injection, the syringe was removed from the holder, filled with 1.0 μl of the injection solution and replaced in the holder. Bregma was measured again, and the needle moved to the injection site on one side and lowered to touch the brain surface. The vertical position of the needle tip was measured, and the dorso-ventral (DV) position of the injection site calculated. The needle was moved slowly to the injection site and left in position for 5 min. A drop of sterile saline (Steri-Amp) was placed on the head incision to prevent dehydration.

The solution was injected into the brain over 10 min, by depressing the plunger by 0.1 μl every minute. Following injection, the needle was left in place for a further 5 min to allow diffusion of the solution, before it was slowly removed from the brain. The syringe was removed and re-filled before further injections.

After all injections had been made, the incision was sutured (Mersilk Black W500; Ethicon, Cornelia, USA) and dusted with Cicatrin™ powder (The Wellcome Foundation, Greenford, UK). The mouse was then removed from the stereotaxic apparatus, given a subcutaneous injection of 1.0 ml sterile saline (Steri-Amp) and left in a warm place to recover. Once awake, the mouse was returned to its home cage.

6.3.2. Elevated plus maze

The elevated plus maze (EPM) is a test of anxiety levels widely used in rodent studies (Handley & Mithani 1984). It exploits the conflict between the animal's innate tendency to explore novel areas with their aversion for heights and open spaces (Montgomery 1955). The maze consists of four raised arms, two of which are enclosed, and two of which are open on all three sides (see Figure 6.3). Animals with higher levels of anxiety will make fewer entries into the open arms, and spend less time in them.

6.3.2.1. Apparatus

The EPM (see Figure 6.3) consisted of four black Plexiglas runways (300 mm [l] × 49 mm [w]) arranged in a cross shape and connected by a square central zone (49 mm × 49 mm).
The maze was raised 300 mm above the floor. Two opposite arms of the apparatus (‘closed arms’) had 150-mm high clear Plexiglas walls surrounding the runways, whilst the remaining two arms (‘open arms’) were not enclosed. A thin rim (2 mm high) of black tape was attached to the edge of the open arms to enable mice to feel the edge of the runway. The EPM was used under low light conditions (4 lx) with light being provided by a single 60 W bulb in the corner of the room.

![Figure 6.3 Elevated plus maze (EPM). View of apparatus from above.](image)

### 6.3.2.2. Procedure

Mice were placed individually on the central portion of the apparatus, facing an open arm. They were left to explore the apparatus for 5 min, and were recorded using a video camera. At the end of the session, the mice were returned to the home cage and the apparatus was thoroughly cleaned with water.

Following recording, the time spent in and the number of entries made into the open and closed arms of the maze were scored manually. An ‘entry’ was defined as movement of all four paws into an arm. Time spent in the central zone of the maze was discounted, with time spent in open arms of the maze expressed as a percentage of the total time spent in
either arm. Similarly, the number of entries into the open arms was calculated as a percentage of the total number of entries into any of the arms.

### 6.4. Preliminary experiments

#### 6.4.1. Optimisation of injection sites

Familiarisation with stereotaxic surgery and optimisation of the sites for injection of SP-SAP were achieved in a preliminary experiment. The red fluorescent dye tetramethylrhodamine (TMR) was injected into the 2 brain regions under study (the NAcc and amygdala), in order to achieve reproducible targeting of these sites.

#### 6.4.1.1. Materials and methods

Six adult male C57BL/6 mice (Harlan) were used in this experiment. They received unilateral injections of 1.0 µl 10.0 % dextran-conjugated TMR (3000 MW, anionic; Molecular Probes, Leiden, The Netherlands) into both the NAcc and the amygdala. One mouse died during surgery. The injection sites were determined from a mouse brain atlas (Franklin & Paxinos 1997), and are given in Table 6.1.

\[
\begin{array}{cccc}
\text{Brain region} & \text{Distance of injection site from Bregma (mm)} \\
& \text{AP} & \text{ML} & \text{DV}^1 \\
\hline
\text{NAcc} & 1.2 & \pm 1.0 & 4.0 \\
\text{Amygdala} & -1.5 & \pm 2.8 & 4.8 \\
\end{array}
\]

Mice were perfused 2 weeks after injection, and their brains were removed, post-fixed, cryoprotected and sectioned (see section 2.4.1). Every 3rd section through the brain was rinsed in 10.0 mM PB (see section 2.2.1), mounted onto gelatine-coated

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1 DV: distance below brain surface.
slides\(^1\) and left to air-dry in the dark. Sections were dehydrated through ethanol solutions of increasing strength (distilled water, 70 % ethanol × 2, 95 % ethanol × 2, 100 % ethanol × 2; 2 min in each), and cleared in Histoclear (2 min × 2; National Diagnostics). Slides were coverslipped in DPX mounting medium (BDH) and left to air-dry in the dark.

### 6.4.1.2. Results

The injection sites were visualised under green illumination (see section 2.4.4), under which TMR fluoresces red. The positions of the injection sites (data not shown) were compared to their desired targets and injection coordinates were adjusted accordingly. The DV position of NAcc injections was increased by 0.2 mm. No adjustment of amygdala injection sites was necessary. The adjusted injection sites are given in Table 6.2.

**Table 6.2 Adjusted injection sites for stereotaxic surgery.**

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Distance of injection site from Bregma (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP</td>
</tr>
<tr>
<td>NAcc</td>
<td>1.2</td>
</tr>
<tr>
<td>Amygdala</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

### 6.4.2. Comparison of targeted toxins

In addition to SP-SAP, an alternative toxin that targets NK1 receptor-expressing cells is available (SSP-SAP). SSP-SAP is formed by the conjugation of synthetic [Sar\(^9\),Met(O\(_2\))\(^{11}\)]-SP with SAP. It is claimed to be highly effective and selective for NK1 receptor-expressing cells with less non-specific damage than SP-SAP when injected at the same concentration (Wiley & Lappi 1999). In this experiment, SP-SAP and SSP-SAP were directly compared.

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1 Gelatine-coated slides:
- Wash Superfrost Microscope Slides (BDH) in warm soapy water.
- Rinse in distilled water.
- Immerse slides in 0.75 % gelatine (Sigma) + 0.05 % chromium potassium sulphate (Sigma) for 30 s.
- Dry at 40 - 60 °C.

2 DV: distance below brain surface.
by injecting them into the striata of mice and comparing the degree of ablation brought about by the two toxins.

6.4.2.1. Materials and methods

Four adult male C57BL/6 mice (Harlan) were used in this experiment. They were injected into the striatum (coordinates relative to Bregma: AP: 0.4 mm; ML: ± 2.0 mm; DV: 2.7 mm): 1.0 μl 1.00 μM SP-SAP (Advanced Targeting Systems, San Diego, USA) was injected into the right side, whilst the left side was injected with 1.0 μl SSP-SAP (Advanced Targeting Systems) at 1.00 μM (n = 2) or 100 nM (n = 2). After three weeks, the mice were perfused, and their brains were removed, post-fixed, cryoprotected and sectioned (see section 2.4.1). Every 3rd section was processed for immunohistochemistry (IHC) with primary antibodies raised against the NK1 receptor (Chemicon International; see section 2.4.2), detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB) or fluorescein isothiocyanate (FITC; see section 2.4.3). Sections were visualised microscopically (see section 2.4.4) and NK1 receptor immunoreactivity compared between areas injected with SP-SAP and SSP-SAP.

6.4.2.2. Results

In general, SP-SAP injection at 1.00 μM was found to cause a clearer loss of NK1-expressing neurones than SSP-SAP at either concentration (data not shown). Neither compound produced necrosis or alterations in gross tissue morphology. 1.00 μM SP-SAP was therefore chosen for use in the remainder of the study.

6.4.3. Confirmation of efficacy of substance P-saporin

Following selection of SP-SAP as the toxin for use in this study, its efficacy and specificity were compared following injection into the two brain regions under study.

6.4.3.1. Materials and methods

Five adult male C57BL/6 mice (Harlan) were used in this experiment. They were injected bilaterally with 1.0 μl 1.00 μM SP-SAP into the NAcc (n = 3) or amygdala (n = 2), according to the coordinates given in Table 6.2. One mouse (NAcc group) died after

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1 DV: distance below brain surface.
surgery. Four weeks after surgery, the mice were perfused and their brains were removed, post-fixed, cryoprotected and sectioned (see section 2.4.1). Every 4th section was processed for immunohistochemistry (IHC) with primary antibodies raised against the NK1 receptor (Chemicon International; see section 2.4.2), detected using DAB or FITC (see section 2.4.3).

The specificity of the ablation was assessed in mice injected with SP-SAP in the NAcc. Since choline acetyltransferase (ChAT) is localised in most NK1-expressing interneurones of the striatum (Aubry et al. 1993; Gerfen 1991; Kaneko et al. 1993), IHC for ChAT (see section 2.4.2) was used in a second set of sections as an additional marker of the loss of NK1 receptor-expressing cells. The remaining sections were processed for IHC for neuronal nuclei (NeuN; see section 2.4.2) to check for non-specific loss of neurones, or glial fibrillary acidic protein (GFAP; see section 2.4.2) to assess the extent of gliosis, a sign of brain injury. Control ("naïve") sections of the NAcc were taken from mice injected in the amygdala.

Sections were visualised microscopically (see section 2.4.4) and immunoreactivity compared between sections from SP-SAP-injected regions and control ("naïve") sections from uninjected regions.

**6.4.3.2. Results**

Injection of SP-SAP into the NAcc resulted in a clear loss of NK1 receptor immunoreactivity in a circular area of approximately 1 mm diameter around the injection site (Figure 6.4A). However, both mice also exhibited loss of NK1 receptor expressing neurones at the dorsomedial edge of the caudate putamen (CPu) adjacent to the lateral ventricle (Figure 6.4B), presumably due to the toxin flowing back as the needle was removed from the brain.
Figure 6.4 Ablation of NK1 receptor-expressing neurones by SP-SAP in the NAcc. Representative sections from the NAcc (A) and dorsomedial CPu (B) of a naïve mouse and a mouse 4 weeks after injection of SP-SAP in the NAcc, stained immunohistochemically for the NK1 receptor (FITC fluorescence). A clear loss of NK1 receptor immunoreactivity was observed in the shell and core subregions in the SP-SAP mouse, but this was coupled with additional loss of NK1 receptor-expressing neurones in the dorsomedial CPu lining the lateral ventricle. ac: anterior commissure; AcbC: nucleus accumbens (core); AcbSh: nucleus accumbens (shell); CPu: caudate putamen; LV: lateral ventricle. Scale bars = 1 mm.
Chapter six Behavioural effects of ablation of neurones expressing the neurokinin-1 receptor

There was no visible reduction in the number of NeuN-labelled nuclei at the injection site in the NAcc, and no increase in GFAP immunoreactivity, indicating that there was little non-specific neuronal damage at the injection site (Figure 6.5). Since the proportion of cells expressing the NK1 receptor in this brain region is estimated to be low (1 - 2 %), the similar density of NeuN-labelled nuclei in SP-SAP-injected and naïve mice is unsurprising. In addition, the ChAT-positive cells of the NAcc were missing at the site of the lesion (data not shown), indicating that the observed loss of NK1 receptor immunoreactivity was probably caused by cell death rather than an inability for the NK1 receptor to be detected immunohistochemically.
Figure 6.5 Effects of SP-SAP injection in the NAcc. High-power images of the NAcc of a naïve mouse and a mouse 4 weeks after injection of SP-SAP into the NAcc, stained immunohistochemically for the NK1 receptor, NeuN or GFAP. SP-SAP caused a clear loss of NK1 receptor immunoreactivity, but no visible changes in NeuN or GFAP staining. SP-SAP also caused loss of ChAT immunoreactivity (data not shown). Scale bar = 50 μm.
In the amygdala, SP-SAP caused a more subtle loss of NK1 receptor immunoreactivity, since the expression of the NK1 receptor is more diffuse in this brain region. However, a reduction in the intensity of staining was seen in the central nucleus of the amygdala, along with a loss of stained dendrites and cell bodies in the basolateral, basomedial, lateral and medial nuclei (Figure 6.6).
Figure 6.6 Ablation of NK1 receptor-expressing neurones by SP-SAP in the amygdala. Representative sections for the amygdala of a naïve mouse and a mouse 4 weeks after injection of SP-SAP into the amygdala. SP-SAP caused a subtle loss of immunoreactivity in the central, basolateral and medial nuclei of the amygdala. BLA: basolateral nucleus of the amygdala; BMA: basomedial nucleus of the amygdala; CeA: central nucleus of the amygdala; LA: lateral nucleus of the amygdala; MeA: medial nucleus of the amygdala. Scale bar = 1 mm.
6.4.4. Localisation of ablated cells' somata in the amygdala

Since the somata of NK1-expressing cells within the amygdala do not seem to express the NK1 receptor, it is difficult to identify which cells are being eliminated by SP-SAP in this brain region using IHC. In order to gain more information about the location of these cells, the reporter gene lacZ was used. Since lacZ is present in the cassette inserted into exon 1 of the NK1 receptor gene in NK1⁻/⁻ mice (see section 2.3.1), the product of this gene, β-galactosidase (βGal), is presumably expressed in same cells that express the NK1 receptor in wild type animals. These cells can be stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), an artificial substrate of βGal. In the presence of βGal, X-Gal forms a blue dimer, which can be visualised under bright field microscopy.

6.4.4.1. Materials and methods

Two adult male NK1⁻/⁻ and 3 adult male heterozygous NK1⁺/⁻ mice were used in this experiment (section 2.3.1). The NK1⁺/⁻ mice were injected unilaterally in the left amygdala with 1.00 μM SP-SAP according to the coordinates given in Table 6.2. Five weeks after injection of the NK1⁺/⁻ mice, all 5 mice were perfused and their brains removed, post-fixed, cryoprotected and sectioned (see section 2.4.1).

Every third section through the brain was stained for βGal activity. Sections were washed twice for 10 min in 0.1 M PB. They were then incubated in staining solution¹ overnight at 37 °C before two more washes in 0.1 M PB. Sections were mounted onto gelatine-coated slides (see page 169) and left to air dry. The sections were counterstained by immersion in Neutral Red solution² for 2 - 3 min. Sections were dehydrated through ethanol sections of increasing strength (distilled water, 70 % ethanol × 2, 95 % ethanol × 2, 100 % ethanol × 2; 30 s in each), and cleared in Histoclear (30 s × 2; National Diagnostics). Slides were

¹ Staining solution:
Phosphate-buffered saline (Dulbecco 'A' solution; Oxoid, Basingstoke, UK), containing:
5 mM K₃Fe(CN)₆ (Sigma)
5 mM K₄Fe(CN)₆ (Sigma)
2 mM MgCl₂ (Sigma)
0.01 % sodium deoxycholate (Sigma)
0.02 % Triton-X-100 (BDH)
2 % X-Gal solution (50 mg.ml⁻¹ in dimethylformamide [BDH]; Calbiochem, San Diego, USA)

² Neutral Red solution:
0.5 % Neutral Red (Gurr Certistain®; BDH)
1 % glacial acetic acid (BDH)
coverslipped in DPX mounting medium (BDH) and left to air-dry before being observed under bright field microscopy (see section 2.4.4). The distribution of βGal-positive cells was observed in the amygdalae of the mice, and the effect of SP-SAP in heterozygous mice was determined by comparing injected and uninjected sides.

6.4.4.2. Results

In the amygdalae of NK1⁻/⁻ mice, βGal-positive nuclei were observed diffusely through the basolateral, basomedial, central, and medial nuclei (Figure 6.7). Stained nuclei were more numerous in the medial nucleus of the amygdala than other subnuclei.

In the amygdala of NK1⁺/− mice, fewer βGal-positive nuclei were seen than in NK1⁻/⁻ mice. This is presumably due to the lower level of expression of the enzyme in mice with only one copy of the gene present. However, the distribution of the stained nuclei was similar to that seen in NK1⁻/⁻ mice. Injection of SP-SAP into the amygdala resulted in a loss of βGal-positive nuclei in the amygdala (Figure 6.8). This suggests that, although it is difficult to assess the precise location of the cells ablated by SP-SAP in the amygdala using immunohistochemistry, their somata are distributed throughout the central, basolateral and medial nuclei of the amygdala, but with a higher density in the medial nucleus.
Figure 6.7 βGal activity in the amygdala of the NK1⁻/⁻ mouse. A: Representative section of the amygdala of a NK1⁻/⁻ mouse, stained with X-Gal for cells with βGal activity (blue) and counterstained with Neutral Red. Blue βGal-positive nuclei can be seen scattered diffusely throughout the amygdala, notably in the medial nucleus. BLA: basolateral nucleus of the amygdala; MeA: medial nucleus of the amygdala; Pir: piriform cortex. Scale bar = 1 mm. B: High-power image of the medial nucleus of the amygdala. Arrows indicate βGal-positive nuclei. Scale bar = 250 μm.

Figure 6.8 Ablation of βGal-positive nuclei in the amygdala of NK1⁺/⁻ mice. Representative sections from a NK1⁺/⁻ mouse five weeks after unilateral injection of SP-SAP into the amygdala, stained with X-Gal for cells with βGal activity (blue) and counterstained with neutral red. Left: low-power photomicrographs of the amygdala. BLA: basolateral nucleus of the amygdala; MeA: medial nucleus of the amygdala; Pir: piriform cortex. Scale bar = 1 mm. Right: high-power photomicrographs of the medial nucleus of the amygdala. Arrows indicate some of the βGal-positive nuclei. Scale bar = 250 μm. On the uninjected ("naïve") side of the brain, βGal-positive nuclei can be seen scattered diffusely though the central, basolateral and medial nuclei of the amygdala, but these are absent on the side of the brain injected with SP-SAP.
6.4.5. **Comparison of behavioural effects of substance P-saporin injection in the nucleus accumbens and amygdala**

Following establishment of the optimal injection coordinates, the efficacy of SP-SAP in selectively ablating NK1 receptor-expressing neurones in the brain, and localisation of the somata of ablated cells in the amygdala, a study was undertaken to compare the behavioural effects of ablation of NK1 expressing cells in these two regions. Following bilateral injection of SP-SAP into either the NAcc or the amygdala, the mice were tested for anxiety, spontaneous locomotor activity and reward to morphine, in order to assess whether loss of these cells in either region brings about similar behaviours to those seen in NK1^−/− mice.

6.4.5.1. **Materials and methods**

6.4.5.1.1. **Mice and surgery**

Fifty-eight adult male C57BL/6 mice (Harlan) were used in this experiment. They were divided into three groups: two of these received bilateral injections of 1.0 µl 1.00 µM SP-SAP into the NAcc (n = 16) or the amygdala (n = 17), according to the coordinates given in Table 6.2. Two mice died during surgery (1 from each group). The 3rd group of 25 mice were used as naïve controls (see below).

6.4.5.1.2. **Behavioural analysis**

Behavioural analysis of mice undergoing SP-SAP injection was begun 5 weeks after surgery. One of the remaining mice from the NAcc group was not tested due to severe injury following fighting in the home cage. Mice were tested in groups of 12. Each mouse underwent three behavioural tests: the EPM; spontaneous locomotor activity in the open field; and CPP to morphine.

Twenty-four hours after testing on the EPM (see above), locomotor activity was assessed using the open field apparatus under normal lighting conditions (see section 2.5.4.1). Mice were placed in the open field arena and ambulatory locomotor activity assessed over 10 min.

Beginning 3 d after locomotor assessment, CPP to morphine (3.0 mg.kg⁻¹) was assessed (see section 2.5.3), with injections given in a volume of 10.0 ml.kg⁻¹. Three mice showed
preference for one of the two compartments during preconditioning (one from the NAcc group and two from the amygdala group), and were omitted from analysis.

6.4.5.1.3. Immunohistochemistry

After the behavioural experiments, mice were perfused, and their brains removed, post-fixed, cryoprotected and sectioned (see section 2.4.1). IHC was carried out on every 4th section through the brain with primary antibodies raised against the NK1 receptor (Chemicon International; see section 2.4.2), detected using DAB or FITC (see section 2.4.3). IHC for ChAT (see section 2.4.2) was used in a second set of sections as an additional marker of the loss of NK1 receptor-expressing cells following injection of SP-SAP in the NAcc. The remaining sections were processed for IHC for NeuN or GFAP (see above).

6.4.5.1.4. Analysis

Examination of immunohistochemically-stained sections was carried out independently by both experimenters who were blinded to the results of behavioural testing. Results were compared and agreed between the two observers.

Sections stained for the NK1 receptor were examined and compared to the staining pattern of control mice. The loss of NK1 receptor immunoreactivity was assessed in each mouse: animals with no visible bilateral loss of NK1 receptor-expressing neurones in the brain region of interest were excluded from analysis, as were mice with visible physical damage to the brain or bilateral loss of NK1 receptor immunoreactivity in any other brain region. In mice injected with SP-SAP in the NAcc, ChAT immunoreactivity was used as a secondary marker of NK1 receptor-expressing cells. Sections stained with NeuN and GFAP were also compared to control mice's sections. Major decreases in neuronal nuclear staining or increases in GFAP immunoreactivity were considered as signs of non-specific damage caused by the injection procedure.

Due to the frequent loss of NK1 receptor-expressing neurones from the dorsomedial portion of the CPu following injection of SP-SAP into the NAcc (see above), mice from this group were not excluded on the basis of additional ablation in this brain region. The NAcc group therefore contains mice that exhibited ablation of these cells in both the NAcc
and the dorsomedial CPus, rather than the NAcc alone. However, the 3 mice that did not exhibit ablation in both these regions were excluded from this group, leaving eleven in this group. In the amygdala group, one mouse was excluded on the basis of histological examination, leaving 15.

For each injection site, the behaviour of mice on the EPM (time measure and numbers of entries), spontaneous locomotor activity levels and CPP scores were compared with those of control mice using 1-way ANOVAs (see section 2.6).

6.4.5.1.5. Control mice

A further group of 25 mice were used as controls in this experiment. These mice did not undergo stereotaxic surgery, but 12 of them underwent the same set of behavioural tests as mice injected with SP-SAP. The remaining 13 mice underwent CPP with saline (10.0 ml.kg⁻¹; see section 2.5.3).

CPP scores from saline- and morphine-treated control mice were compared using a 1-way ANOVA (see section 2.6). Due to considerable variability in the data, the one value that fell more than 2 standard deviations (SDs) from the group mean (saline group) was omitted from analysis.

6.4.5.2. Results

6.4.5.2.1. Nucleus accumbens

Mice with loss of NK1 receptor-expressing neurones in the NAcc and dorsomedial CPu exhibited similar behaviours to naïve mice on the EPM (Figure 6.9). One-way ANOVAs indicated that there were no significant differences between the groups in the time spent in the open arms ($F_{1,21} = 0.17; P = 0.680$), or in the number of entries made into them ($F_{1,21} = 0.16; P = 0.697$).
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Figure 6.9 Effects of SP-SAP in NAcc: EPM behaviour. Mean ± SEM time spent in (A) and number of entries made into (B) the open arms of the EPM by naive mice \( n = 12 \) and mice 5 weeks after injection of SP-SAP into the NAcc \( n = 11 \). Ablation of NK1 receptor-expressing neurones in the NAcc and dorsomedial CPu did not affect behaviour on the EPM.

Mice with ablation in these regions also exhibited similar levels of locomotor activity to naive mice in the open field (Figure 6.10). A 1-way ANOVA revealed no significant difference in the amount of ambulatory activity between the two groups \( F_{1,21} = 0.06; P = 0.813 \).

Figure 6.10 Effects of SP-SAP in NAcc: locomotor activity. Mean ± SEM ambulatory locomotor activity in the open field test by naive mice \( n = 12 \) and mice 5 weeks after injection of SP-SAP into the NAcc \( n = 11 \). Ablation of NK1 receptor-expressing neurones in the NAcc and dorsomedial CPu did not affect locomotor activity.

These mice also exhibited similar degrees of CPP to morphine \( (3.0 \text{ mg.kg}^{-1}) \) to naive mice. A 1-way ANOVA revealed no significant difference between groups in the scores achieved in the procedure \( F_{1,20} = 0.39; P = 0.537 \).
Figure 6.11 Effects of SP-SAP in NAcc: morphine CPP. Mean ± SEM CPP scores following conditioning with 3.0 mg.kg$^{-1}$ morphine in naïve mice ($n = 12$), and mice 5 weeks after injection of SP-SAP into the NAcc ($n = 10$). Ablation of NK1 receptor-expressing neurones in the NAcc and dorsomedial CPu did not affect CPP to morphine.

Loss of NK1 receptor-expressing neurones in the NAcc therefore did not have significant effects on anxiety levels, locomotor activity or morphine reward at the dose tested.

6.4.5.2.2. Amygdala

Mice with loss of NK1 receptor immunoreactivity in the amygdala exhibited similar behaviour to naïve mice in the EPM (Figure 6.12). Although mice injected with SP-SAP into the amygdala showed a tendency to spend more time in the open arms than naïve mice, a 1-way ANOVA revealed that this difference was not statistically significant ($F_{1,25} = 3.44; P = 0.075$). There was also no significant difference between the groups in the number of entries made into the open arms ($F_{1,25} = 0.14; P = 0.713$).
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Figure 6.12 Effects of SP-SAP in amygdala: EPM behaviour. Mean ± SEM time spent in (A) and number of entries made into (B) the open arms of the EPM by naïve mice (*n* = 12) and mice 5 weeks after injection of SP-SAP into the amygdala (*n* = 15). Ablation of NK1 receptor-expressing neurones in the amygdala did not affect behaviour on the EPM.

Mice with ablation in the amygdala exhibited slightly lower levels of spontaneous locomotor activity than naïve mice in the open field test (Figure 6.13). However, a 1-way ANOVA revealed that this difference did not reach statistical significance (*F*$_{1,24}$ = 3.04; *P* = 0.093).

Figure 6.13 Effects of SP-SAP in amygdala: locomotor activity. Mean ± SEM ambulatory locomotor activity in the open field test by naïve mice (*n* = 12) and mice 5 weeks after injection of SP-SAP into the amygdala (*n* = 15). Ablation of NK1 receptor-expressing neurones in the amygdala did not affect locomotor activity.

Mice with ablation of NK1 receptor-expressing neurones in the amygdala exhibited lower CPP scores than naïve mice following conditioning with 3.0 mg.kg$^{-1}$ morphine (Figure 6.14). A 1-way ANOVA revealed that the scores achieved by mice with loss of NK1...
receptor-expressing neurones in the amygdala were significantly lower than those of naive mice ($F_{1,24} = 4.88; P = 0.037$).

![Figure 6.14 Effects of SP-SAP in amygdala: CPP to morphine.](image)

These results suggest that ablation of NK1 receptor-expressing neurones in the amygdala, but not the NAcc, led to reductions in morphine reward, without affecting anxiety levels or locomotor activity.

### 6.4.5.2.3. Control mice

Following completion of the behavioural experiments involving morphine CPP, the behaviour of naive mice following CPP to saline was examined. Mice undergoing saline conditioning in both compartments of the apparatus exhibited CPP scores that tended to be lower than those treated with morphine (3.0 mg.kg$^{-1}$). However, due to the variability in the data, the scores of saline- and morphine-treated mice did not differ significantly (1-way ANOVA: $F_{1,22} = 3.03; P = 0.096$).
6.5. **Behavioural effects of ablation of neurokinin-1 receptor-expressing neurones in the amygdala**

Following the preliminary observation that ablation of NK1 receptor-expressing neurones in the amygdala, but not in the NAcc and dorsomedial CPu of mice brought about deficits in morphine reward behaviour, a set of experiments was designed to confirm and extend this finding. Firstly, a different strain of mouse was used, and tested for morphine CPP in the naïve state before the ablation experiment was begun. A hybrid C57BL/6 × 129/sv line was chosen, as this was similar to the wild type mice used in the original paper describing the effects of genetic ablation of NK1 receptors on morphine CPP (Murtra et al. 2000b). The observation of reduced morphine CPP following ablation of NK1 receptor-expressing neurones in the amygdala was then confirmed in a set of controlled experiments. These were designed to compare the effects of NK1 receptor-expressing neurone ablation on the CPP induced by morphine and saline. Control injections of the constituent molecules of SP-SAP were also attempted. SP was injected in order to control for the activation of NK1 receptors at the site of injection, whereas SAP injections were used to control for the non-specific effects of toxin administration on cells other than those which express NK1 receptors. The injection of both substances also acted as controls for the stress and pain associated with handling, surgery and the behavioural tasks undertaken. Following surgery, the mice were tested for anxiety behaviour, CPP to morphine or saline, as well as the locomotor response to an acute injection of saline or morphine, in order to compare the effects of ablation of NK1 receptor-expressing
neurones in this brain region to the morphine-induced behaviours observed in NK1−/− mice.

6.5.1. Materials and methods

6.5.1.1. Mice

Adult male C57BL/6 and female 129S2/Sv mice were purchased from Harlan and housed as breeding pairs or trios (2 females per male). Their male F1 offspring (n = 220) were used in these experiments. Experiments were begun when mice were 6 - 8 weeks old.

6.5.1.2. Morphine experiments

6.5.1.2.1. Surgery

One hundred and eighty-two mice were used in these experiments. Mice received bilateral injections into the amygdala under stereotaxic surgery, according to the coordinates given in Table 6.2. Injections were of 1.0 μl 1.00 μM SP-SAP (n = 78), 1.00 μM SP (n = 28; Sigma) or 1.00 μM SAP (n = 40; Advanced Targeting Systems). Thirty-six mice did not undergo surgery and were used as naïve controls. Two mice died during surgery (one each from the SP-SAP and SP groups), and three mice died after surgery (two from the SP-SAP group and one from the SAP group).

6.5.1.2.2. Behavioural analysis

Beginning 5 weeks after surgery, each of the four groups of mice (SP-SAP; SP; SAP; naïve) was subdivided into two further groups. The number of mice in each group is given in Table 6.3.
Table 6.3 Numbers of mice used in morphine experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group I</th>
<th>Group II</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-SAP</td>
<td>38</td>
<td>37</td>
<td>75</td>
</tr>
<tr>
<td>SP</td>
<td>15</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>SAP</td>
<td>21</td>
<td>18</td>
<td>39</td>
</tr>
<tr>
<td>Naïve</td>
<td>18</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>92</strong></td>
<td><strong>85</strong></td>
<td><strong>177</strong></td>
</tr>
</tbody>
</table>

Mice in both groups were assessed for anxiety behaviour using the EPM (see above). Thereafter, the two groups were treated separately: Group I mice underwent morphine CPP and the locomotor response to saline, whilst Group II mice underwent saline CPP and testing for the locomotor response to acute morphine. The order of the two tests was swapped between the two groups to ensure that morphine injections occurred during the final behavioural test. Mice underwent surgery and were tested in groups of 12 over a period of approximately 18 months — each group contained mice from any or all of the four treatment groups.

One day after testing on the EPM, mice in Group I underwent testing of the locomotor response to an acute injection of saline in the open field (see section 2.5.4.1). Mice were given an injection of sterile saline (Steri-Amp; 10.0 ml.kg⁻¹) and returned to the home cage. Thirty minutes after injection, locomotor activity was monitored for 10 min. Beginning 3 – 5 d after locomotor assessment, they were then tested for CPP to morphine (3.0 mg.kg⁻¹), given in a volume of 6.67 ml.kg⁻¹ (see section 2.5.3). Thirty-eight animals demonstrated a preference for one of the compartments during preconditioning (13 from the SP-SAP group, 6 from the SP group, 13 from the SAP group and 6 from the naïve group) and were excluded from the remainder of the behavioural experiment.

Beginning 1 d after testing on the EPM, Group II mice underwent CPP with saline (6.67 ml.kg⁻¹; see section 2.5.3). Mice which demonstrated a preference for one of the two compartments during preconditioning (14 from the SP-SAP group, 3 from the SP group, 9
from the SAP group and 4 from the naïve group) were not removed from the experiment, but assigned to receive the first injection of saline in one of the two compartments at random. They underwent the entire CPP procedure but their CPP scores were omitted from statistical analysis. Three to five days after the test session, the locomotor effects of an acute injection of morphine (10.0 mg.kg\(^{-1}\)) were assessed in the open field (see section 2.5.4.1). The mice were given an injection of morphine in a volume of 10.0 ml.kg\(^{-1}\) and returned to the home cage. Thirty minutes after injection, locomotor activity was monitored for 10 min.

6.5.1.2.3. Immunohistochemistry

After the behavioural experiments, mice were perfused, and their brains removed, post fixed, cryoprotected and sectioned (see section 2.4.1). IHC was carried out for the NK1 receptor, NeuN and GFAP as described above.

6.5.1.2.4. Analysis

Examination of immunohistochemically-stained sections was carried out independently by both experimenters who were blinded to the results of behavioural testing. Results were compared and agreed between the two observers.

The position and extent of loss of NK1 receptor-expressing neurones in the amygdala was assessed in the brains of mice from the SP-SAP group. The criteria for inclusion in the analysis were more stringent than in the preliminary experiments described above: animals were omitted from analysis unless they had a clear reduction of NK1 receptor immunoreactivity throughout the amygdala on both sides of the brain. Animals with visible necrosis or bilateral loss of NK1 receptor-expressing neurones in any other brain region were also omitted. For the remaining three groups of mice (SP, SAP and naïve), the integrity of NK1 receptor immunoreactivity was checked throughout the brain. In all groups, non-specific loss of neurones was assessed in sections processed for NeuN IHC, whilst gliosis was sought in sections processed for GFAP.

Forty-eight mice from the SP-SAP group were omitted from analysis on the basis of the above criteria (23 from Group I and 25 from Group II). No mice were omitted from the SP or SAP groups.
The two measures of behaviour on the EPM (time in open arms and number of entries into open arms) were compared across groups using 1-way ANOVAs with treatment (SP-SAP, SP and SAP) as the between-subjects factor (see section 2.6). Post hoc Tukey comparisons were used following significant main effects. Two mice from the SP group were omitted: one did not move from the central zone of the maze during the 5 min recording, and the other jumped from the maze soon after the start of the trial. For logistical reasons, 12 mice from the naïve group were not tested on the EPM. The final numbers of mice in each group are given in Table 6.4.

CPP scores from animals without preference during preconditioning and which were not omitted on the basis of histology were compared using a 2-way ANOVA with treatment and drug as between-subject factors. Post hoc analysis was carried out using Tukey comparisons (see section 2.6). Due to considerable variability in the data, CPP scores that fell more than 2 SDs from the group mean were excluded from analysis (one from the morphine SP group and two from the morphine SAP group). As two further mice from the morphine SAP group were given injections in the wrong compartment of the CPP apparatus during conditioning, their scores were also excluded from analysis. The final numbers of mice in each group are given in Table 6.4.

The measure of locomotor activity was analysed using a 2-way ANOVA with treatment and drug as between-subject factors, followed by post hoc Tukey comparisons (see section 2.6). The locomotor recording equipment malfunctioned on many of the trials. As such, the number of mice included in the final analysis was low. Data from 3 mice were also excluded from analysis as they fell more than 2 SDs from the group mean (one from the morphine naïve group, and two from the morphine SP-SAP group). The final numbers of mice in each group are given in Table 6.4.
Table 6.4 Final numbers of mice in analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EPM</th>
<th>CPP</th>
<th>Locomotor assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
</tr>
<tr>
<td></td>
<td>(morphine)</td>
<td>(saline)</td>
<td>(morphine)</td>
</tr>
<tr>
<td>SP-SAP</td>
<td>27</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>SP</td>
<td>25</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>SAP</td>
<td>39</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Naïve</td>
<td>24</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>36</td>
<td>40</td>
</tr>
</tbody>
</table>

6.5.1.3. Conditioned place preference to cocaine

Thirty-eight mice were used in this experiment. Twenty-two mice underwent bilateral injection of 1.0 μl 1.00 μM SP-SAP into the amygdala, and 16 were used as naïve controls. Two mice died during surgery. Beginning 5 weeks after surgery, both groups of mice were tested for CPP to cocaine (5.0 mg.kg⁻¹) injected in a volume of 10.0 ml.kg⁻¹ (see section 2.5.3). None of the mice displayed preference for one of the two compartments during preconditioning. After the behavioural experiments, the mice were perfused and processed for IHC as described above. Sections were observed microscopically (see section 2.4.4) and loss of NK1 receptor-expressing neurones was assessed in the SP-SAP group, resulting in the omission of 7 mice from analysis. CPP scores were compared to saline controls from Group II of the morphine experiment using a 2-way ANOVA with drug and treatment (naïve vs. SP-SAP) as between-subject factors (see section 2.6).

6.5.2. Results

6.5.2.1. Histology

Injection of SP-SAP into the amygdala caused bilateral reductions of NK1 receptor immunoreactivity restricted to the subnuclei of the amygdala in 42 (43.3 %) of the 97 mice injected. Injection of SP or SAP into the amygdala did not cause any visible changes in NK1 receptor immunoreactivity (Figure 6.16).
Naive

SP-SAP

SP

SAP

Figure 6.16 NK1 receptor immunoreactivity in the amygdala following injection of SP-SAP, SP or SAP. Representative sections from the amygdala of naïve mice, or mice around 7 weeks after injection of SP-SAP, SP or SAP into the amygdala, immunohistochemically stained for the NK1 receptor and labelled with DAB. SP-SAP caused a visible loss of NK1 receptor-expressing neurones in the basolateral, basomedial and medial nuclei, and a reduction in the intensity of staining in the central nucleus. This does not occur following injection of SP or SAP alone into the amygdala. BLA: basolateral nucleus of the amygdala; CeA: central nucleus of the amygdala; MeA: medial nucleus of the amygdala. Scale bar = 500 μm.
Immunohistochemical detection of NeuN did not reveal any visible alterations in the density of neuronal nuclei in the amygdalae of injected mice, suggesting that SP-SAP, SP and SAP did not have non-specific toxic effects on neurones within the amygdala (Figure 6.17). Immunohistochemistry for GFAP revealed slightly greater levels of gliosis in the amygdalae of mice injected with SP-SAP than those injected with SP or SAP. The staining in the latter two groups was similar to that in naïve controls (Figure 6.18). Furthermore, in sections where the path taken by the needle was visible, there were numerous GFAP-positive astrocytes visible around the needle position. This is probably due to the physical damage experienced in this region, but was similar in mice receiving each of the three compounds. The non-specific damage caused by the needle was therefore experienced to similar degrees in the three groups of mice, although SP-SAP may have caused slight increases in gliosis, possibly due to the death of NK1 receptor-expressing cells.
Figure 6.17 NeuN immunoreactivity in the amygdala following injection of SP-SAP, SP or SAP. Representative sections from the amygdala of naïve mice, or mice around 7 weeks after injection of SP-SAP, SP or SAP into the amygdala, immunohistochemically stained for NeuN and labelled with Texas Red. Left: low-power photomicrographs of the amygdala. Note needle track visible in SP-SAP and SAP sections. Scale bar = 500 μm. Right: high-power image of the medial nucleus of the amygdala near the injection target site. Scale bar = 250 μm. There is no visible loss of neuronal nuclei in the amygdala following injection of any of the three compounds.
Figure 6.18 GFAP immunoreactivity in the amygdala following injection of SP-SAP, SP or SAP. Representative sections from the amygdala of naïve mice, or mice around 7 weeks after injection of SP-SAP, SP or SAP into the amygdala, immunohistochemically stained for GFAP and labelled with FITC. Left: low-power photomicrographs of the amygdala. Note needle track visible in SP-SAP and SAP sections, surrounded by numerous GFAP-positive astrocytes. Scale bar = 500 μm. Right: high-power image of the medial nucleus of the amygdala near the injection target site. Scale bar = 100 μm. Injection of SP-SAP causes a slight increase in the density of GFAP-positive astrocytes, which persists at least 7 weeks, but this is not observed in SP- or SAP-injected mice.
6.5.2.2. Behaviour of naïve mice

On the EPM, naïve mice spent 37.7 ± 4.0% of the time in the open arms of the maze, and 41.2 ± 2.8% of the entries they made were into the open arms.

Naïve mice exhibited a clear CPP to morphine (3.0 mg.kg⁻¹; Figure 6.19A). A 1-way ANOVA revealed a significant effect of drug (saline vs. morphine) on CPP score (F[1,24] = 6.35; P = 0.019). They also exhibited locomotor stimulation 30 min after an acute injection of morphine (10.0 mg.kg⁻¹), as assessed in the open field test of ambulatory locomotor activity (1-way ANOVA: F[1,22] = 26.3; P < 0.001; Figure 6.19B).

Naïve mice also exhibited a strong CPP to cocaine (5.0 mg.kg⁻¹), which was greater than that seen with morphine (Figure 6.20). A 1-way ANOVA revealed a significant effect of drug on CPP score (F[1,28] = 29.7; P < 0.001).
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Figure 6.20 Behaviour of naïve mice: cocaine CPP. Mean ± SEM CPP scores achieved by naïve male C57BL/6 × 129/sv following conditioning with saline (n = 14) or cocaine (5 mg.kg⁻¹; n = 16). Naive mice showed a strong CPP to this dose of cocaine. ***P < 0.001 vs. saline (1-way ANOVA).

6.5.2.3. Elevated plus maze behaviour

On the EPM, mice with ablation of NK1 receptor-expressing neurones in the amygdala spent less time in the open arms, and made fewer entries into the open arms than naive mice. When compared to mice with control injections of SAP or SP into the amygdala, they tended to spend less time in the open arms and make fewer entries into them than both control groups (Figure 6.21).

A 1-way ANOVA of the time spent in the open arms by the three groups of mice revealed a significant main effect of treatment ($F_{2,88} = 5.32; P = 0.007$). Post hoc Tukey comparisons revealed a significant difference in the amount of time spent in the open arms between mice injected with SP-SAP and SP ($P = 0.006$), but the difference between SP-SAP- and SAP-injected mice narrowly missed statistical significance ($P = 0.058$). There was no difference in the time spent in the open arms in SP- and SAP-injected mice ($P = 0.476$).

A 1-way ANOVA of the number of entries into the open arms also revealed a significant effect of treatment ($F_{2,88} = 4.74; P = 0.011$). Post hoc Tukey comparisons revealed that the number of entries made by mice injected with SP-SAP was lower than that made by those injected with SP ($P = 0.013$) and SAP ($P = 0.048$), but there was no difference in the number of entries made by the latter two groups ($P = 0.696$).

Taken together, ablation of NK1 receptor-expressing neurones in the amygdala caused an increase in anxiety as assessed on the EPM, which was manifest in both the time spent in the open arms of the maze, and the number of entries made into them.
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6.5.2.4. Conditioned place preference

Mice with ablation of NK1 receptor-expressing neurones in the amygdala exhibited similar CPP scores following conditioning with morphine (3.0 mg.kg\(^{-1}\)) and saline. However, the CPP scores achieved by control mice injected with SP or SAP were higher following conditioning with morphine than with saline (Figure 6.22).

A 2-way ANOVA revealed a significant main effect of drug (\(F_{1,44} = 10.81; P = 0.002\)) and a significant drug by treatment interaction (\(F_{2,44} = 3.96; P = 0.026\)), but no significant main effect of treatment (\(F_{2,44} = 1.83; P = 0.172\)). *Post hoc* Tukey comparisons revealed that CPP scores achieved by morphine- and saline-conditioned mice injected with SP differed significantly (\(P = 0.038\)). A similar effect was seen in mice injected with SAP into the amygdala (\(P = 0.046\)), but mice injected with SP-SAP did not display any such difference (\(P = 1.000\)). However, comparison across the three treatment groups failed to reveal significant differences in CPP scores following morphine conditioning (\(P > 0.9\)), or saline conditioning (\(P > 0.1\)). Mice with ablation of NK1 receptor-expressing neurones in the amygdala did not therefore exhibit a significant CPP to morphine at a dose of 3.0 mg.kg\(^{-1}\): this seems to be due to a combined effect of a decrease in morphine CPP scores, and an increase in the scores achieved by mice following saline conditioning following SP-SAP injection.
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Figure 6.22 Effects of ablation of NK1 receptor-expressing neurones in the amygdala: morphine CPP. Mean ± SEM CPP scores following conditioning with saline or morphine (3.0 mg.kg⁻¹) by mice 5 weeks after injection of SP-SAP (saline: n = 8; morphine: n = 10), SP (saline: n = 9; morphine: n = 8) or SAP (saline: n = 9; morphine: n = 6) into the amygdala. SP-SAP-injected mice did not exhibit CPP to morphine, but SP- and SAP-injected mice showed a clear preference for a morphine-associated context. *P < 0.05 vs. saline (post hoc Tukey comparisons).

Conversely, mice with ablation of NK1 receptor-expressing neurones in the amygdala exhibited a clear CPP to 5.0 mg.kg⁻¹ cocaine, with greater CPP scores than saline-conditioned mice (Figure 6.23). A 2-way ANOVA revealed a significant main effect of drug (F_{1,46} = 28.3; P < 0.001), but no significant main effect of treatment (naïve vs. SP-SAP; F_{1,46} = 1.10; P = 0.300) or drug by treatment interaction (F_{1,46} = 1.83; P = 0.183). Reward to cocaine was therefore unaffected by the ablation of NK1 receptor-expressing neurones in the amygdala, with both naïve and SP-SAP-injected mice demonstrating CPP to cocaine at this dose.
Figure 6.23 Effects of ablation of NK1 receptor-expressing neurones in the amygdala: cocaine CPP. Mean ± SEM CPP scores following conditioning with saline or cocaine (5.0 mg.kg⁻¹) by naive mice (saline: n = 14; cocaine: n = 16), and mice 5 weeks after injection of SP-SAP into the amygdala (saline: n = 8; cocaine: n = 15). Both groups of mice showed a clear preference for a cocaine-associated context.

6.5.2.5. Locomotor effects of morphine

Mice with ablation of NK1 receptor-expressing neurones in the amygdala exhibited similar levels of ambulatory locomotor activity after an acute injection of saline to control mice injected with SP or SAP. Following an injection of morphine (10.0 mg.kg⁻¹), SP- and SAP-injected mice showed increases in locomotor activity, but this increase was reduced in SP-SAP-injected mice (Figure 6.24).

A 2-way ANOVA revealed significant main effects of drug ($F_{1,32} = 61.5; P < 0.001$), treatment ($F_{2,32} = 6.39; P = 0.005$) and a significant drug by treatment interaction ($F_{2,32} = 5.59; P = 0.008$). Post hoc Tukey comparisons revealed that morphine caused increases in ambulatory locomotor behaviour relative to saline in mice injected with SP ($P = 0.010$) and SAP ($P < 0.001$), but this did not occur in SP-SAP-injected mice ($P = 0.285$). Furthermore, locomotor activity levels following morphine administration were lower in mice injected with SP-SAP than those injected with SAP ($P = 0.007$), but not those injected with SP ($P = 0.257$). The reason for this latter finding is probably the low number of animals in the SP morphine group ($n = 2$), due to failure of the automatic recording equipment on many of this group’s trials.
Figure 6.24 Effects of ablation of NK1 receptor-expressing neurones in the amygdala: locomotor effects of morphine. Mean ± SEM ambulatory locomotor activity 30 min after an acute injection of saline or morphine (10 mg·kg$^{-1}$) measured for 10 min in the open field, in mice 5 weeks after injection of SP-SAP (saline: $n = 13$; morphine: $n = 4$), SP (saline: $n = 3$; morphine: $n = 2$) or SAP (saline: $n = 9$; morphine: $n = 7$) into the amygdala. SP-SAP-injected mice did not exhibit the locomotor stimulant effects of morphine observed in SP- and SAP-injected mice. **$P < 0.01$, ***$P < 0.001$ (post hoc Tukey comparisons).

These results suggest that ablation of NK1 receptor-expressing neurones in the amygdala also brings about reductions in the locomotor stimulant effects of acute morphine administration.

6.6. Discussion

The ultimate purpose of the studies described in this chapter was to examine the behavioural effects of the ablation of NK1 receptor-expressing cells from discrete brain regions in the mouse, and to compare these behaviours to those observed in NK1$^{-/-}$ mice. The data presented provide strong evidence for a role of these neurones within the amygdala in the mediation of morphine reward behaviours, as well as the modulation of anxiety levels. Furthermore, they suggest that these neurones within the NAcc may be less important than those in the amygdala, since ablation of these neurones in the NAcc did not bring about significant alterations in behaviour, whilst ablation of those in the amygdala led to a similar behavioural phenotype to that observed in NK1$^{-/-}$ mice.

6.6.1. Selection of substance P-saporin

Following optimisation of the stereotaxic injection procedure, ensuring reproducible targeting of the NAcc and amygdala, the efficacy of SP-SAP and its analogue SSP-SAP
were compared. These two compounds had previously been compared \textit{in vitro} in KNRK cells transfected with the NK1 receptor and \textit{in vivo} following injection into the rat striatum. These studies revealed that while SP-SAP caused incomplete lesions of NK1 receptor-expressing cells, even at doses which produced non-specific damage (Wiley & Lappi 1997), the conjugate of SAP with SSP, which is more stable than SP and has a longer-lasting effect at NK1 receptors, was found to cause selective ablation of NK1 receptor-expressing cells at lower doses than SP-SAP without loss of other striatal neurones (Wiley & Lappi 1999). In the mouse, we observed loss of NK1-expressing neurones in the striatum following injection of SSP-SAP at the two concentrations tested. However, SP-SAP caused a clearer destruction of these cells, without the visible necrosis reported in the rat striatum (Wiley & Lappi 1997). The differential effect of the two neurotoxins may be due to a species difference, possibly in the binding kinetics of the compounds to the NK1 receptor. However, without more thorough investigation, conclusions based on these findings are somewhat speculative. Based on the observation of a more reliable ablation with SP-SAP in this experiment, this compound was chosen for the remainder of the study.

The efficacy and specificity of NK1 receptor-expressing neurone ablation was confirmed in the NAcc and amygdala of mice. Although SP-SAP caused a reduction in NK1 receptor immunoreactivity in a circular area around the injection site in both brain regions, its additional loss in the dorsomedial portion of the CPu following injection of SP-SAP into the NAcc is hypothesised to be due to the neurotoxin flowing back into the lateral ventricles during retraction of the needle during surgery. Since this phenomenon occurred in the majority of mice tested, mice with ablation in both the NAcc and this region were included in the 'NAcc' group during subsequent behavioural experiments. The specificity of SP-SAP's actions in the mouse was confirmed by NeuN, GFAP and ChAT IHC, demonstrating that the observed loss of NK1 receptor immunoreactivity was due to loss of the NK1 and ChAT-expressing neurones of the NAcc, without non-specific loss of neurones or significant gliosis. SP-SAP is therefore of similar efficacy in the mouse and rat (Wiley & Lappi 1997), although with less apparent non-specific damage at the dose used.

\textit{6.6.2. Preliminary findings}

In the preliminary behavioural experiment comparing the effects of NK1 receptor-expressing cell ablation in the NAcc (including the dorsomedial CPu) and amygdala, it was
found that destruction of these cells in neither brain region brought about significant alterations in EPM behaviour or spontaneous locomotor activity. However, following SP-SAP injection into the amygdala, but not the NAcc, mice demonstrated reductions in morphine CPP scores, suggesting that loss of these cells brings about a reduction in the drug's rewarding properties. Unfortunately, a subsequent analysis of the CPP behaviour of naïve mice of this strain (C57BL/6) following conditioning with saline in both compartments revealed considerable variability in their behaviour on this task. Saline- and morphine-conditioned mice therefore did not display significantly different scores, hence it cannot be stated that they exhibit a significant CPP to morphine at this dose (3.0 mg.kg\(^{-1}\)). This dose of morphine was chosen on the basis of Murtra et al.'s (2000b) findings — in this study this dose of morphine brought about a strong CPP to morphine. In hindsight, it would have been more prudent to assess the rewarding nature of a range of doses of morphine in these mice before commencing the lesion experiment. Nevertheless, given the trend towards higher scores in morphine-conditioned mice, and the fact that the difference between the groups is not far from significance (\(P = 0.096\)), this dose is probably adequate for the comparison of the two lesion sites in a preliminary study. Furthermore, it is noteworthy that the mean CPP scores achieved by mice injected with SP-SAP in the amygdala is very similar to that of saline-conditioned naïve mice, adding weight to the tentative conclusion that NK1 receptor-expressing cells in the amygdala are more important for morphine CPP behaviour than those in the NAcc and dorsomedial CPu. Whilst the possibilities that compensatory mechanisms across the five-week period between surgery and testing, or the incomplete nature of the cell ablation could account for the lack of a significant reduction in CPP scores between naïve mice and those injected with SP-SAP into the NAcc, this comparison suggested that a fuller analysis of the effects of ablation of these cells in the amygdala was more likely to be a successful avenue for further investigation.

6.6.3. Ablation of neurokinin-1 receptor-expressing cells in the amygdala

The preliminary experiments were therefore followed by a study to confirm and extend the observation of a reduction in CPP scores after ablation of NK1-receptor expressing neurones in the amygdala. Following the disappointing observation of a lack of a significant CPP to morphine at 3.0 mg.kg\(^{-1}\) in the C57BL/6 mice used in the preliminary
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behavioural experiments, hybrid C57BL/6 × 129/sv were used. These mice exhibited a clear CPP to morphine at 3.0 mg.kg⁻¹, and a strong hyperlocomotor effect of 10.0 mg.kg⁻¹ morphine, and so were chosen for use in the remainder of the experiment. Injection of SP-SAP into the amygdala caused a slight increase in GFAP immunoreactivity, which was not observed in preliminary studies. This mild gliosis is probably a consequence of the cell death occurring as a result of SP-SAP's actions on NK1 receptor-expressing cells. This increase was generally minor, but those mice displaying dramatic increases in immunoreactivity, which were usually coupled to visible physical damage and necrosis around the injection site, were omitted from analysis.

6.6.3.1. Effects on anxiety-related behaviour

Ablation of NK1 receptor-expressing neurones in the amygdala brought about changes in EPM behaviour characteristic of an increase in anxiety-related behaviours relative to SP- and SAP-injected controls. This finding is different to that observed in the preliminary experiments described above: in this experiment, SP-SAP-injected mice made fewer entries into and spent less time in the open arms of the maze than naïve mice, as well as SP- and SAP-injected mice. There are many possible reasons for this discrepancy, including the different background strain of the mice affecting the relative influence of the ablation. However, 129/sv mice are characterised by high levels of anxiety (e.g. Homanics et al. 1999) – the introduction of more 129/sv in the mice of the current experiment would therefore be expected to mask such a lesion-induced increase in anxiety-related behaviours. It is more likely that differences in the extent and specificity of the SP-SAP-induced lesions are responsible for the observed difference between experiments: in the present experiment, which included two separate groups of control mice, care was taken to ensure that all animals remaining in the SP-SAP group had bilateral loss of NK1 receptor immunoreactivity throughout the amygdala, whereas inclusion in the preliminary experiment was determined on the basis of less stringent criteria. It is therefore possible that the more complete loss of NK1 receptor-expressing cells in the present study is necessary for the observed increases in anxiety behaviours on the EPM.

The amygdala is known to be critically involved in the mediation of behaviours related to anxiety, particularly in the association of environmental stimuli with fearful events, and the expression of such associations (Phillips & LeDoux 1992). NK1 receptor-expressing
neurones within the amygdala have been directly linked to anxiety: maternal separation of guinea pig pups from their mothers, a highly stressful and ethologically relevant event, causes endocytosis of the receptor within neurones in the basolateral nucleus of the amygdala (Kramer et al. 1998; Steinberg et al. 2001), as does immobilisation stress in gerbils (Smith et al. 1999), suggesting that substance P is released within this brain region in response to anxiogenic stimuli. This internalisation is blocked by the systemic administration of NK1 receptor antagonists (Smith et al. 1999; Steinberg et al. 2002), whilst intra-amygdala injections of the antagonist L 760 735 reduce the amount of separation-induced vocalisation emitted (Boyce et al. 2001). The anxiogenic nature of cell ablation is, however, acting in an opposite direction to the anxiolytic effects of intra-amygdala injections of NK1 receptor antagonists. This discrepancy may be due to species differences, since the current experiments were carried out in the mouse, or the different nature of the two approaches: the SP-SAP technique involves removal of entire cells, resulting in the chronic loss of NK1 receptor-mediated transmission and also co-expressed receptors and neurotransmitters and inter-neuronal connections, whereas microinjection of the antagonist brings about acute pharmacological blockade of the receptor. Despite this, the present findings still highlight the importance of these neurones, although the nature of the participation of the NK1 receptor-expressing cells remains to be determined. NK1 receptor-expressing neurones in other areas of the brain have also been implicated in the modulation of anxiety-related behaviours, including the nucleus basalis magnocellularis (Hasenohrl et al. 1998b), dorsal periaqueductal grey (Aguiar & Brandão 1996) and lateral septal nucleus (Gavioli et al. 1999). These areas remain potential sites for further investigation with this technique.

6.6.3.2. Effects on conditioned place preference behaviour

The analysis of CPP behaviour was attempted in order to confirm the preliminary findings in a fully controlled experiment: not only were controls for surgery and the non specific effects of the toxin's constituent molecules included in this study (SP- and SAP-injected mice), but saline-conditioned groups were also included within each treatment group to control for the morphine-independent aspects of the CPP task. Ablation of NK1 receptor-expressing cells in the amygdala did not bring about the dramatic reduction in morphine CPP scores observed in the preliminary study. However, the small reduction relative to
control groups, coupled with a larger increase in saline CPP scores within this group led to an overall finding that, relative to saline controls, mice with loss of these neurones in the amygdala did not exhibit a significant CPP to morphine. Conversely, both groups of control mice did show such a preference. This finding suggests that the lesion prevented mice from exhibiting CPP to morphine, at least at this dose.

The reason for the observed increase in saline CPP scores brought about by the ablation is unclear. Although the difference in saline CPP scores between SP-SAP, SP and SAP groups did not reach statistical significance in post hoc Tukey comparisons, the clear trend for an increased saline CPP scores following ablation is of importance in bringing about the observed lack of CPP to morphine in these mice. The negative CPP scores achieved by control SP- and SAP-injected mice (and naïve mice) probably come about as a result of the CPP protocol employed here. When mice are being conditioned with a drug stimulus, they receive the drug on the first, third and fifth days of conditioning, with vehicle on alternate days. The compartment in which the mouse receives the first injection during the conditioning procedure is therefore the same compartment that is to be paired with the putative rewarding stimulus. Increases in the amount of time spent in this compartment during the test session are interpreted as a CPP, whereas decreases indicate a conditioned place aversion (CPA). When vehicle (e.g. saline) is substituted for the drug in such control experiments, the mouse will receive its first injection before being introduced to this compartment. If it is assumed that this first injection (which is usually the first injection experienced by the animal) is aversive, and that it is more aversive than the injections on subsequent days (i.e. no sensitisation to this aversion takes place across the conditioning period), it will bring about a mild conditioned place aversion (CPA) to that compartment. Rewarding drug stimuli, on the other hand, presumably counteract this tendency towards CPA in order to bring about positive CPP scores.

The increase in saline CPP scores observed in mice of the SP-SAP group suggests that this putative CPA-like effect brought about by the first injection does not occur, and may even be reversed to a mild CPP. Since these mice show increases in anxiety-related behaviours on the EPM, it is unlikely to be due to a simple modulation of anxiety levels, since such an effect would be mediated by a decrease in the anxiety associated with the first injection procedure. However, it remains possible that these mice's increased anxiety levels could
modulate the aversion brought about by this first intraperitoneal (IP) injection, perhaps by decreasing the animals' sensitivity to painful or stressful stimuli.

It is important to stress that in this experiment, the morphine- and saline-conditioned mice had been treated differently prior to the CPP task. In order to make the most efficient use of mice, the same mice were tested for both CPP and the locomotor effects of acute drug administration. However, to avoid treating a mouse with morphine before carrying out another task, the tasks involving morphine were carried out last of all. The mice undergoing morphine CPP had therefore experienced a single saline injection before starting the CPP task, whereas those in the saline CPP groups had not previously been injected. It is possible that this previous injection could affect the aversive nature of the first injection during the CPP procedure, thereby elevating morphine CPP scores.

Despite these caveats, it is clear that morphine at a dose of 3.0 mg.kg⁻¹ is unable to elicit a CPP greater than the mild preference-like effect observed in the saline-conditioned mice. The analysis of a higher dose of morphine would be of use in assessing whether this phenomenon is more general. Since there is only a small reduction in CPP scores to morphine in the SP-SAP group when compared to its control groups, it remains possible that the lack of a difference between saline- and morphine-conditioned groups is due ultimately to the increase in saline CPP scores described above, rather than a true reduction in the rewarding effect of morphine.

When conditioned with 5.0 mg.kg⁻¹ cocaine, mice injected with SP-SAP displayed similar CPP scores to those of naïve mice. There is therefore no reduction in CPP to cocaine at this dose after ablation of NK1 receptor-expressing neurones in the amygdala. However, the increase in saline CPP scores casts some doubt on this conclusion: a lower dose of cocaine, bringing about smaller CPP scores, may have led to the saline- and cocaine-induced CPP scores being similar, in which case the conclusion from the experiment would have been that ablation of these neurones in the amygdala brings about deficits in both morphine and cocaine CPP. The dose of cocaine used in this study (and that of morphine) was chosen on the basis of Murtra et al.'s (2000b) findings: these doses gave rise to similar, high CPP scores in wild type mice of a similar genetic background to those used in this experiment. Here, however, 3.0 mg.kg⁻¹ morphine induced lower CPP scores than 5.0 mg.kg⁻¹ cocaine. Clearly, these issues can only be resolved by carrying out a dose-response
analysis of the effect of NK1 receptor-expressing cell ablation on morphine and cocaine reward. Given the relatively low rate of success in producing bilateral lesions and unbiased behaviour in the preconditioning session of the CPP task, this would require a long period of research time and large numbers of mice. As they stand, the CPP results presented here suggest that NK1 receptor-expressing neurones in the amygdala may play a role in the mediation of CPP to morphine in the mouse, but further questions need to be addressed before this conclusion can be fully justified.

6.6.3.3. Effects on morphine-induced locomotor behaviour

The observation that morphine hyperlocomotion is significantly reduced in mice with ablation of NK1 receptor-expressing neurones in the amygdala adds weight to the suggestion that the amygdala may indeed be a site for the NK1 receptor’s effects in mediating morphine reward behaviours. Unlike in the CPP experiments described above, the interpretation of the reduction in hyperlocomotion by acute morphine was not complicated by differences in behaviour following vehicle administration. However, since the mice tested for morphine hyperlocomotion had previously been tested for saline CPP, and undergone six IP injections, whereas the vehicle controls had not yet undergone testing for CPP to morphine, the groups of mice had very different experiences prior to this test. Although this may have influences behaviour in this task, consideration of the behaviour elicited by the three groups injected with morphine alone suggests that this effect is more likely to be genuine: SP-SAP caused a reduction relative to both control groups, although the three groups had been treated identically after surgery.

6.6.3.4. Possibilities for further study

When taken together with the CPP findings above, this observation suggests that the neurones expressing NK1 receptors in the amygdala are of importance in morphine reward-related behaviours. As with the CPP results, a dose-response analysis of this phenomenon would be of use in examining how general this effect is, as would an analysis of cocaine’s locomotor effects following SP-SAP-mediated ablation. An analysis of the other morphine-related behaviours that are disrupted in NK1−/− mice, such as conditioned place aversion (CPA) to naloxone-precipitated withdrawal, withdrawal-induced jumping and locomotor sensitisation, would also be of interest in assessing the generality of this
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effect. Furthermore, despite the observed separation between the effects of ablation in the NAcc and amygdala observed in the preliminary study described above, a more thorough investigation of the effects of ablation within the NAcc would be of interest. This is of particular importance in the light of the unusual effect on saline CPP behaviour observed following ablation of NK1 receptor-expressing neurones in the amygdala.

6.6.4. Identity of NK1 receptor-expressing cells in the amygdala

Assuming that the results described above do indicate a role for NK1 receptor-expressing neurones within the amygdala in morphine-reward (and anxiety) behaviours, an important question is what their exact identity is. Because of the small size of the mouse brain, the approach taken here is not able to distinguish between ablation of these neurones within the separate subnuclei of the amygdala in this species, although when taken together with the c-Fos results (Chapter 5), a role for the basolateral nucleus seems more likely than the central nucleus. However, there are few visible NK1 receptor-expressing neurones within the basolateral nucleus in the mouse (see Figure 6.16), whilst in the medial nucleus, dendrites expressing the receptor are more prominent and the central nucleus is characterised by a diffuse NK1 receptor immunoreactivity. Staining for βGal-positive nuclei within the brains of NK1−/− and heterozygous NK1+/− mice suggested that the somata of these cells are distributed throughout these subnuclei, but are more numerous within the medial nucleus, and that these cells are ablated following intra-amygdala injection of SP-SAP. Although the existence of only a few neurones does not preclude their importance in bringing about the observed behavioural changes, the more extensive cell loss from the medial nucleus of the amygdala suggests that this area may also be of importance in the behavioural changes observed. Clearly, the question of the exact identity of these cells is important in our understanding of their role in the behaviours assessed in this study: a possible method with which it could be further tackled is using *in situ* hybridisation to identify the cell nuclei expressing NK1 receptor mRNA throughout the mouse brain, and to assess the influence of SP-SAP injection on this expression profile. Additionally, the use of more precise injection apparatus may enable more specific targeting of amygdala subnuclei: in these experiments. The steel needle used to inject the neurotoxin was 0.22 mm in diameter: the use of a glass micropipette may enable the more accurate targeting of subnuclei and the injection of a smaller volume of SP-SAP.
There is also uncertainty surrounding the neurochemical identity of the NK1 receptor-expressing neurones in the amygdala, which further complicates the interpretation of these findings. In the basolateral nucleus of the amygdala of the guinea pig, the NK1 receptor is colocalised with glutamic acid decarboxylase, a marker for the inhibitory neurotransmitter γ-aminobutyric acid (GABA), and local SP microinjections increase the frequency of inhibitory postsynaptic potentials in most cells recorded from this region (Maubach et al. 2001). Interestingly, the CB1 cannabinoid receptor is expressed on the cholecystokinin-positive, parvalbumin-negative GABAergic interneurones of the basolateral nucleus, at least in the rat and the mouse (Katona et al. 2001; Marsicano & Lutz 1999; McDonald & Mascagni 2001). Although there are only a few NK1 receptor-expressing neurones within this brain region in the mouse, it is possible that the CB1 and NK1 receptors are localised on the same neurones within this nucleus of the amygdala — since the CB1 receptor is found only at low levels in many of the brain regions in which the expression of the NK1 receptor is prominent, such as the NAcc and the VTA (Marsicano & Lutz 1999), the amygdala may be one of the few areas of significant overlap of the two receptors. Since mice lacking CB1 receptors display similar morphine-related behaviours to NK1−/− mice (Ledent et al. 1999; Martin et al. 2000; Murtra et al. 2000b; Ripley et al. 2002) and mice with ablation of NK1 receptor-expressing neurones in the amygdala, it is conceivable that these amygdala neurones are not only crucial for the NK1 receptor's effects in mediating morphine reward behaviours, but also those of the CB1 receptor. Furthermore, CB1 knockout mice show an anxiogenic-like profile in the light/dark box test (Martin et al. 2002). Although this is an opposite finding to that observed in NK1−/− mice (Santarelli et al. 2001), it is similar to the anxiogenic-like response on the EPM observed by ablation of NK1 receptor-expressing neurones within the amygdala described above. However, in contrast to NK1 receptors, the activation of CB1 receptors is inhibitory and, in the amygdala, causes a disinhibition of glutamatergic projection neurones (Katona et al. 2001). Although there is some evidence that inhibitory CB1 receptors are also expressed presynaptically on these projection neurones (McDonald & Mascagni 2001; Ong & Mackie 1999), a simple synergistic relationship between the NK1 and CB1 receptors in mediating the behavioural responses to morphine administration is unlikely. A fuller histological analysis of the cellular expression pattern of the NK1 receptor in this brain region is required in order to resolve these issues. In particular, the degree of coexpression of the NK1 and CB1 receptors within GABAergic neurones would be of use in determining...
whether these neurones are key to the similar behaviours observed in the two knockout lines: this could be achieved using dual labelling IHC or in situ hybridisation, or a combination of the two techniques, in order to fully categorise the NK1 receptor-expressing cell population.

6.6.5. Source of substance P in the amygdala

A further question that needs to be addressed is the origin of the ligand for the NK1 receptor in the amygdala. Few studies have addressed the issue of the origin of SP in the amygdala. Despite claims that the amygdala does not receive SP projections from without (Emson et al. 1978), retrograde tracing studies have demonstrated that the ventrolateral hypothalamus sends a modest SP projection to the medial nucleus of the amygdala, at least in the guinea pig (Ricciardi & Blaustein 1994), whereas the central nucleus receives SP projections from the parabrachial and caudal laterodorsal tegmental nuclei (Block et al. 1989; Petrov et al. 1994; Yamano et al. 1988). SP is expressed in cell bodies and fibres within the medial and central nuclei of the rat and cat amygdala (Cassell et al. 1986; Cassell & Gray 1989; Emson et al. 1978; Malsbury & McKay 1989; Roberts et al. 1982). These neurones may make SP synapses onto NK1 receptor expressing cells within the same or other subnuclei in the amygdala: the presence of a short SP projection from the medial to the central nucleus of the amygdala has been suggested, although by an indirect analysis of the effect of knife cuts of the connections between these two nuclei on levels of SP (Emson et al. 1978). Efferent SP projections from the amygdala are better characterised: the medial nucleus sends SP projections to areas of the brain involved in defensive rage and predatory attack behaviours (Han et al. 1996; Shaikh et al. 1993), whilst the pressor response is mediated by SP projections from the central nucleus to the locus coeruleus, parabrachial nucleus, periaqueductal grey and lateral and ventromedial hypothalamic nuclei (Lu et al. 1997; Wu et al. 1999).

6.6.6. Role of the amygdala in opiate reward

The present results add further evidence for a role of the amygdala in opiate reward, and suggest that NK1 receptor-expressing neurones may be more important in the amygdala than the NAcc. This distinction between these two areas' roles in reward behaviour supports previous findings: although the NAcc clearly plays a crucial role in the mediation
of the rewarding impact of psychostimulant drugs such as cocaine and amphetamine, the importance of this brain region in opiate reward is less clear. Indeed, a recent study has examined the effects of ablating the cholinergic cells of the NAcc, using an immunotoxin to target these cells, which express the human interleukin-2 receptor in a transgenic mouse line. Loss of these neurones increased the mice’s sensitivity to cocaine, as assessed by acute locomotor stimulation, locomotor sensitisation and CPP (Hikida et al. 2001). Since the NK1 receptor is expressed on the cholinergic neurones of the striatum which are ablated by SP-SAP (Aubry et al. 1993; Gerfen 1991; Kaneko et al. 1993), the cell loss achieved in the present study is probably very similar to that of Hikida et al. (2001), but without any observable deficits in morphine CPP. These findings therefore add further evidence for the separation of the effects of the two classes of drug.

The mechanism by which loss of the NK1 receptor-expressing neurones in the amygdala brings about reductions in morphine reward-related behaviour remains a matter of speculation. Loss of these cells, which may be GABAergic, would be expected to bring about a disinhibition of their target cells: such cells may include the amygdala’s projection neurones. Although the location of the crucial cells within the subnuclei of the amygdala remains to be clarified, a possible projection is that from the basolateral nucleus of the amygdala to the core of the NAcc: this connection has been shown to be critical in mediating the control of behaviour by discrete, reward-related stimuli which act as conditioned reinforcers, in a dopamine-dependent manner (Cador et al. 1989; Everitt et al. 1989, 1991; Fuchs et al. 2002; Parkinson et al. 2001). The CPP paradigm involves such a process, since the morphine-associated compartment of the apparatus acquires motivational significance during the conditioning phase, subsequently bringing about approach behaviour during the final choice session (Bardo & Bevins 2000). A modulation of this output of the amygdala by ablation of neurons expressing the NK1 receptor could therefore be linked to the observed behavioural findings. However, excitotoxic lesions of the basolateral amygdala do not impair the acquisition of intravenous heroin self-administration under a continuous schedule of reinforcement, or with a second-order schedule, in which rats learn to work in order to receive a cue previously associated with the drug stimulus (Alderson et al. 2000). Although the reinstatement of heroin-seeking behaviour by conditioned drug-associated cues or drug priming can be abolished by inactivation of this brain region (Fuchs & See 2002), some doubt is cast on the relevance of
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this connection to opiate reward behaviour, since these studies suggest that the basolateral amygdala may not be necessary for the acute rewarding effects of opiates, or for the cue-mediated drug seeking behaviour. Although the loss of the few NK1 receptor expressing cells from this region, as opposed to all of the region's cell bodies may explain the discrepancy between these present findings and those of Alderson et al. (2000), it still remains possible that such cells in other subnuclei of the amygdala may be more crucial. For example, the lateral nucleus of the amygdala is known to be of importance in CPP to amphetamine (Hiroi & White 1991), as well as the conditioned cue preference task, in which a light cue is paired repeatedly with a food reward before a choice procedure (McDonald & White 1993). The central nucleus of the amygdala is connected to the core of the NAcc and receives dense reciprocal dopaminergic afferents from the VTA (Ben-Ari et al. 1975; Ungerstedt 1971; Wallace et al. 1992): it is therefore well placed to control the dopaminergic input to the NAcc, and possibly influence reward-related behaviours. Indeed, blockade of dopamine D2 receptors in this region reduces the acquisition and expression of CPP to morphine (Rezayof et al. 2002), whilst acute injection with morphine causes increases in the expression of SP here (Cantarella & Chahl 1996). Clearly, a more detailed analysis of the role of NK1 receptor-expressing neurones in the subnuclei of the amygdala is required in order to better understand the mechanism of the observed effects.

6.6.7. Further possible brain regions for a neurokinin-1 receptor-mediated effect

Although the NK1 receptor-expressing neurones in the amygdala may be of importance in morphine reward-related behaviours, it is likely that neurones in other areas of the brain expressing the receptor are also of importance. Although the present results provide some evidence that the NAcc and dorsomedial CPu may not be a crucial site for these effects, other areas are potential sites for further investigation – in particular, the locus coeruleus, the nucleus basalis magnocellularis and the tegmental pendunculopontine nucleus.

6.6.7.1. The locus coeruleus

The locus coeruleus has been heavily implicated in opiate dependence, particularly in the mediation of withdrawal (Nestler et al. 1993, 1994, 1999; Nestler & Aghajanian 1997). NK1 receptors are heavily expressed in this region, on noradrenergic cells which project to the forebrain (Chen et al. 2000; Moyse et al. 1997; Santarelli et al. 2001). The loss of NK1
receptors from these cells in NK1-/- mice may be of importance in the mediation of the mouse's behavioural alterations, not only in terms of morphine, but the antidepressant-like profile they exhibit. However, the small size of this brain region in the mouse makes it a difficult area to target using stereotaxic injections of SP-SAP. This could possibly be overcome by using the rat for studies of the effects of NK1 receptor-expressing cell ablation in this region, or by using alternative delivery strategies for SP-SAP, as discussed above.

6.6.7.2. The nucleus basalis magnocellularis

Secondly, the nucleus basalis magnocellularis is a promising candidate for further study of the role of substance P in reward behaviours. This region of the ventral pallidum supplies the main cholinergic innervation to wide areas of the cortex (Lehmann et al. 1980). It contains a dense network of SP-immunoreactive fibres (Ljungdahl et al. 1978a), and expresses NK1 receptors (Chapter 3). Importantly, microinjections of SP into this region support place preference in a dose-dependent manner (Hasenöhrl et al. 1998a; Holzhäuer-Oitzl et al. 1988), and have been linked to increased dopamine release within the NAcc, at least on the contralateral side of the brain (Boix et al. 1995). Although the role of this region in reward to opiates is unclear, it clearly plays an important SP-mediated role in reward-related behaviours. Further analysis of the role of NK1 receptor-expressing neurones in this region in reward to morphine would therefore be of interest. However, it is important to note that although SP injections here, and in other regions of the brain such as the lateral hypothalamus and lateral septal nucleus are rewarding (Stäubli & Huston 1985), this does not necessarily imply that NK1 receptors in these regions are involved in reward to opiates. Indeed, microinjections of SP into the amygdala or NAcc do not support place preference (Lenard & Kertes 2002; Schildein et al. 1998; Stäubli & Huston 1985), despite the evidence presented here for a role for NK1 receptor-expressing neurones in the amygdala in morphine CPP.

6.6.7.3. The tegmental pendunculopontine nucleus

A third possible target for further studies is the tegmental pendunculopontine nucleus. This nucleus is well connected to regions of the brain involved in the translation of the emotional aspects of reward into behaviour: its cholinergic neurons are a principal source
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of excitatory input to dopamine neurones in the substantia nigra pars compacta which project to the dorsal striatum (Blaha et al. 1996; Blaha & Winn 1993; Charara et al. 1996; Futami et al. 1995; Jackson & Crossman 1983; Saper & Loewy 1982; Takakusaki et al. 1996), whilst it also acts as a site of convergence of information from the dorsal and ventral striatum, directing the flow of sensorimotor and incentive-motivational information to the brainstem in order to bring about appropriate behavioural and autonomic responses (Inglis et al. 1994; Inglis & Winn 1995; Winn et al. 1997). Although NK1 receptor immunoreactivity in this brain region is only moderate (Chapter 3), the behavioural effects of lesions of this nucleus are strikingly similar to those of genetic ablation of the NK1 receptor. Specifically, at least in non-dependent (drug naïve) rats, lesions of this nucleus prevent the acquisition of CPP to morphine (Bechara & van der Kooy 1989, 1992a; Leri & Franklin 2000; Nader & van der Kooy 1997; Olmstead & Franklin 1993, 1994; Parker & van der Kooy 1995), heroin (Nader et al. 1994) and amphetamine (Bechara & van der Kooy 1989; Olmstead & Franklin 1994), but not cocaine (Parker & van der Kooy 1995), and they reduce heroin self-administration (Olmstead et al. 1998). These lesions also block the locomotor stimulant effects of amphetamine, and the conditioned hyperactivity produced by amphetamine or morphine, but without affecting the analgesic properties of morphine in the tail-flick or formalin tests (Bechara & van der Kooy 1992b; Olmstead & Franklin 1993). Although lesions of this nucleus also disrupt CPP to food in sated rats (Bechara & van der Kooy 1992a), a behaviour that is intact in NK1°° mice (Murtra et al. 2000b), this region may represent a further site of interaction of the NK1 receptor and the mediation of morphine reward mechanisms.

Nevertheless, the fact that lesioning this nucleus and genetic manipulation of the NK1 receptor bring about similar alterations in behaviour does not necessarily imply that it is a site of the receptor's effects in mediating morphine reward-related behaviours: it is possible that this nucleus and the site or sites of the NK1 receptor's effects are distinct but lie in a common neural pathway. Although the direct anatomical connection from this nucleus to the central and medial nuclei of the amygdala is minimal (Hallanger & Wainer 1988), an intriguing possibility is that these connections contain SP, either in direct connections or via a polysynaptic pathway. Such a connection could provide a link between the observed effects of lesions of this nucleus and the ablation of NK1 receptor-expressing neurones in these subnuclei of the amygdala. This hypothesis may be supported by the observation
that a few of the connections from the tegmental pendunculopontine nucleus to the amygdala are cholinergic in the rat (Hallanger & Wainer 1988), whilst in man, SP is coexpressed with acetylcholine in this nucleus (Gai et al. 1993; Halliday et al. 1990). Further work is required to assess the importance and neurochemical profile of such anatomical connections, and to reveal more clues as to the links between this nucleus' role and that of the NK1 receptor.

6.7. Conclusions

The experiments described in this chapter were carried out in an attempt to identify potential loci for the NK1 receptor's effects in morphine reward behaviours. In preliminary experiments the neurotoxin SP-SAP was used to selectively ablate NK1 receptor-expressing neurones from either the NAcc or amygdala of mice, and their behaviour in the morphine CPP procedure were monitored, suggesting a more important role for these neurones in the amygdala. This finding was confirmed and extended in a controlled experiment. These results, although they would benefit from further experimentation, suggest that NK1 receptor-expressing neurones in the amygdala are involved in the mediation of morphine reward-related behaviours. Furthermore, loss of these neurones brings about an anxiogenic-like effect on the EPM. These behaviours are similar to those observed in mice lacking cannabinoid CB1 receptors, which, along with NK1 receptors, are expressed on the GABAergic interneurones of the basolateral nucleus of the amygdala. Although the origin of the SP innervation of the amygdala is not known, it may include afferent connections from the tegmental pendunculopontine nucleus. The efferent projections of the amygdaloid subnuclei have been implicated in the control of reward-related behaviours: these results therefore add further evidence for their contribution. However, the potential involvement of additional sites of NK1 receptor expression, such as the locus coeruleus and nucleus basalis magnocellularis, in the receptor's role in morphine reward and possibly addiction cannot be overlooked, and await further investigation.
CHAPTER SEVEN

LEARNING AND MEMORY BEHAVIOURS IN
NEUROKININ-1 RECEPTOR KNOCKOUT MICE
Chapter seven  Learning and memory behaviours in neurokinin-1 receptor knockout mice

7. LEARNING AND MEMORY BEHAVIOURS IN NEUROKININ-1 RECEPTOR KNOCKOUT MICE

7.1. Introduction

In this chapter, the role of substance P (SP) and its receptor in learning and memory processes are discussed. Results are presented from a range of learning and memory tasks in neurokinin-1 (NK1) receptor knockout (NK1⁻⁻) mice, and these are discussed in relation to the published literature.

7.2. Background

7.2.1. Rationale for the analysis of learning and memory in neurokinin-1 receptor knockout mice

7.2.1.1. Effects of substance P in learning and memory tasks

The behaviour of NK1⁻⁻ mice in learning and memory tasks was investigated for three reasons. Firstly, SP and NK1 receptors are expressed in a range of brain regions known to be of importance in certain types of learning and memory, such as the amygdala (see Chapter 3). The loss of NK1 receptors from these regions may therefore bring about alterations in learning and memory behaviour. A number of studies have examined the role of exogenously applied SP on learning and memory, most of which suggest that it has a memory-enhancing effect. Post-training injections of SP either peripherally or into the nucleus basalis magnocellularis, medial septal nucleus or lateral hypothalamus of the rat produce long-lasting facilitation of one-trial uphill avoidance or step-down avoidance learning (Gerhardt et al. 1992; Hasenöhrl et al. 1990a; Kafetzopoulos et al. 1986; Staubli & Huston 1979, 1980; Tomaz et al. 1997; Tomaz & Huston 1986; Tomaz & Nogueira 1997). In mice, a similar enhancement of passive avoidance was demonstrated following post-training peripheral injections of SP (Schlesinger et al. 1983), whilst in ageing rats, chronic peripheral administration of SP before training has been shown to reduce deficits in the Morris water maze (Hasenöhrl et al. 1990b, 1994). Conversely, injections of SP into the substantia nigra or amygdala have been shown to produce retrograde amnesia in avoidance learning (Huston & Stäubli 1978, 1979), suggesting that the site of injection determines the effect of the peptide on behaviour. However there is little available data on the effects of
endogenous SP in learning and memory processes: analysis of the behaviour of NK1\(^{-}\) mice in a range of learning and memory tasks was therefore of interest in examining its role under more physiological conditions.

### 7.2.1.2. Examination of classical conditioning ability

Secondly, the learning ability of NK1\(^{-}\) mice was of interest since much of the work presented in this thesis stems from the observation that they do not exhibit CPP to morphine (Murtra et al. 2000b). Since the CPP paradigm ultimately depends on the animal under test forming an association between an environmental cue and the rewarding nature of the stimulus of interest, normal behaviour on such tasks, especially those examining Pavlovian (classical) conditioning, would confirm that this observation was not confounded by deficits in learning ability.

### 7.2.1.3. Hippocampal adaptations

The third reason for an interest in learning and memory behaviour is based on the observation that NK1 antagonists are effective antidepressant drugs in humans (Kramer et al. 1998) and in preclinical animal trials (Harrison et al. 2001; Kramer et al. 1998; Papp et al. 2000; Rupniak et al. 2000). The dentate gyrus of the hippocampus is one of a few areas in the brain where the production of new neurones occurs in adulthood, in a wide range of mammalian species, including humans (Altman & Das 1965, 1967; Cameron et al. 1993; Eriksson et al. 1998; Gould et al. 1997, 1998, 1999b; Gueneau et al. 1982; Kaplan & Bell 1983; Kaplan & Hinds 1977): this neurogenesis can be modulated by a variety of genetic factors and naturally occurring or artificial environmental stimuli, including chronic antidepressant treatment. Hence chronic, but not acute administration of antidepressant drugs including 5-hydroxytryptamine (5-HT) reuptake inhibitors, noradrenergic reuptake inhibitors and monoamine oxidase inhibitors, as well as chronic electroconvulsive seizure, has been shown to increase the proliferation of new neurones in the dentate gyrus of the rat (Malberg et al. 2000; Manev et al. 2001) possibly via the enhanced synthesis of brain-derived neurotrophic factor (BDNF; Chen et al. 2001a; Nibuya et al. 1995; Vaidya & Duman 2001).

In order to investigate whether NK1\(^{-}\) mice exhibited similar increases in hippocampal neurogenesis, Morcuende et al. (submitted) injected NK1\(^{-}\) and wild type mice with the
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Thymidine analogue 5-bromo-2'-deoxyuridine (BrdU), which is incorporated into the DNA of dividing cells (Nowakowski et al. 1989). Immunohistochemical detection of BrdU revealed that NK1−/− mice had around 30% more new cells than wild type mice in the dentate gyrus one day after the last injection, and that around 80% of these new cells co-expressed a marker for neuronal nuclei (NeuN). However, the number of new cells was not significantly different between genotypes 7, 14 or 28 d after the last injection of BrdU. These results suggest that NK1−/− mice have enhanced proliferation of new neurones in the dentate gyrus. However, these new cells therefore exhibit a compensatory decrease in survival in NK1−/− mice, since the numbers of labelled cells are the same in the two genotypes 7 d after BrdU injection. This increase in proliferation was paralleled by a two-fold enhancement of the expression of BDNF in the hippocampus, thereby mimicking the effect seen in rats after chronic antidepressant treatment.

7.2.1.3.1. Functions of hippocampal neurogenesis

The function of new neurones in the dentate gyrus is unknown (Gage 2002; Kempermann 2002). The new cells are capable of migrating from the subgranular zone to the granule cell layer, where they can form the features of mature granule cells, both morphologically and functionally: adult-generated neurones send dendritic processes into the molecular layer and axons into the CA3 region of the hippocampus, express markers of mature neurones, receive synaptic inputs from perforant path cells and display similar resting potential, input resistance, threshold potential for spiking and firing rate to mature granule cells (Kaplan & Bell 1984; Markakis & Gage 1999; Stanfield & Trice 1988; van Praag et al. 2002). An intriguing possibility is that these new cells confer a greater scope for synaptic plasticity within the hippocampus, an area of the brain crucial for certain types of learning and memory. This pool of new neurones provides a supply of new synaptic space, potentially allowing the animal to achieve greater network complexity in response to environmental novelty (Kempermann 2002).

A link between hippocampal neurogenesis and an increased capacity for learning is supported by a number of studies demonstrating that the survival, but not proliferation, of granule cells born one to two weeks before training is enhanced by hippocampus-dependent learning and memory paradigms, such as the spatial version of the Morris water maze and trace eye blink conditioning (Gould et al. 1999a). In addition, many stimuli that
cause increases in neurogenesis also give rise to beneficial effects on hippocampus-dependent learning and memory. These include environmental enrichment, which causes increases in the survival of new neurones (Kempermann et al. 1997; Nilsson et al. 1999) and voluntary exercise, which enhances both proliferation and survival (van Praag et al. 1999b), as well as enhancing perforant path to dentate gyrus long-term potentiation (LTP), a cellular correlate of learning and memory (van Praag et al. 1999a). Both these stimuli improve the acquisition of water maze learning, although they fail to bring about differences in probe trial performance. Conversely, stimuli which decrease the proliferation of new neurones, such as stress (Gould et al. 1997, 1998), ageing (Gould et al. 1999b; Kuhn et al. 1996), elevated corticosterone levels (Cameron & Gould 1994), and chronic opiate administration (Eisch et al. 2000) have deleterious effects on such tasks (Bodnoff et al. 1995; Gallagher et al. 1993; Gallagher & Nicolle 1993; Krugers et al. 1997; Spain & Newsom 1991). Similarly, the reduction of granule cell synthesis using the cytostatic drug methylazoxymethanol acetate (MAM) led to reductions in trace eye blink conditioning, a hippocampus-dependent task, while sparing the hippocampus-independent delay version of this task (Shors et al. 2001).

The analysis of the behaviour of NK1 knockout mice therefore provides information not only on the role of substance P and NK1 receptors themselves, but also on the role of granule cell proliferation in the dentate gyrus, on learning and memory. NK1 knockout mice were tested in a variety of hippocampus-dependent and -independent tasks, which make demands on short- and long-term memory.

7.2.2. Learning and memory tasks

7.2.2.1. The Morris water maze

Firstly, spatial learning and memory was examined in NK1 knockout mice using the Morris water maze (Morris 1981, 1984; Morris et al. 1982). This classic paradigm tests the ability of the subject to learn the position of a platform hidden just below the surface of a pool of water, using extra-maze cues. Mice are trained by placing them in the water and allowing them to swim until they locate and climb onto the platform. Following a number of training trials, the ability of the mice to remember the position of the platform is assessed in ‘probe trials’, during which the platform is removed and the swimming behaviour of the mice is
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monitored. Mice with better spatial memory search closer to the platform’s position and spend more time in its vicinity. Spatial learning and memory depend critically on hippocampal function (Morris et al. 1982; O'Keefe & Nadel 1978; Sutherland et al. 1982; Whishaw 1987). Although NK1 receptor expression is low in the mouse hippocampus (Chapter 3), it was of interest to examine the effects of enhanced granule cell proliferation in the dentate gyrus on performance in this task.

7.2.2.2. Fear conditioning

7.2.2.2.1. Contextual fear conditioning

Secondly, contextual fear conditioning was examined, in which the mouse is exposed to a novel context that is paired with a mild foot shock. Upon re-exposure to the same context after a delay, the formation of an association between the context and the aversive shock is assessed by examining the defensive responses of the animal, including ‘freezing’, the adoption of a hunched, immobile posture (Bolles 1970; Fanselow 1980). Although the association of aversive events with environmental stimuli probably occurs within the basolateral nucleus of the amygdala (Fanselow & LeDoux 1999), the hippocampus is crucial for the integration of multiple sensory inputs in the formation of contextual representations, which are subsequently transmitted to the amygdala for association with unconditioned stimuli (USs; Anagnostaras et al. 2001; Fanselow 2000; LeDoux 2000; Maren 2001b; Maren & Fanselow 1996). Electrolytic or neurotoxic lesions of the dorsal hippocampus impair contextual fear conditioning in both rats (Anagnostaras et al. 1999; Kim et al. 1993; Kim & Fanselow 1992; Maren et al. 1997; Maren & Fanselow 1997; Phillips & LeDoux 1992) and mice (Chen et al. 1996). Such lesions produce more reproducible effects when made after training, in which case they are time-dependent: lesions made later after conditioning have a smaller effect on conditioned freezing behaviour, possibly because of the transfer of stored information from the hippocampus to the neocortex. However, in some studies, lesions made before training do not abolish contextual fear conditioning (e.g. Maren et al. 1997). This is hypothesised to be due to conditioning to unimodal cues in the context, a process that relies less on the hippocampal memory system.

Despite concern that the hippocampus may be involved in the unconditioned expression of freezing, and hence that hippocampal lesions may simply disrupt the expression of
conditioned fear, rather than fear conditioning itself (see Gewirtz et al. 2000), the majority of the available evidence indicates that the hippocampus is certainly involved in the formation of unified representations of context. However, certain experimental approaches occasionally yield results that suggest that contextual fear conditioning is spared following hippocampal damage. The exact conditions under which hippocampal integrity is required remain to be clearly elucidated, although it is becoming accepted that the association of contextual stimuli with aversive events requires the hippocampus when the they are in the ‘background’ of an animal’s perception (Phillips & LeDoux 1994): when the experimental set-up biases the animal towards being conditioned to specific cues in the environment, they are treated as explicit conditioned stimuli (CSs), and the task becomes more hippocampus-independent (Anagnostaras et al. 2001; Gewirtz et al. 2000).

7.2.2.2.1. Extinction of conditioned fear

The extinction of conditioned fear was also examined in NK1−/− mice, by re-exposing them to the fear-associated context repeatedly, but without US presentation. Such a procedure results in the gradual loss of the ability of the CS to elicit fear responses. Based on the observation that extinguished conditioned responses can spontaneously recover, extinction is believed to be the formation of new memories, rather than an erasure of the existing CS-US association. It is dependent upon NMDA receptors in the amygdala (Falls et al. 1992; Lee & Kim 1998), and some studies have shown a slowing of extinction following lesion of the ventromedial prefrontal cortex (Morgan et al. 1993).

7.2.2.2. Cued fear conditioning

The analysis of contextual fear conditioning was followed by the examination of cued fear conditioning in a delay protocol. This task involves the association of a foot shock with a discrete cue; in this case a tone. The association of the tone and shock is assessed by comparing the freezing response of mice in a novel context before and after tone presentation: association of the two stimuli will bring about increases in freezing following the tone. This paradigm is independent of hippocampal function (Anagnostaras et al. 1999; Kim & Fanselow 1992; McEchron et al. 1998; Phillips & LeDoux 1992), but requires the integrity of the amygdala (Phillips & LeDoux 1992). Since the NK1 receptor is expressed in the central and medial nuclei, and sparsely in the basolateral, and lateral nuclei of the
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amygdala (Chapters 3 & 6), the examination of cued fear conditioning was carried out in order to investigate the role of this receptor system in cued fear conditioning behaviour.

7.2.2.2.3. Neuroanatomy of fear conditioning

In terms of both contextual and cued fear conditioning, the amygdala can be divided into two subsystems: one comprising of the lateral, basolateral and basomedial nuclei, termed the basolateral complex, and the other comprising the central nucleus (Campeau & Davis 1995b). The basolateral complex acts as the primary sensory interface for the amygdala, receiving inputs from various cortical and subcortical structures, such as the auditory cortex and thalamus for auditory cues (Campeau & Davis 1995a; LeDoux et al. 1986; Romanski & LeDoux 1992), and the hippocampus for contextual cues (Maren &Fanselow 1995). The pathways for the US are less well defined, but it has been demonstrated that the lateral nucleus receives inputs from thalamic areas that receive afferents from the spino-thalamic tract and are responsive to nociceptive stimulation (Romanski et al. 1993). Selective neurotoxic lesions of the basolateral complex attenuate the acquisition of contextual or cued fear conditioning when made before training (Cahill et al. 2000; Cousins & Otto 1998; Helmstetter 1992), and can abolish the expression of conditioned fear responses when made up to one month after training (Cousens & Otto 1998; Lee et al. 1996; Maren et al. 1996a), even after extensive overtraining (Maren 1998, 1999, 2001a). Furthermore, pretraining infusions of the γ-aminobutyric acid (GABA) receptor subunit A agonist muscimol (Helmstetter & Bellgowan 1994; Muller et al. 1997; Wilensky et al. 1999) or the N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV) into the basolateral complex of the amygdala prevent conditioned freezing (Fanselow & Kim 1994; Kim et al. 1991; Lee & Kim 1998; Maren et al. 1996b). Based on this evidence, it is believed that the basolateral complex is the site of CS-US association and storage during fear conditioning (Fanselow & LeDoux 1999).

The lateral nucleus of the amygdala is of particular interest in the study of cued fear conditioning, since selective lesions of this subnucleus (Amorapanth et al. 2000; LeDoux et al. 1990; Nader et al. 2001) or injections of the NMDA receptor subunit 2B antagonist ifenprodil into it impair auditory fear conditioning (Rodrigues et al. 2001). Cells in the dorsal subdivision of this nucleus are responsive to both auditory and nociceptive stimuli (Romanski et al. 1993), and single-unit recordings have shown that auditory fear
conditioning induces short-term plasticity in this region, with cells exhibiting enhanced spike firing in response to acoustic CSs following pairing with a footshock (Maren 2000; Quirk et al. 1995, 1997; Repa et al. 2001). Although the precise neurochemical basis of this brain region’s storage capacity remain unclear, it is likely that some form of LTP occurs here. LTP has been observed at thalamo-amygdalar synapses in the lateral amygdala (Clugnet & LeDoux 1990; McKernan & Shinnick-Gallagher 1997; Rogan et al. 1997; Rogan & LeDoux 1995; Weisskopf et al. 1999), and has been shown to be sensitive to the same stimulus contingencies as fear conditioning itself (Bauer et al. 2001). However, the dependence of this form of plasticity on NMDA receptors is uncertain (Mahanty & Sah 1998), since they seem to play a role in normal synaptic transmission in the amygdala (Fendt 2001; Lee et al. 2001; Li et al. 1995). Two types of LTP can be produced at thalamic input synapses in the lateral amygdala, depending on the protocol used: an L-type voltage-gated calcium channel (VGCC)-dependent and NMDA receptor-independent form (Weisskopf et al. 1999), and a NMDA receptor-dependent, VGCC-independent form (Bauer et al. 2002). Blockade of NMDA receptors prevented both short- and long-term fear memory, whilst VGCC blockade impaired only long-term memory, suggesting that these two forms of plasticity may have differential roles in memory formation and consolidation in vivo (Bauer et al. 2002).

In contrast, contextual fear conditioning seems to depend upon plasticity within the basolateral nucleus of the amygdala. Repeated high-frequency stimulation of the ventral angular bundle, which carries projections from the hippocampal formation to the basolateral nucleus of the amygdala, causes NMDA receptor-dependent LTP of field potentials in the basolateral nucleus (Maren & Fanselow 1995). Post-training lesions of either of these two areas bring about deficits in contextual fear conditioning (Maren 1999; Maren & Fanselow 1995).

During the expression of fear memories, the central nucleus acts as the amygdala’s interface to fear response systems (Campeau & Davis 1995b), receiving information from the lateral or basolateral nucleus and sending projections to the areas of the hypothalamus and brainstem that control behavioural, autonomic and endocrine conditioned responses (LeDoux et al. 1988). Lesions of this nucleus prevent the acquisition and expression of
conditioned fear (Kapp et al. 1979; Nader et al. 2001), but this seems to be due to a deficit in performance rather than association (Amorapanth et al. 2000).

### 7.2.2.2.4. Trace fear conditioning

Cued fear conditioning was also tested using a trace protocol. In this task, the CS (a tone) and US (a foot shock) are separated by a time interval. In contrast to delay paradigms, in which the CS and US are presented contiguously, trace paradigms require the integration of discontiguous sets of stimuli, since the subject is required to maintain a representation or ‘trace’ of the cue within working memory in order for an association with the US to be made. Such a process is dependent upon the integrity of the hippocampus, whereas delay protocols are independent of hippocampal function (McEchron et al. 1998; McEchron & Disterhoft 1999; Thompson & Kim 1996). Furthermore, mice lacking the NMDA receptor subunit 1 in hippocampal CA1 pyramidal cells are impaired in trace fear conditioning (Huerta et al. 2000). Since the demonstration of reduced learning following pharmacological reduction in hippocampal neurogenesis was achieved using a trace paradigm, albeit with eye blink conditioning (Shors et al. 2001), it was of interest to examine the effects of increased neuronal proliferation in the dentate gyrus on performance in a trace paradigm.

### 7.2.2.2.5. Involvement of amygdalar neurokinin-1 receptors in stress

The analysis of amygdala-dependent fear conditioning behaviours was also of interest on the basis of observations that NK1 receptors are internalised in response to stressful stimuli in the basolateral nucleus of the amygdala (Boyce et al. 2001; Kramer et al. 1998; Smith et al. 1999), and focal injections of the NK1 receptor antagonist L 760 735 into the same brain region prevent separation-induced vocalisations in guinea-pig pups (Boyce et al. 2001). Substance P is therefore probably released in this brain region in response to stressful stimuli, such as noxious foot shocks, leading to NK1 receptor internalisation. The absence of these receptors in NK1/- mice may therefore lead to alterations in fear conditioning behaviour.
7.2.2.3. Working memory tasks

Two tests of short-term working memory were also attempted. These were spontaneous alternation and the spontaneous novelty-preference test of object recognition, both of which exploit a rodent's natural tendency to explore novel places or objects, but in a spatial or non-spatial manner respectively.

7.2.2.3.1. Spontaneous alternation

In the free trial version of the spontaneous alternation task, the animals are allowed to explore a T- or Y-maze, and are briefly contained within the first of the two end compartments that they enter. Following a short delay, a second exploration is begun, and the behaviour of the animal is monitored: rodents will tend to enter the alternate box to that in which they were contained during the conditioning session (Dember & Fowler 1958, 1959). In addition to the hippocampus (Isseroff 1979), spontaneous alternation behaviour is sensitive to lesions of the septum, the medial mammillary bodies and various thalamic regions, as well as the prefrontal cortex, dorsomedial caudate putamen (CPu) and cerebellum, although the involvement of these latter structures is more likely to be via indirect effects on neophobia, behavioural inhibition or motor coordination rather than spatial working memory (see Lalonde 2002). The deficits in spontaneous alternation behaviour induced by the administration of scopolamine, a muscarinic acetylcholine receptor antagonist, are reversed by intracerebroventricular administration of tachykinins, including SP (Kameyama et al. 1998). Although injections of SP alone do not affect performance on the task, tachykinin agonists probably counteract scopolamine's affect by activating cholinergic neurones expressing tachykinin receptors, such as in the lateral dorsal tegmentum (Ukai et al. 1995).

7.2.2.3.2. Object recognition

In the object recognition task, exploratory behaviour is monitored during exposure to two objects. One of the objects is then replaced, and a second trial is begun: during this trial, one of the objects is familiar, having been present in the first exposure, whilst the other object is novel. Rodents tend to spend more time exploring the novel object in the first few minutes of exposure, thus indicating that they recognise that the object is not the same as the one previously in its place (Ennaceur & Delacour 1988). Recognition memory
seems to involve hippocampal function, since damage to this brain region produces object recognition deficits in rodents (Mumby et al. 1996; Myhrer 1988) and humans (Reed & Squire 1997; Zola-Morgan et al. 1986). Additionally, the object recognition task itself has been shown to be dependent upon NMDA receptors in the hippocampus in the mouse: genetic ablation of the NMDA receptor 1 subunit from the CA1 region leads to deficits in the task (Rampon et al. 2000). Similarly, in the rat, infusion of APV impairs object recognition memory when tested 3 h, but not 5 min, after training (Baker & Kim 2002). Recently, however, the necessity of the hippocampus in this task has been brought into question, since lesions of this brain region do not always prevent object recognition from taking place (see Mumby 2001). In contrast, lesions of other brain regions of the temporal lobe produce more reliable deficits, particularly the perirhinal and postrhinal (or parahippocampal) cortices (Bussey et al. 1999; Ennaceur et al. 1996; Ennaceur & Aggleton 1997; Mumby et al. 2002b). These areas do however have dense reciprocal connections with the hippocampus (Deacon et al. 1983; Naber et al. 1999, 2001; Suzuki & Amaral 1990; Yukie 2000), suggesting that in the intact animal, hippocampal function may play a role in the task, possibly in attaching contextual or spatial significance to the memory of objects (Mumby et al. 2002a).

7.2.2.4. Rota-Rod test

Finally, the motor coordination and motor learning ability of NK1−/− mice was tested using the Rota-Rod (Dunham & Miya 1957) in the accelerating mode (Jones & Roberts 1968). This task measures how long a mouse can stay on a rotating horizontal drum as its speed of rotation is increased, to assess motor coordination. Motor learning is assessed by the degree to which the fall times increase over repeated trials. Performance on this task is sensitive to manipulations of the striatum (Emerich et al. 1993), and it increases dopamine metabolism in the striatum (Bertolucci-D’Angio et al. 1990), whilst mice with mutations causing degeneration of cerebellar granule and Purkinje cells show impairments in both motor coordination and learning in this paradigm (Caston et al. 1995; Lalonde et al. 1995). The striatum is rich in NK1 receptors, so their loss in NK1−/− mice may affect motor coordination. Moreover, it is prudent to verify that the NK1−/− mice do not exhibit gross alterations in motor coordinative ability, as similar motor function between experimental...
groups is necessary to allow fair interpretation of the results of behavioural tests involving locomotion.

7.3. Materials and methods

7.3.1. Mice

Adult (2 - 6 months old) NK1⁻/⁻ (n = 79) and wild type mice (n = 80) from heterozygous breeding were used in these experiments (see section 2.3.1). Within each experimental group, mice were matched for age and sex.

7.3.2. Morris water maze

7.3.2.1. Apparatus

The Morris water maze consisted of a circular tank (1.5 m diameter) positioned in the centre of a room. White curtains were hung around the walls of the room on three sides, 0.55 m from the edge of the tank at the closest point. The curtains on the fourth side were left open, with the wall 1.20 m from the tank’s edge. Various large, distinct objects were hung from the curtains at positions around the room, including a child’s orange plastic chair, a large black paper triangle and a pair of footballs. These objects remained in the same positions throughout the 16-d procedure. The tank was filled with water made opaque with non-toxic white paint (1:1760 dilution; Ready Mix Paint 44800 White 284; The Early Learning Centre, Swindon, UK) and maintained at 22 °C. A Plexiglas platform (100 mm diameter) was positioned in the pool, and the water level adjusted to 5 mm above the platform. The rim of the tank was approximately 0.19 m above the water level. The platform was placed in one of two opposite positions in the pool; both centred 300 mm from the edge of the tank and designated SW and NE. The experimenter sat in the same place in the room throughout all trials. The apparatus is illustrated in Figure 7.1.
Behavioural observations were made via a camera mounted on the ceiling above the pool, connected to a PC running HVS Water 2020 (HVS Image Ltd., Hampton, UK) via an HVS2020 Tracker (HVS Image Ltd.), monitor and VCR. Recordings were begun and ended using an HVS air squeeze (HVS Image Ltd.).

Mice ($n = 16$ per genotype) were trained in two groups of 16. Conditions were identical in both experimental runs.
7.3.2.2. Habituation

Prior to exposure to the water maze, mice were handled for 2 min every day for 11 d. Mice were picked up by the tail and placed on the experimenter's hands 2 - 3 times before being returned to the home cage. Handling in this manner, which is similar to the handling that is experienced during training, reduces stress levels by acclimating the mice to the experimenter. Failure to do so may result in mice simply floating in the pool and not swimming and searching for the platform.

On the first day of training, each mouse was acclimated to the water and given a chance to swim and climb on to the platform before the first trial. Each mouse was placed on the platform (in the SW position; see Figure 7.1) for 30 s, before being retrieved and placed in the pool, away from the platform. It was allowed to swim for 30 s, before being retrieved and given three practice climbs onto the platform. This was achieved by placing the mouse directly in front of the platform and guiding it until it climbed on. Each practice climb was done from a different side. The mouse was left on the platform for a further 30 s to rest, before the first trial was immediately begun.

7.3.2.3. Training

Mice were trained individually in the water maze. Each mouse was given 4 trials per day, with an intertrial interval of 60 s. At the beginning of the trial block, each mouse was placed on the platform in the SW position for 60 s. At the start of a trial, the mouse was retrieved from the platform and carried below the edge of the pool, to one of the four starting positions (N, S, E or W; see Figure 7.1). It was then placed in the pool, facing outwards towards the edge and allowed to swim. The path taken by the mouse was recorded using HVS Water for Windows, and the time taken to find and climb onto the platform recorded. Mice that did not climb onto the platform in 90 s were retrieved and carried around the edge of the pool to the platform. Escape latencies for these mice were recorded as 91 s. At the end of the block of trials, each mouse was left on the platform for 60 s before being returned to the home cage. Mice that jumped from the platform before, between or after trials were retrieved and returned to the platform immediately. 'Escape latency' was calculated as the mean time taken to find and climb onto the platform by each mouse over the four trials of one day.
Mice were trained in daily blocks of four trials for 12 consecutive days, with probe trials (see below) on days 4, 8 and 12.

7.3.2.4. Reversal learning

Following 12 d of training with the platform in the SW position, 4 d of training were carried out with the platform in the opposite quadrant of the maze, i.e. NE (see Figure 7.1). Training was carried out exactly as described above from days 13 to 16, with a probe trial (see below) on day 16.

7.3.2.5. Probe trials

Probe trials were carried out every 4 d. Following the training sessions, each mouse was placed on the platform for 60 s. It was retrieved from the platform, and held outside the tank below the surface as the platform was removed from the water. The mouse was then carried around the edge of the tank to a position slightly offset from opposite where the platform was during training, i.e. when the platform was in the SW, the starting position was NNE (see Figure 7.1). The mouse was released, facing the wall of the tank, and swimming behaviour monitored for 60 s before being returned to the home cage.

The computer software was used to calculate a variety of parameters relating to the mice's behaviour (see Figure 7.2):

- Mean swimming speed (m.s⁻¹);
- Amount of time spent swimming slowly (< 0.1 m.s⁻¹; %);
- Amount of time spent in thigmotaxis (swimming in the outermost 75 mm of the pool's diameter; %);
- Amount of time spent in each quadrant (NE, SE, SW, NW; %);
- Number of times the mouse crossed an area surrounding the platform position (200 mm diameter), and equivalent positions relative to each quadrant;
- Amount of time spent in an area surrounding the platform position (200 mm diameter), and equivalent positions relative to each quadrant (%);
- Mean distance of the mouse from the centre of the platform position, and equivalent positions relative to each quadrant (m).
Chapter seven Learning and memory behaviours in neurokinin-1 receptor knockout mice

**Figure 7.2 Parameters for probe trial data.** View of MWM from above, showing the four quadrants of the pool (TQ: Target Quadrant; AL: Adjacent Left; AR: Adjacent Right; OP: Opposite), the platform positions and equivalent positions in the other quadrants, and the regions in which swimming is considered as 'crossing' the platform position and in which it is considered as 'thigmotaxic'.

**7.3.2.6. Statistical analysis**

The escape latencies were analysed separately for the initial training and reversal learning, using 2-way repeated-measures ANOVAs, with genotype as the between-subjects factor and day as the within-subjects factor (see section 2.6).

Swim speeds, slow swimming times and thigmotaxis times from probe trial data were analysed separately for each day using 1-way ANOVAs with genotype as the between-subjects factor. The four measures of spatial memory (time in quadrants, number of platform crossings, time at platforms and proximity) were analysed in two ways. Firstly, within-genotype analyses were carried out, in which 1-way ANOVAs were performed on the data for each genotype with quadrant as the
factor'. *Post hoc* Dunnett's comparisons were carried out following significant main effects to compare parameters in the target quadrant to other quadrants. Secondly, parameters for the target quadrant were compared across probe trials on days 4, 8 and 12 using 2-way repeated-measures ANOVAs, with genotype as the between-subjects factor and day as the within-subjects factor. Parameters from target quadrants on day 16 were compared using 1-way ANOVAs with genotype as the between-subjects factor. One mouse of each genotype was excluded from analysis due to a cataract (wild type) and unusually high levels of thigmotaxic swimming behaviour during probe trials (NK1<sup>−/−</sup>).

### 7.3.3. Fear conditioning

#### 7.3.3.1. Apparatus

The training chamber for fear conditioning consisted of an aluminium box (Mouse Shuttle Box; 270 mm [w] × 110 mm [l] × 130 mm [h]; Campden Instruments, Loughborough, UK) with a Plexiglas door at the front. The chamber was housed within a sound-attenuating chamber (684 mm [w] × 450 mm [l] × 487 mm [h]; Campden Instruments) fitted with a fan to mask external noises and positioned in a dimly lit room. The chamber's grid floor consisted of 32 parallel stainless steel rods of 2.5 mm diameter, separated by 10 mm, connected to a constant current shock source (521/C; Campden Instruments) via a shock scrambler (521/S; Campden Instruments), set to deliver scrambled alternating current (AC) at 0.55 mA. A removable tray beneath the grid floor was in place to catch faeces and urine. The training chamber contained two white lights (one on each end wall) and a speaker mounted in the ceiling set to produce a tone at 2.9 kHz and 85 dB. The tone and shock were computer-controlled by a PC running Animal Behavior Environment Test (ABET) System (Lafayette Instrument Company, Lafayette, USA). All training and testing sessions were videotaped using a camcorder attached to a videocassette recorder.

Before each trial, the chamber and grid floor were cleaned thoroughly; first with water and then with 70% ethanol to remove any faeces and urine, and to ensure that ethanol was the

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1 It is unclear whether data such as these should be analysed with conventional ANOVA or repeated-measures ANOVA. Although the data for the four quadrants are collected from the same group of animals, the data for each mouse for each quadrant depends on the value of the other three, at least for the measure of time spent in each quadrant. Use of conventional (non-repeated-measures) ANOVA was chosen here, as this is the method used in most published reports of Morris water maze behaviour (e.g. Giese *et al.* 1998).
predominant smell during training. Mice used in fear conditioning experiments were transported to a room adjacent to the experimental room at least 1 h before use. Each mouse was transported from its home cage to the training chamber individually in a clean mouse cage.

7.3.3.2. **Contextual fear conditioning**

The protocol for contextual fear conditioning is illustrated in Figure 7.3.

7.3.3.2.1. **Conditioning**

Mice \((n = 24\) per genotype) were placed individually in the training chamber and the door closed. The door to the sound-attenuating chamber was closed and the training chamber lights were switched on. After 2 min, the tone was played for 30 s, the last 2 s of which were co-presented with a foot shock. The mouse was left for a further 30 s in the chamber before the lights were switched off and the mouse returned to its home cage.

7.3.3.2.2. **Testing**

After 24 h, half of the mice \((n = 12\) per genotype) were tested for contextual fear. The apparatus was set up exactly as it was for conditioning, and each mouse was placed individually in the chamber. The lights were switched on for 5 min. During the first minute of re-exposure, observations of the mouse’s behaviour were made instantaneously every 5 s. Behaviour was scored as ‘freezing’ or ‘not freezing’, where ‘freezing’ is defined as a lack of movement other than that caused by respiration. Each observation was made without considering what happened before or after it.

The second group of mice were tested for contextual fear 15 d after conditioning and observed as described above.

7.3.3.2.3. **Extinction of contextual fear**

To examine the extinction of contextual fear, the mice tested at 24 h after conditioning were re-exposed to the training chambers three more times for 5 min at 24 h intervals, and their freezing behaviour observed (see above).
Figure 7.3 Protocol for contextual and delay fear conditioning. Mice were trained on day 1. Twenty-four hours later, one group of mice was tested for contextual fear conditioning, before being re-exposed to the testing chamber three more times on days 3–5 in the test of the extinction of conditioned fear. A second group of mice were tested for contextual fear conditioning 15 d after training, and for cued fear conditioning on day 16.
7.3.3.3. **Cued fear conditioning**

7.3.3.3.1. **Delay fear conditioning**

The same mice, which had been tested for contextual fear conditioning at 15 d after training, were tested for cued fear conditioning 24 h later, i.e. 16 d after conditioning. The protocol for delay fear conditioning is illustrated in Figure 7.3.

The mice \((n = 12\) per genotype) were placed individually into a chamber, which differed from the training chamber in shape, wall composition, floor texture, lighting colour and smell. It consisted of a semicircular chamber \((170 \text{ mm diameter} \times 130 \text{ mm} [h]; \text{Campden Instruments})\) made of Plexiglas, with a door at the front identical to that of the training chamber. It contained 2 red lights in place of the white lights of the training chamber, and a roughened Plexiglas floor in place of the training chamber's grid floor. An identical speaker to that of the training chamber was present in the ceiling, and was computer-controlled. The test chamber was placed in the same sound-attenuating chamber as the training chamber. Before each test session, it was cleaned with water and then a dilute solution of lemon-scented detergent.

Each mouse was transported as described above and placed individually in the test chamber. The lights were illuminated, and after 3 min, the tone was presented for a further 3 min. Freezing behaviour was observed throughout the 6-min test session. At the end of the session, each mouse was returned to the home cage.

7.3.3.3.2. **Trace fear conditioning**

The mice used in this experiment had previously been trained and tested in the Morris water maze. The protocol for trace fear conditioning is illustrated in Figure 7.4.

Mice \((n = 8\) per genotype) were transported individually to the training chamber (see above). The chamber lights were switched on, and after 1 min, the tone was presented for 15 s. Two minutes after the termination of the tone, the shock was presented for 2 s. This sequence was repeated a further three times with a 4-min intertrial interval. Mice were then returned to their home cages.
Twenty-four hours after conditioning, each mouse was transported to the cued test chamber (see above). One minute after the lights were switched on, the tone was presented for 15 s. After a further 2 min, the mouse was returned to the home cage. Observations were made every 5 s throughout the test period.

![Training](image)

![Test of trace fear conditioning](image)

Figure 7.4 Protocol for trace fear conditioning. Mice were trained with four tone-shock presentations with a delay of 2 min between tone and shock and a 4-min intertrial interval. Twenty-four hours after training, fear conditioning in response to the tone was assessed in a novel chamber.

7.3.3.4. **Statistical analysis**

Freezing times were expressed as percentages. For contextual fear conditioning, freezing time in the first minute of re-exposure was analysed using a 2-way ANOVA, with retention time and genotype as the between-subjects factors (see section 2.6). The data from extinction of conditioned fear was analysed using a 2-way repeated-measures ANOVA, with genotype as the between-subjects factor and day as the within-subjects factor. *Post hoc*
comparisons were carried out using 1-way ANOVAs of each day’s data with genotype as the between-subjects factor.

For delay and trace fear conditioning data, freezing behaviour was analysed using 2-way repeated-measures ANOVAs with genotype as the between-subjects factor and pre / post CS as the within-subjects factor. To assess how freezing behaviour changed in the time period following CS presentation, the post-CS freezing behaviour of mice following trace fear conditioning was also divided into 15-s bins and analysed using a 2-way repeated-measures ANOVA with genotype as the between-subjects factor and time as the within-subjects factor.

7.3.4. Spontaneous alternation

Spontaneous alternation behaviour was assessed in a black Plexiglas Y-maze consisting of a runway (346 mm [l] × 138 mm [w] × 141 mm [h]) connected to two end compartments (146 mm [l] × 138 mm [w] × 141 mm [h]). The first 146 mm of the runway (the ‘start box’) and the end compartments could be closed by guillotine doors. The apparatus was cleaned with 70 % ethanol before each habituation or test session.

Mice (NK1⁻ /⁻ ; n = 25; wild type: n = 26) were habituated to the apparatus twice for 10 min, separated by a 24-h interval. On the third day, each mouse was placed in the ‘start box’ with the guillotine door in place. After 30 s, the door was removed and the mouse was allowed to explore the apparatus. As soon as it entered one of the end compartments with all four paws, the guillotine door was closed and the mouse was contained within the compartment for 30 s. It was then returned to the ‘start box’, where it was contained for either 30 s (NK1⁻ /⁻ ; n = 15; wild type: n = 14) or 10 min (NK1⁻ /⁻ ; n = 10; wild type: n = 12). The guillotine door was then removed and the mouse allowed to explore the apparatus for 5 min. The first compartment entered by the mouse was recorded. EthoVision (see section 2.5.2) was used to monitor the mouse’s behaviour and calculate the amount of time spent in and number of entries made into each of the end compartments.

The time spent in the alternate compartment (i.e. the opposite compartment to that entered during the initial exploration) and the number of entries made into it were expressed as percentages of total time and total number of entries respectively. Both parameters were analysed using 2-way ANOVAs with genotype and time as between-subject factors (see
section 2.6). The numbers of mice entering the same and alternate compartment in the first entry of the test session was compared between genotypes within each retention time point using $\chi^2$ tests. Due to immobility during the test, 6 NK1$^{-/-}$ mice (from the 30 s group) and 6 wild type mice (4 from the 30 s group and 2 from the 10 min group) were omitted from analysis. A further three wild type mice (1 from the 30 s group and 2 from the 10 min group) were omitted from the analyses of entry and time data, due to failure of the recording equipment.

**7.3.5. Object recognition**

Object recognition was assessed in NK1$^{-/-}$ and wild type mice ($n = 20$ per genotype), which had already been tested for spontaneous alternation (see above). The test arena was identical in size, shape and colour to the open field arena (see section 2.5.4.1).

Mice were habituated to the arena twice for 20 min, at 24 h intervals. On the 3rd day, the conditioning session was carried out. Two small objects of similar size (a glass slide holder and a wooden toy) were placed in the arena, near opposite corners of the box. The mouse was introduced to the arena and allowed to explore the objects for 5 min. It was then returned to the home cage for 10 min before a second 5-min exposure to the same objects. The arena and objects were thoroughly cleaned with 70 % ethanol before each session.

The mice were returned to the home cage for 10 min or 24 h ($n = 10$ per genotype per time point). One of the two objects was replaced with a third, novel object (a blue plastic bottle lid). During the test session, the mice were allowed to explore the arena and the objects for two sessions of 5 min, separated by 10 min, as above.

During each session, the amount of time spent exploring each object was monitored visually. ‘Exploration’ was defined as the mouse being within 40 mm of the object and oriented towards it. Times spent exploring the object to be replaced (during the conditioning session) or the novel object (during the test session) were expressed as percentages of the total time spent exploring either object in the arena. This value was compared between genotypes and times using a 3-way repeated-measures ANOVA with genotype and retention time as between-subject factors and session (conditioning vs. test) as the within-subject factor (see section 2.6). Four wild type mice (one from the 10 min group and three from the 24 h group) were excluded from analysis following experimental
error. One NK1⁻/⁻ mouse's data point (10 min group) fell more than 2 standard deviations (SDs) from the group means in both conditioning and test sessions, and was also excluded.

7.3.6 Rota-Rod test

Motor coordination and learning were assessed in mice (n = 14 per genotype) that had previously been tested for contextual fear conditioning (see above). Mice were placed on the rotating drum of a Rota-Rod Treadmill for Mouse (Accelerating Model; Ugo Basile, Comerio, Italy), set to accelerate from 5 to 40 rpm over 5 min. The time taken by each mouse to fall was recorded. Each mouse underwent two trials on the Rota-Rod per day for 4 d, with a 1-h intertrial interval. Mice that remained on the drum for the full 5 min were removed. Mice were returned to the home cage between trials, and the Rota-Rod was cleaned with 70% ethanol then water and dried thoroughly between trials.

For each mouse, the time to fall from the rod was expressed as the mean of the day's two measurements. These values were compared using a 2-way repeated-measures ANOVA with genotype as the between-subjects factor and day as the within-subjects factor (see section 2.6). The data from 1 NK1⁻/⁻ mouse were omitted since it had been severely injured by a cage mate.

7.4 Results

7.4.1 Morris water maze

7.4.1.1 Initial learning

7.4.1.1.1 Escape latencies

Mice of both genotypes learned to find the hidden platform rapidly during training trials with the platform in the SW quadrant of the maze (Figure 7.5). A 2-way repeated-measures ANOVA revealed that there was a significant effect of training day on escape latency (F₁₁,₃₀₈ = 35.2; P < 0.001), but no significant effects of genotype (F₁,₃₁₈ = 0.20; P = 0.659) or interaction between day and genotype (F₁₁,₃₀₈ = 1.75; P = 0.062).
Figure 7.5 Escape latencies during Morris water maze initial learning (days 1 - 12) and reversal learning (days 13 - 16). Mean ± SEM escape latencies for NK1−/− and wild type mice. Arrows indicate days on which probe trials were performed.

7.4.1.1.2. Probe trials

Probe trials were performed on days 4, 8 and 12 of initial training. There were no differences between genotypes in swimming speed (1-way ANOVAs: \( P > 0.5 \)), the amount of time spent swimming slowly (1-way ANOVAs: \( P > 0.9 \)) or the amount of time spent swimming around the edge of the pool (1-way ANOVAs: \( P > 0.1 \)), suggesting that NK1−/− mice did not have alterations in swimming performance or anxiety levels during the probe trials (data not shown).

During probe trials, the swimming behaviour of each mouse was monitored for 60 s with the platform removed from the water. After 4 d of training, both wild type and NK1−/− mice demonstrated a spatial bias in their swimming behaviour in terms of the time spent in each quadrant of the maze (1-way ANOVAs: \( P < 0.03 \); Figure 7.6A). However, post hoc Dunnett’s comparisons revealed that only NK1−/− mice displayed a significant preference for the target quadrant (TQ; \( P < 0.005 \)). Wild type mice spent longer in the TQ quadrant than adjacent left (AL; \( P = 0.016 \)) and opposite (OP) quadrants (\( P = 0.038 \)), but similar amounts of time in the TQ and the adjacent right (AR) quadrant (\( P = 0.099 \)). On days 8 and 12, mice of both genotypes displayed a clear spatial strategy, spending more time in the target quadrant than the other three (post hoc Dunnett’s comparisons: \( P < 0.001 \)). Analysis
of the time spent in the target quadrant over the three probe trials (Figure 7.6B) revealed a significant effect of day ($F_{2,56} = 19.2; P < 0.001$), but no significant effects of genotype ($F_{1,28} = 0.54; P = 0.469$) or day by genotype interaction ($F_{2,56} = 0.48; P = 0.619$).
Figure 7.6 Probe trial data (initial learning): time spent in quadrants. A: Mean ± SEM times spent in TQ, AR, AL and OP quadrants by wild type and NK1⁻/⁻ mice during probe trials on days 4, 8 and 12. *P < 0.05; **P < 0.01; ***P < 0.001 (vs. TQ). B: Mean ± SEM times spent in TQ by wild type and NK1⁻/⁻ mice across probe trials on days 4, 8 and 12.
Spatial learning was also assessed by analysing the number of times the mice crossed the platform position and equivalent positions in the other quadrants (see Figure 7.2). On day 4, wild type mice did not display a statistically significant bias in their crossing behaviour ($F_{3,56} = 1.58; P = 0.204$; Figure 7.7). However, NK1$^{-/-}$ mice did display such a bias ($F_{3,56} = 2.97; P = 0.040$) but there was no significant difference between the number of crossings in the TQ and AL (post hoc Dunnett's comparison: $P = 0.060$) or AR quadrants ($P = 0.091$). On days 8 and 12, both genotypes crossed the platform position more than equivalent positions in the other three quadrants (wild type: post hoc Dunnett's comparisons: $P < 0.02$; NK1$^{-/-}$: Mann-Whitney tests with Bonferroni's correction: $P < 0.005$). Analysis of the number of crossings over the platform position across the three probe trials (Figure 7.7B) revealed a significant effect of day ($F_{2,56} = 14.7; P < 0.001$), but not genotype ($F_{1,28} = 0.33; P = 0.569$) or day by genotype interaction ($F_{2,56} = 0.20; P = 0.818$).
Figure 7.7 Probe trial data (initial learning): crossings. A: Mean ± SEM number of crossings made over platform positions in TQ, AR, AL and OP quadrants by wild type and NK1/− mice during probe trials on days 4, 8 and 12. *P < 0.05; **P < 0.01; ***P < 0.001 (vs. TQ). B: Mean ± SEM number of crossings over platform position in TQ by wild type and NK1/− mice across probe trials on days 4, 8 and 12.
The amount of time spent in a circular area surrounding the platform position and equivalent positions in the other quadrants (see Figure 7.2) revealed a similar pattern to the time and crossings measures (Figure 7.8). On day 4, wild type mice did not exhibit a statistically significant bias in the amount of time spent in the four positions ($F_{3,56} = 1.64; P = 0.191$). NK1$^{-/-}$ mice did show such a bias ($F_{3,56} = 2.98; P = 0.039$), although the differences between the amount of time spent in the TQ position and AL (post hoc Dunnett's comparison: $P = 0.053$) or AR positions ($P = 0.069$) narrowly missed statistical significance. On day 8, wild type mice did show a bias in their behaviour ($H_1 = 8.50; P = 0.037$), but there were no differences in time spent in the TQ position and AL (Mann-Whitney test with Bonferroni's correction: $P = 0.084$) or AR positions ($P = 0.057$). Conversely, NK1$^{-/-}$ mice showed clear selectivity for the TQ on day 8 (post hoc Dunnett's comparisons: $P < 0.007$). On day 12, both genotypes spent more time in the platform position than equivalent positions in the other three quadrants (wild type: Mann-Whitney tests with Bonferroni's correction: $P < 0.001$; NK1$^{-/-}$: post hoc Dunnett's comparisons: $P < 0.001$). Analysis of time spent in the TQ position across probe trials (Figure 7.8B) revealed a significant effect of day ($F_{2,56} = 15.4; P < 0.001$), but not genotype ($F_{1,28} = 0.11; P = 0.740$) or day by genotype interaction ($F_{2,56} = 0.23; P = 0.793$).
Figure 7.8 Probe trial data (initial learning): times spent at platform positions. A: Mean ± SEM times spent at platform positions in TQ, AR, AL and OP quadrants by wild type and NK1−/− mice during probe trials on days 4, 8 and 12. *P < 0.05; **P < 0.01; ***P < 0.001 (vs. TQ). B: Mean ± SEM times spent at platform position (TQ) by wild type and NK1−/− mice across probe trials on days 4, 8 and 12.
The final measure used to assess spatial memory in probe trials was the mean proximity of each animal to the platform position, and equivalent positions in the other three quadrants (Gallagher et al. 1993). During the probe trial on day 4, wild type mice did not demonstrate a statistically significant bias in their proximity to these positions ($F_{3,56} = 2.49; P = 0.070$), whereas NK1$^{-/-}$ mice did show such a bias ($F_{3,56} = 5.14; P = 0.003$). However, post hoc Dunnett's comparisons in the NK1$^{-/-}$ group revealed that although the mice swam significantly closer to the platform position than equivalent positions in the OP ($P < 0.001$) and AR quadrants ($P = 0.044$), their proximity to the position in the AL quadrant did not differ significantly from that in the TQ ($P = 0.076$; Figure 7.9A). On days 8 and 12, both genotypes displayed bias in their swimming behaviour, swimming closer to the platform position than equivalent positions in the other three quadrants (post hoc Dunnett's comparisons: $P < 0.03$). Analysis of the proximity to the platform position over the three probe trials (Figure 7.9B) revealed a significant effect of day ($F_{2,56} = 25.7; P < 0.001$), but no significant effects of genotype ($F_{1,28} = 0.04; P = 0.850$) or day by genotype interaction ($F_{2,56} = 0.85; P = 0.431$).
Figure 7.9 Probe trial data (initial learning): proximity. A: Mean ± SEM proximity to platform positions in TQ, AR, AL and OP quadrants by wild type and NK1⁻/⁻ mice during probe trials on days 4, 8 and 12. *P < 0.05; **P < 0.01; ***P < 0.001 (vs. TQ). B: Mean ± SEM proximity to platform position (TQ) by wild type and NK1⁻/⁻ mice across probe trials on days 4, 8 and 12.
Taken together, these results suggest that wild type and NK1\(^{-/-}\) mice spent similar amounts of time swimming close to the platform position, made similar numbers of crossings over its position and tended to swim at similar distances from it across the three probe trials. However, during the first probe trial on day 4, NK1\(^{-/-}\) mice showed a subtle increase in selectivity for the TQ across all four probe trial parameters, suggesting that they form spatial memory sooner than wild type controls.

7.4.1.2. Reversal learning

7.4.1.2.1. Escape latencies

From days 13 to 16, the platform was moved to the NE quadrant (see Figure 7.1). Both groups of mice rapidly learned to find the platform (Figure 7.5). A 2-way repeated-measures ANOVA revealed a significant effect of training day on escape latency \((F_{3,84} = 20.2; P < 0.001)\), but no significant effects of genotype \((F_{1,28} = 0.73; P = 0.401)\) or interaction between day and genotype \((F_{3,84} = 0.01; P = 0.999)\).

7.4.1.2.2. Probe trial

A probe trial was carried out after 4 d of reversal learning. There were no significant differences between genotypes in swimming speed \((F_{1,28} = 0.13; P = 0.724)\), the amount of time spent swimming slowly \((F_{1,28} = 0.10; P = 0.758)\) or the amount of time spent swimming around the edge of the pool \((F_{1,28} = 3.46; P = 0.074)\) suggesting that NK1\(^{-/-}\) mice did not have alterations in swimming performance or anxiety levels during the probe trial (data not shown).

Both genotypes displayed spatial strategies in the probe trial on day 16, spending more time in the target quadrant than the other three (post hoc Dunnett’s comparisons: \(P < 0.001\); Figure 7.10). There was no difference between genotypes in the time spent in the target quadrant \((F_{1,28} = 0.24; P = 0.630)\).
Both genotypes also displayed a bias in their crossing behaviour (wild type: Kruskal-Wallis test: $H_1 = 12.5; P = 0.006$; NK1$^{-/-}$: 1-way ANOVA: $F_{3,56} = 6.77; P = 0.001$), but only NK1$^{-/-}$ mice made more crossings over the platform position than equivalent positions in the other three quadrants of the maze (post hoc Dunnett’s comparisons: $P < 0.005$; Figure 7.11): wild type mice made more crossings over the platform position than equivalent positions in the AR (Mann-Whitney test with Bonferroni’s correction: $P = 0.011$) and AL (P = 0.034) quadrants, but not the OP quadrant (P = 0.068). There was no difference between genotypes in the number of crossings over the platform position ($F_{1,28} = 0.05; P = 0.824$).

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**Figure 7.10** Probe trial data (reversal learning): time spent in quadrants. Mean ± SEM times spent by wild type and NK1$^{-/-}$ mice in TQ, AR, AL and OP quadrants during probe trial on day 16. ***P < 0.001 (vs. TQ).

**Figure 7.11** Probe trial data (reversal learning): crossings. Mean ± SEM number of crossings made over platform positions in TQ, AR, AL and OP quadrants by wild type and NK1$^{-/-}$ mice during probe trial on day 16. *P < 0.05; **P < 0.01; ***P < 0.001 (vs. TQ).
Analysis of the time spent at each of the four positions revealed a statistically significant bias in both genotypes (NK1\(^{-/-}\): 1-way ANOVA: \(F_{3,36} = 8.20\); \(P < 0.001\); wild type: Kruskal-Wallis test: \(H_3 = 14.8\); \(P = 0.002\); Figure 7.12). Only NK1\(^{-/-}\) mice spent more time in the platform position than the other three positions (post hoc Dunnett’s comparisons: \(P < 0.002\)); wild type mice spent longer in the TQ position than the AR (Mann-Whitney test with Bonferroni’s correction: \(P = 0.008\)) and AL positions (\(P = 0.011\)) but not the OP position (\(P = 0.133\)). There was no difference between genotypes in the time spent at the platform position (\(F_{1,28} = 0.03; P = 0.862\)).

![Figure 7.12 Probe trial data (reversal learning): time spent at platform positions.](image)

Finally, analysis of the proximity measure revealed that both genotypes displayed a bias in their swimming behaviour (Kruskal-Wallis tests: \(P < 0.001\)). Neither group of mice swam closer to the platform position than the equivalent position in the AR quadrant (Mann-Whitney tests with Bonferroni’s correction: \(P > 0.08\); Figure 7.13). There was no significant difference in proximity to the platform position between genotypes (\(F_{1,28} = 0.78; P = 0.385\)).
7.4.2. Fear conditioning

7.4.2.1. Contextual fear conditioning

Upon re-exposure to the training chamber at 24 h or 15 d after conditioning, mice of both genotypes exhibited bouts of freezing behaviour. A 2-way ANOVA revealed a significant main effect of retention time on time spent freezing during the first minute of re-exposure ($F_{1,44} = 12.7; P = 0.001$), but no significant effects of genotype ($F_{1,44} = 0.47; P = 0.496$) or time by genotype interaction ($F_{1,44} = 0.62; P = 0.434$), indicating that mice of both genotypes froze more at 15 d after conditioning than 24 h, but that this did not differ between genotypes (Figure 7.14A).

7.4.2.1.1. Extinction of contextual fear memory

Re-exposure of mice in the 24-h group to the training chamber for 5 min at 24-h intervals over 4 d resulted in a gradual extinction of contextual fear memory in both genotypes (Figure 7.14B). A 2-way repeated-measures ANOVA revealed a significant main effect of day on time spent freezing in the first minute of each exposure ($F_{3,66} = 13.7; P < 0.001$), but no significant effects of genotype ($F_{1,22} = 1.24; P = 0.278$) or time by genotype interaction ($F_{3,66} = 0.55; P = 0.642$).
interaction ($F_{3,66} = 2.39; P = 0.076$). This indicates that both genotypes extinguished contextual fear memory at similar rates, despite a tendency for NK1$^{-/-}$ mice to freeze less than wild type mice on days 3 and 4.

![Figure 7.14 Contextual fear conditioning. A: Mean ± SEM times spent freezing by wild type and NK1$^{-/-}$ mice in the first minute of re-exposure to the shock-associated context at 24 h or 15 d after training. B: Extinction of conditioned fear. Mean ± SEM times spent freezing by wild type and NK1$^{-/-}$ mice in the first minute of repeated re-exposures to the shock-associated context for 5 min at 24-h intervals.](image)

**7.4.2.2. Cued fear conditioning**

Sixteen days after training, the mice from the 15-d contextual fear-conditioning group were tested for cued fear memory. Both groups of mice exhibited low levels of freezing behaviour upon exposure to the novel testing chamber, but this increased during tone presentation (Figure 7.15). A 2-way repeated-measures ANOVA revealed a significant effect of tone presentation on freezing behaviour ($F_{1,22} = 183; P < 0.001$), but no significant effects of genotype ($F_{1,22} = 1.23; P = 0.280$) or tone by genotype interaction ($F_{1,22} = 1.47; P = 0.237$), indicating that mice of both genotypes behaved similarly in the test of delay fear memory, with both groups demonstrating a CS-induced freezing response.
7.4.2.3. Trace fear conditioning

Mice undergoing trace fear conditioning were tested for shock-associated freezing 24 h after conditioning. Both groups of mice exhibited some freezing behaviour upon exposure to the novel context, but this increased after the tone was played (Figure 7.16A). A 2-way repeated-measures ANOVA revealed a significant main effect of CS presentation on freezing behaviour ($F_{1,14} = 20.0; P = 0.001$), but no significant effect of genotype ($F_{1,14} = 0.19; P = 0.666$) or genotype by CS interaction ($F_{1,14} = 0.04; P = 0.842$). This indicates that CS presentation caused increases in freezing behaviour in both genotypes (i.e. conditioning had occurred), but that neither the generalised freezing response before CS presentation nor the conditioned freezing response differed between genotypes.

The behaviour of mice during the 15 s of tone presentation and the subsequent 2 min was analysed following division into 15-s bins (Figure 7.16B). A 2-way repeated-measures ANOVA revealed a significant main effect of time on freezing behaviour ($F_{8,112} = 2.88; P = 0.006$), but no significant effects of genotype ($F_{1,14} = 1.44; P = 0.250$) or time by genotype interaction ($F_{8,112} = 1.28; P = 0.261$), suggesting that the pattern of freezing behaviour was similar in the two genotypes. These data do not therefore provide any evidence for a difference between genotypes in the ability to freeze at the appropriate time delay after tone presentation (2 min).
Chapter seven  Learning and memory behaviours in neurokinin-1 receptor knockout mice

![Figure 7.16 Trace fear conditioning. A: Mean ± SEM times spent freezing by wild type and NK1+/ mice in the first minute of exposure to a novel context (pre-CS), and in the minute during and after tone presentation (post-CS) at 24 h after training. B: Mean ± SEM times spent freezing by wild type and NK1+/ mice in the 135 s following tone presentation, divided into 15-s bins. The grey bar indicates the time of CS presentation. The arrow indicates the time at which the shock US was given during training.]

7.4.3. Spontaneous alternation

Thirty seconds or 10 min after a 30-s containment in one arm of a Y-maze, NK1+/ and wild type mice were re-introduced to the maze and their behaviour was monitored. Analysis of the time spent in the alternate compartment (the opposite compartment to the one in which each mouse had previously been contained) during the 5-min test session showed that there were no significant effects of genotype ($F_{1,32} = 0.07; P = 0.798$), time ($F_{1,32} = 1.57; P = 0.219$) or genotype by time interaction on this parameter ($F_{1,32} = 0.03; P = 0.854$; Figure 7.17A). However, overall the mice spent more than 50% of the time in the alternate compartment (median = 65.3 %; Wilcoxon signed rank test: $W_{36} = 553; P = 0.001$). Similarly, analysis of the number of entries into the alternate compartment showed that there were no significant main effects of genotype ($F_{1,32} = 1.32; P = 0.260$), time ($F_{1,32} = 1.62; P = 0.212$) or genotype by time interaction on this parameter ($F_{1,32} = 0.16; P = 0.695$; Figure 7.17B). Overall, the mice made more than 50% of their entries into the alternate compartment ($56.3 ± 2.0 %; \text{Student's } t \text{ test: } T_{35} = 3.28; P = 0.002$). Together these results indicate that both genotypes exhibited alternation behaviour, but this did not differ between retention times or genotypes.
At both time points similar numbers of NK1⁻/⁻ and wild type mice made their first entry into the alternate compartment (30 s: NK1⁻/⁻: 6 of 9 [66.7 %]; wild type: 7 of 10 [70.0 %]; χ² = 0.024; P = 0.876; 10 min: NK1⁻/⁻: 5 of 10 [50.0 %]; wild type: 5 of 10 [50.0 %]; χ² = 0.000; P = 1.000; Figure 7.17C). However, fewer mice alternated on the first entry at 10 min than 30 s, suggesting a reduction in alternation behaviour on the first entry over time.

Figure 7.17 Spontaneous alternation. A: Mean ± SEM times spent in the alternate compartment by wild type and NK1⁻/⁻ mice at 30 s or 10 min after containment, during the 5-min test session. B: Mean ± SEM number of entries made into the alternate compartment during the 5-min test session. C: Numbers of mice entering the alternate compartment in the first entry of the test session. Dotted lines indicate chance (50 %).

7.4.4. Object recognition

Object recognition was assessed by analysing the amount of time spent exploring one of two objects during the initial conditioning sessions, and a novel object in the test session.
The results of a 3-way repeated-measures ANOVA are given in Table 7.1. Only the ‘session’ term in the analysis was significant, indicating that, overall, the mice spent more time investigating the novel object than the object previously in the same position, i.e. that object recognition had occurred. The lack of a significant main effect of genotype or retention time, or any interaction term, indicates that the level of object recognition was similar in the two genotypes and was not significantly affected by the time delay between conditioning and testing (Figure 7.18). Additionally, they indicate that the levels of motivation, curiosity and interest were similar in both genotypes. However, the mice tended to spend less than 50% of their exploration time investigating the object to be replaced and the novel object, indicating a preference for the object that was not replaced. This is probably due to the fact that this position was further from the experimenter, such that the object to be replaced (and the novel object in the test session) was not approached as readily because of an anxiety effect.

Table 7.1 Results of 3-way repeated-measures ANOVA of object recognition data. The significant P value is underlined.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Session (S)</td>
<td>1</td>
<td>33.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genotype (G)</td>
<td>1</td>
<td>0.28</td>
<td>0.598</td>
</tr>
<tr>
<td>Retention time (RT)</td>
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<td>1.47</td>
<td>0.234</td>
</tr>
<tr>
<td>S × G</td>
<td>1</td>
<td>3.82</td>
<td>0.060</td>
</tr>
<tr>
<td>S × RT</td>
<td>1</td>
<td>0.79</td>
<td>0.380</td>
</tr>
<tr>
<td>G × RT</td>
<td>1</td>
<td>0.57</td>
<td>0.457</td>
</tr>
<tr>
<td>S × G × RT</td>
<td>1</td>
<td>2.58</td>
<td>0.118</td>
</tr>
<tr>
<td>Error</td>
<td>31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7.18 Object recognition. Mean ± SEM times spent by wild type and NK1⁻/⁻ mice exploring an object during the conditioning session and following replacement with a novel object in the same position during the test session, at 10 min or 24 h after training. Time values are expressed as a percentage of the total time spent exploring either of the two objects in the arena.

7.4.5. Rota-Rod test

Mice of both genotypes learned to remain on the drum of the Rota-Rod with two daily trials over 4 d (Figure 7.19). A 2-way repeated-measures ANOVA of mean daily times to fall revealed significant main effects of genotype ($F_{1,25} = 5.75; P = 0.024$) and day ($F_{3,75} = 40.6; P < 0.001$), but no significant genotype by day interaction ($F_{3,75} = 0.98; P = 0.406$). These results suggest that NK1⁻/⁻ mice exhibit a mild performance enhancement on the Rota-Rod task, but that motor learning occurs at a similar rate in both genotypes.
7.5. Discussion

The behaviour of NK1\(^{-}\) mice was examined in a range of learning and memory tasks. These can be broadly divided into those that involve the hippocampus (the Morris water maze, contextual and trace fear conditioning, spontaneous alternation and object recognition), and those that are independent of hippocampal function (delay fear conditioning and the Rota-Rod test). The requirements of each of these tasks vary within each grouping, in terms of the nature of the learning (e.g. spatial or associative) and their duration.

7.5.1. Behaviour of neurokinin-1 receptor knockout mice in learning and memory tasks

7.5.1.1. Morris water maze

In the Morris water maze, mice were trained to find a platform hidden below the surface of the pool. This classic test of spatial learning and memory is critically dependent upon hippocampal function (Morris et al. 1982; O'Keefe & Nadel 1978; Sutherland et al. 1982; Whishaw 1987). In the experiments described here, mice of both genotypes learned to find the platform. There were no differences between genotypes in the time taken to find the platform across the 12 d of initial learning, and the subsequent 4 d of reversal learning, during which the position of the platform was moved. However, in probe trials, NK1\(^{-}\)
mice showed a subtle increase in selectivity for the target quadrant after 4 d of training relative to wild type controls and following 4 d of reversal learning, as assessed by the four separate measures of swimming behaviour. Despite this mild within-group difference, statistical comparisons failed to reveal any differences in these parameters between the two genotypes with respect to the TQ. Taken together, these results demonstrate that both genotypes learned the task rapidly, but that the NK1<sup>−/−</sup> mice did so marginally more rapidly than the wild type mice. This result, although subtle, is in apparent contrast to those of Hasenöhrl et al. (1990b, 1994), who showed that peripheral administration of SP reduced the age-induced impairments in the Morris water maze. However, this difference is somewhat unsurprising, since the present experiments did not use aged animals, and the previous studies involved the exogenous application of SP.

### 7.5.1.2. Fear conditioning

Three fear conditioning paradigms were attempted in NK1<sup>−/−</sup> mice. Firstly, contextual fear conditioning was examined, with formation of contextual fear memories being assessed 24 h or 15 d after training. NK1<sup>−/−</sup> mice behaved in a similar manner to their littermate wild type controls at both time points, suggesting that there are no differences in the formation of contextual fear memories between the two genotypes. There were also no differences in the extinction of the freezing response between genotypes, suggesting that the extinction of contextual fear memory occurs at similar rates in both genotypes.

Intriguingly, in both genotypes, the amount of freezing at 15 d after training was significantly greater than that at 24 h, although the mice were trained identically. This may be an effect of consolidation, the process in which short-term memory is transferred into stable long-term memory. Although the neurochemical mechanisms for this process remain a matter of controversy, it is possible that an LTP-based mechanism could be responsible, in which consolidation represents the substitution of the mRNA- and protein-synthesis independent 'early' LTP with the protein-synthesis-dependent 'late' form (Schafe et al. 2001). However, the requirement for mRNA and protein synthesis in long-term memory formation, and putatively in 'late' LTP lies in the hours rather than days following training (Bailey et al. 1999; Schafe & LeDoux 2000). The observation of an increased freezing response between 24 h and 15 d after training in this study therefore suggests that an alternative mechanism is in place to bring about this effect. It is believed that
consolidation is a long process extending over several days or weeks (Dudai 1996; McGaugh 2000): such an extended period of consolidation for contextual fear memory could therefore explain the strengthening of the memory trace observed in this experiment.

In the test of cued fear conditioning using a delay protocol, a hippocampus-independent task, mice of both genotypes displayed conditioned freezing in response to the tone, but there were no differences between genotypes. Similarly, there were no differences between genotypes in trace fear conditioning, although both groups displayed significant freezing in response to the tone CS.

Much of the previous published work on the role of SP in learning and memory employed one-trial avoidance tasks, which are similar to fear conditioning, as they involve the administration of a footshock as an aversive stimulus. These studies indicated that post-trial injection of SP either peripherally or into the nucleus basalis magnocellularis or medial septal nucleus bring about facilitation of the task (Kafetzopoulos et al. 1986; Schlesinger et al. 1983; Stäubli & Huston 1980; Tomaz & Huston 1986). More recent studies have utilised NK1 receptor antagonists to examine fear conditioning in the gerbil, a species with human-like NK1 receptor pharmacology (Beresford et al. 1991; Gitter et al. 1991). In one study, MK-869 was found to inhibit foot tapping behaviour, a species-specific sign of fear, in response to footshock-associated discrete cues (Ballard et al. 2001), whilst Rupniak et al. (unpublished observations) used a four plate apparatus to measure fear conditioning: in this paradigm, foot tapping in response to re-exposure to the shock apparatus was inhibited by the antagonist L 760 735. However, in both these studies the inhibitory effect of NK1 receptor antagonism is explained on the basis of its acute anxiolytic effect, rather than a direct modulation of mnemonic processes, in agreement with previous findings in the rat (Mongeau et al. 1998).

7.5.1.3. Working memory

In the test of spontaneous alternation, both genotypes exhibited alternation behaviour on the first entry of the test session at the 30 s retention time point. Both groups of mice also made more entries into and spent more time in the alternate compartment over the entire 5-min test session at both retention time points. In the object recognition paradigm, mice of both genotypes spent more time exploring a novel object than they did exploring a
familiar object previously in the same position. There were therefore no alterations in working memory in NK1−/− mice in the spatial or non-spatial tasks used, and no deficits in object recognition ability.

7.5.1.4. Rota-Rod test

Finally, motor coordination and learning were assessed in the Rota-Rod test. A mild enhancement in performance was observed in NK1−/− mice, although the motor learning rate was similar in the two genotypes. Although differences in motor performance can confound the findings of other behavioural experiments, the small enhancement observed here, and the lack of a profound deficit in motor coordination in NK1−/− mice suggest that this performance difference is unlikely to have a major impact on other tasks. Interestingly, the Rota-Rod results presented here mirror the published demonstrations of increased proliferation of dentate gyrus granule cells in mice undergoing periods of voluntary exercise (van Praag et al. 1999a,b). In this study, an improvement of motor performance has been demonstrated in mice with enhanced proliferation of these cells. Although these two observations cannot be causally linked, this correlation suggests that there may a bi-directional link between exercise and hippocampal neurogenesis.

7.5.1.5. Summary

In summary, NK1−/− mice display normal behaviour in a battery of tests of learning and memory, including hippocampal and non-hippocampal tasks. The only detectable difference between genotypes was a subtle increase in selectivity during probe trial behaviour in NK1−/− mice, suggesting that they may be marginally quicker at demonstrating spatial memory. In addition, the mild enhancement of motor ability observed in the Rota-Rod, although not modulated by differences in motor learning between genotypes, may play a minor role in certain tasks involving locomotion. These data provide more information about the involvement of SP and the NK1 receptor in learning and memory processes, either directly, or via their modulatory influence on hippocampal neurogenesis.
7.5.2. Role of substance P and the neurokinin-1 receptor in learning and memory

7.5.2.1. Comparison with previous studies

In terms of the role of endogenous SP and the NK1 receptor in learning and memory processes, these results argue against the necessity for NK1 receptors in normal performance on these tasks. In contrast, the studies examining the effects of NK1 antagonism on fear conditioning behaviour suggest that the receptor is necessary for these behaviours. However, the additional reductions in non-conditioned fear brought about by NK1 antagonists suggest that these effects are not mnemonic in nature, but due to the drugs' anxiolytic effects. Furthermore, there is an extensive literature examining the effects of exogenous SP administration on learning behaviour, most of which indicate a memory-enhancing effect of the molecule. These studies imply that the NK1 receptor plays a role in the modulation of certain learning and memory tasks, whereas the present results from NK1<sup>−/−</sup> mice argue against a crucial role for the SP-NK1 receptor system. However, since NK1<sup>−/−</sup> mice do not express NK1 receptors throughout life, one cannot exclude the possibility that developmental compensations for lack of NK1 receptor signalling may occur, and compensate for the deficit. The levels of SP and of the other tachykinin receptors are normal in the brains of NK1<sup>−/−</sup> mice (De Felipe et al. 1998), suggesting that tachykinin signalling other than that mediated by the NK1 receptor is unaffected by the gene knockout. Increases in 5-HT and noradrenaline tone have been observed in NK1<sup>−/−</sup> mice (Froger et al. 2001; Santarelli et al. 2001), and may account for the observed increase in hippocampal neurogenesis. These results indicate that these alterations in monoamine function do not influence learning and memory significantly, although further work is required to identify whether these systems are compensating for a lack of substance P signalling in the tasks used here.

7.5.2.2. Effects of anxiety

Performance in learning and memory tasks can be affected by the emotional state of the animal being tested. In particular, differences between experimental groups in anxiety behaviour could severely impede assessment of learning in fear conditioning, since the signs of conditioned fear are used as the learning index. Similarly, in the Morris water maze differences in anxiety can affect swimming behaviour and motivation to find and climb
onto the platform. Despite the preclinical and clinical data suggesting a role for the NK1 receptor in depression and anxiety, the mice used in this study display normal behaviour in tests of anxiety, such as the elevated plus maze (Murtra et al. 2000b). Furthermore, there were no differences in thigmotaxis or amount of time spent swimming slowly in the probe trials of the water maze task, suggesting that NK1−/− mice did not display anxiety-induced wall hugging or floating. The strain of NK1−/− mouse produced in the laboratory of René Hen has been shown to have reduced anxiety levels in a variety of tests (Santarelli et al. 2001), but there have been no published reports of this strain’s behaviour in learning and memory paradigms.

7.5.2.3. Implications for conditioned place preference findings

Importantly, NK1−/− mice did not demonstrate any deficits in associative learning ability, in spatial learning (Morris water maze) or Pavlovian conditioning (fear conditioning protocols). This observation therefore adds weight to the finding that these mice do not exhibit CPP to morphine (Murtra et al. 2000b): since CPP is a learning-based task, any generalised deficits in learning ability can interfere with the expression of a reward-induced place preference.

7.5.3. Role of hippocampal adaptations

7.5.3.1. Neurogenesis

Finally, the lack of a clear memory-enhancing effect in NK1−/− mice provides information on the role of increased proliferation of hippocampal granule cells. A number of studies have claimed to show correlations between enhanced hippocampal neurogenesis and learning and memory behaviours in rodents. These include the effects of environmental enrichment, which causes increases in the survival of new granule cells (Kempermann et al. 1997; Nilsson et al. 1999), and voluntary exercise, which causes increases in both proliferation and survival (van Praag et al. 1999b), on Morris water maze behaviour (van Praag et al. 1999a). However, in these studies, conclusions regarding spatial learning behaviour are made on the basis of shorter escape latencies in the enriched or runner groups: Kempermann et al. (1997) saw no differences between enriched and control mice in their probe trial after 6 d of training, whereas a parallel study in the rat did not assess probe trial behaviour (Nilsson et al. 1999). Van Praag et al. (1999) failed to demonstrate spatial
learning in mice with access to a running wheel or control animals in a probe trial, but claimed that running enhances acquisition on the water maze task on the basis of a stronger trend towards spatial learning in the runner mice along with decreased escape latencies during training. The differences observed in escape latencies could be confounded by differences in the procedural aspects of the task, such as motivation to find the platform. In the absence of data demonstrating equivalence in such behaviours, such as with a visible version of the task, and without demonstrating spatial learning in probe trials, the conclusions of these studies must be treated with caution. In the present study, the lack of differences in escape latencies during training would argue against a difference in the motivational aspects of the task between the two mouse groups.

Recently, an attempt has been made to correlate the genetic variability in baseline hippocampal neurogenesis between mouse strains to performance in the Morris water maze (Kempermann & Gage 2002). In this study, the behaviour of ten strains of recombinant inbred mice based on C57BL/6 and DBA/2 was compared, and correlated to the survival of newly born neurones assessed four weeks after BrdU injection. The authors claim that the observed correlation between neurogenesis and the slope of the learning curves during training indicates that adult neurogenesis is involved in the acquisition of new information. However, their data do not support their conclusions for two reasons. Firstly, there is considerable variability in the behaviour of the twelve strains (including the two parental strains) in the visible version of the water maze task, suggesting that they differ in motivation to find the platform. It is therefore impossible to correlate the differences in escape latency during the hidden platform version of the task with differences in acquisition of spatial learning. Secondly, the authors fail to demonstrate spatial learning in the probe trials, since none of the mouse strains develops a significant bias for the target quadrant by the fifth day of training. A correlation between neurogenesis and spatial learning is therefore not possible.

Despite these caveats, there are still two lines of evidence that suggest that the production of new neurones within the dentate gyrus may influence learning and memory behaviour. Firstly, van Praag et al. (1999) claimed that mice given access to a running wheel demonstrate better spatial learning in probe trials. Secondly, hippocampus-dependent learning and memory tasks have been shown to enhance the survival of one- to two-week
old neurones in the hippocampus (Gould et al. 1999a). However, in these studies the emphasis was placed on the effects of the long-term survival of new granule cells in the dentate gyrus on learning and memory behaviour. The effect of such tasks on the survival of new neurones in NK1^−/− mice has not been assessed, but under baseline conditions they exhibit enhanced proliferation of dentate gyrus granule cells, with the number of new cells falling back to wild type values by 7 d. This suggests that there is a compensatory increase in death of these new cells in NK1^−/− mice. NK1^−/− mice therefore only have more young granule cells, i.e. less than 7 d old, but they have normal numbers of older adult-generated cells. The behavioural results presented here suggest that these young cells, which are presumably poorly differentiated and not integrated into the local circuitry, have only a weak influence on hippocampus-dependent learning and memory.

To date, the clearest demonstration of a functional link between hippocampal neurogenesis and learning has been the demonstration of a reduction in trace eye blink conditioning following the pharmacological inhibition of cell division (Shors et al. 2001). Here I have examined the effects of enhanced proliferation of dentate gyrus granule cells in a trace paradigm, but using fear conditioning. In contrast to the effects observed in the rat with eye blink conditioning, I failed to see any alterations in response to the CS. Although it is possible that the difference in paradigm is responsible for this discrepancy, the role of the hippocampus in associating temporally separated events in trace paradigms in general would argue against this being the cause. In the eye blink experiment, the cytostatic drug MAM was administered chronically for 14 d, maintaining neurogenesis at a low level throughout the training period, beginning 2 d after the cessation of drug treatment. In contrast, since NK1^−/− mice do not exhibit alterations in long-term survival of new neurones, it is suggested that the survival of new neurones may be more crucial in the control of hippocampus-dependent memory tasks. Nevertheless, a number of practical differences between my approach and that of Shors et al. (2001) could have affected the dependence of the tasks on hippocampal neurogenesis, including our longer interval between CS and US (2 min vs. 500 ms) and fewer training trials (4 vs. 800).

7.5.3.2. Antidepressant administration

There have been a number of studies examining the effects of antidepressant treatment on performance in hippocampus-dependent learning and memory tasks. In young rats,
chronic treatment with the tricyclic antidepressant amitriptyline, which inhibits 5-HT and noradrenaline reuptake, enhances spatial learning in the water maze after two months of treatment, possibly via increases in hippocampal mineralocorticoid receptor gene expression (Yau et al. 1995). In aged rats, eight months of such treatment was necessary to bring about a mild improvement in the age-induced impairment on the task, by preventing the development of raised glucocorticoid levels and reducing anxiety (Yau et al. 2002). Conversely, the acute administration of the monoamine oxidase inhibitor phenelzine has been shown to increase retention in an inhibitory avoidance task, but increase escape latencies and path lengths in the Morris water maze, although the potential effect of this drug on the motivational aspects of the task were not addressed (Parent et al. 1999).

7.5.3.3. Brain-derived neurotrophic factor

The increased expression of BDNF in the hippocampus observed after antidepressant treatment suggests a further link between antidepressants and learning and memory. BDNF levels in the CA1 region of the hippocampus are increased after a variety of hippocampus-dependent learning tasks, including contextual fear conditioning (Hall et al. 2000) and the Morris water maze (Kesslak et al. 1998). Furthermore, the reduction of BDNF expression using antisense oligonucleotides has been shown to impair spatial learning in a radial arm maze task (Mizuno et al. 2000), and in the Morris water maze using antibodies to BDNF (Mu et al. 1999). None of these studies have addressed the possibility that increased neurogenesis within the dentate gyrus may be involved in their observations, but they may provide support for a general role of antidepressants in bringing about improvements in hippocampus-dependent learning. However, heterozygous BDNF knockout mice do not have a deficit in the water maze (Montkowski & Holsboer 1997) while overexpression of BDNF resulted only in a deficit in passive avoidance and this was thought to reflect increased levels of BDNF in striatal rather than hippocampal regions of the brain (Croll et al. 1999). Finally, continuous intracerebroventricular infusion of BDNF failed to improve spatial memory in aged rats (Fischer et al. 1994). The link between antidepressant treatment, BDNF expression and learning and memory is therefore not simple: the present observations add weight to the notion that, despite dramatic increases in hippocampal BDNF expression, the effect on learning and memory is minor.
7.6. Conclusions

The data presented in this chapter indicate that NK1⁻/⁻ mice do not exhibit gross changes in learning and memory behaviours in a variety of tasks. They argue against a necessary role for endogenous substance P in learning and memory, although the results of other studies suggest that it may be involved in the mediation of emotional learning and memory behaviours. This finding also strengthens the conclusion that the lack of morphine CPP in NK1⁻/⁻ mice is due to a failure of reward detection rather than associative learning ability. Finally, they provide more information on the role of neurogenesis in learning and memory behaviours, suggesting that the proliferation of new neurones in the dentate gyrus is less important than their long-term survival.
CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS
8. GENERAL DISCUSSION AND CONCLUSIONS

8.1. Introduction

The results presented in this thesis have been discussed extensively within each chapter. The purpose of this chapter is to summarise the findings of the work presented and to provide a broader overview of their significance. A section indicating possible future directions for research within this area is also included.

8.2. Summary of findings

Much of the work presented in this thesis was based upon the findings of Murtra et al. (2000b), who demonstrated that mice lacking the neurokinin-1 (NK1) receptor (NK1\(^-\)) are insensitive to the rewarding properties of morphine. The primary aim of this work was to confirm and extend these findings, and to identify regions of the brain in which the NK1 receptor is crucially involved in the mediation of reward-related behaviours, as well as to examine the effects of genetic disruption of the receptor on learning and memory behaviour.

In Chapter 3, the distribution of the NK1 receptor was examined in the mouse brain using immunohistochemistry (IHC). Although there are no published systematic studies of the distribution of the receptor in the mouse brain, a similar distribution of the receptor was found to that observed in the rat (Nakaya et al. 1994). The highest levels of immunoreactivity were observed in the striatum, the habenular nucleus, the lateral geniculate nucleus, the intergeniculate leaflet, the parabrachial nucleus and the locus coeruleus. Although, in contrast to the rat, immunoreactivity was not observed in the hippocampus of the mouse, a similar pattern of distribution was found in many areas of the brain known to be of importance in mediating reward-related behaviours, such as the nucleus accumbens (NAcc), the ventral tegmental area (VTA) and the subnuclei of the amygdala, suggesting that they may be of importance in the reward-related effects of the NK1 receptor.

Murtra et al.’s (2000b) findings were confirmed in NK1\(^-\) mice of a different genetic background, demonstrating not only that the original findings were reproducible in the background strain available for the remainder of the experimental work presented here, but
also that the observed phenotype was likely to be due to absence of the receptor rather than the effects of loci linked to the targeted allele (Gerlai 1996). However, the use of a range of doses of morphine in these experiments led to the conclusion that NK1\(^{-/-}\) mice show a reduced sensitivity to morphine in reward-related behavioural tasks, rather than the insensitivity reported by Murtra et al. (2000b). This observation was extended by analysing locomotor sensitisation in response to repeated morphine or cocaine administration, a behavioural phenomenon which has been linked to the development of drug-seeking behaviour, craving and relapse. The observed lack of locomotor sensitisation to morphine in NK1\(^{-/-}\) mice, along with their reported lack of self-administration of the drug (Ripley et al. 2002), suggests that the receptor is not only involved in the acute rewarding properties of morphine, but also in the long-term adaptations brought about by repeated morphine exposure. These effects were not confounded by alterations in the metabolism of morphine in NK1\(^{-/-}\) mice.

These behavioural observations suggest that NK1 receptors play an important role in addiction to morphine and possibly other opiates. Whilst the treatment of opiate addicts is controversial, it is possible that pharmacological blockade of the NK1 receptor may prevent the development of dependence, or possibly reduce the craving and relapse associated with an addicted state. Reducing dependence to opiates may also represent a useful clinical application of these findings, particularly in the treatment of patients with severe or chronic pain.

Two approaches were used to attempt to identify the locus or loci of the NK1 receptor's effects in morphine reward. Firstly, the expression of the immediate early gene c-fos was examined in the brains of NK1\(^{-/-}\) mice following morphine administration and the morphine conditioned place preference (CPP) paradigm. Although acute or chronic injections of morphine brought about upregulation in c-Fos protein expression in a few areas of the brain, this was not altered in mice lacking NK1 receptors. However, disruptions in the expression pattern were observed in NK1\(^{-/-}\) mice after the CPP procedure in the basolateral nucleus of the amygdala, the motor and somatosensory cortices and the dentate gyrus, suggesting that these areas form part of the neural system whose activity is disrupted in NK1\(^{-/-}\) mice during this behavioural task. In a second approach, NK1 receptor-expressing neurones were ablated from either the NAcc or the
amygdala of wild type mice, and their morphine-related behaviours observed. Whilst accumbal ablation of these neurones had minimal effects on behaviour, destruction of NK1 receptor-expressing neurones in the amygdala brought about reductions in reward to morphine as assessed in the CPP paradigm, and the locomotor stimulant effect of the drug, as well as increasing anxiety levels. These results therefore strongly imply that the amygdala, notably the basolateral nucleus, may be an important site for the NK1 receptor's effects in mediating morphine reward. These findings would benefit from systematic analysis of further areas of the brain, using both c-Fos and cell ablation techniques, but are indicative of an important role of this brain region in the receptor's effects, which is in agreement with this nucleus' known role in the conditioned aspects of reward.

Finally, the learning and memory behaviour of NK1$^{-/-}$ mice was assessed in a range of hippocampus-dependent and -independent paradigms. No major alterations in learning and memory behaviour were observed, thereby confirming that the lack of CPP to morphine observed in NK1$^{-/-}$ mice was not confounded by reductions in learning ability. These data also demonstrate that there is no simple correlation between the enhanced proliferation of new neurones in the dentate gyrus or raised hippocampal levels of brain-derived neurotrophic factor (BDNF) and enhanced performance in hippocampus-dependent learning and memory paradigms. Correlations between neurogenesis and memory observed in other studies may therefore be more dependent upon the long-term survival of these new neurones. Furthermore, these findings argue against a crucial role for the NK1 receptor in mnemonic processes, although previous findings suggest that substance P may play a modulatory role in certain forms of learning.

8.3. **Future directions**

The behavioural studies presented in this thesis have provided information about the role of the NK1 receptor, and, by inference, substance P in reward and learning and memory processes. As detailed within each chapter, many of the studies presented here would benefit from further experimentation, typically in the use of a range of drug doses, or the examination of further brain regions. However, more generally, these findings could usefully be extended by examining the responses of NK1$^{-/-}$ mice to other drugs of abuse, particularly amphetamine, for which a reduction in CPP has already been observed (Murtra et al. 2000a), as well as heroin, ethanol, nicotine or cannabis. A fuller understanding of the
role of the NK1 receptor may also be achieved by the analysis of a wider range of drug-associated behavioural paradigms in NK1$^{-/-}$ mice and in mice with region-specific ablation of NK1 receptor-expressing neurones, including the analysis of tolerance to morphine's analgesic effects, cross-sensitisation between different drugs and conditioned reward, locomotion, tolerance, sensitisation and taste aversion.

Understanding of the mechanisms underlying the NK1 receptor's participation in the motivational aspects of opiates would be greatly enhanced by the use of DNA microarray technology. Since they demonstrate a huge reduction in sensitivity to the reward-related effects of morphine, NK1$^{-/-}$ mice represent a convenient system in which the rewarding effects of morphine can be analysed at a molecular level. A genome-wide comparison of gene expression in the brains of NK1$^{-/-}$ and wild type mice following morphine administration or reward-related behavioural tasks may indicate some of the molecular processes that are related to the rewarding or addictive properties of the drug. As such, this analysis may provide information about the NK1 receptor-dependent aspects of the plasticity underlying the development of addiction, and suggest molecules or systems that could act as targets for future research in this field. Based on the findings of the work presented here, the amygdala may be one area in which NK1 receptor-dependent alterations in gene expression would be found, although, as demonstrated in the c-Fos experiments, such changes occur in numerous regions of the brain, which, ideally, would be analysed individually.

Further understanding of the processes underlying the behavioural differences between genotypes could also be achieved in vivo. Intracerebral microdialysis following the administration of morphine, or during behavioural tasks related to reward and addiction may indicate differences in the neurochemical processes between genotypes, such as in the behaviour of the monoamine systems. Alternatively, electrophysiological examination may indicate brain regions or cell types exhibiting different neuronal behaviour, which may be related to the behavioural alterations of NK1$^{-/-}$ mice. Such studies could target any of a large number of brain regions, potentially revealing further information about the neural processes that are responsible for the NK1 receptor's effects following drug exposure.

The relationship between the NK1 receptor and the motivational aspects of opiate administration remains an area with many possible avenues of research and clinical
applications. While our understanding of the involvement of the receptor and substance P in emotional behaviours is increasing, much remains to be discovered and these aspects of the tachykinin system remain a fruitful area of research.
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acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide

References


References


APPENDIX

Protocol 2.3.1.1. Genotyping of mice.

All equipment and solutions are autoclaved at 121 °C and 1.03 kPa for 15 min before use.

- Remove 5 mm of the tail tip using a clean razor blade and place in a 1.5 ml microfuge tube (Sarstedt) on ice. Punch holes in mouse’s ears for identification.
- Add 500 µl tail lysis buffer\(^1\) and 12.5 µl Proteinase K (20 mg.ml\(^{-1}\); Sigma) to each sample and vortex briefly. Incubate overnight at 55 °C, briefly vortexing two to three more times. If necessary, store at -20 °C.
- Defrost samples if necessary. Vortex briefly and centrifuge for 3 min at 13 000 rpm. Transfer the supernatant to clean 1.5 ml microfuge tubes and add 200 µl Protein Precipitation Solution (Puregene, Minneapolis, USA). Vortex for 20 s and centrifuge for 3 min at 13 000 rpm.
- Mix the supernatant with 600 µl isopropyl alcohol (Sigma) in clean 1.5 ml microfuge tubes, and centrifuge for 3 min at 13 000 rpm.
- Remove the supernatant, and wash the pellet in 300 µl 70 % ethanol. Centrifuge for 3 min at 13 000 rpm.
- Remove the supernatant and leave the tubes open to allow the ethanol to evaporate.
- Add 100 µl TE\(^2\) to each. Samples can be stored at 4 °C.

---

\(^1\) Tail lysis buffer:
- 50 mM Tris pH 8 (Sigma)
- 100 mM EDTA pH 8 (Sigma)
- 10 mM NaCl (BDH)
- 1% SDS (Sigma)

\(^2\) TE:
- 10 mM Tris pH 8 (Sigma)
- 1 mM EDTA pH 8 (Sigma)
• Make up PCR reaction mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra pure water</td>
<td>32.50 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase 10 × reaction buffer</td>
<td>5.00 μl</td>
</tr>
<tr>
<td>(Promega, Southampton, UK)</td>
<td></td>
</tr>
<tr>
<td>25 mM MgCl₂ (Promega)</td>
<td>3.00 μl</td>
</tr>
<tr>
<td>10 μM dATP (Promega)</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>10 μM dCTP (Promega)</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>10 μM dGTP (Promega)</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>10 μM dTTP (Promega)</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>NeoF¹ (0.5 μg.μl⁻¹, Sigma Genosys, Cambridge, UK)</td>
<td>1.00 μl</td>
</tr>
<tr>
<td>NK1-F² (0.5 μg.μl⁻¹; Sigma Genosys)</td>
<td>1.00 μl</td>
</tr>
<tr>
<td>NK1-R³ (0.5 μg.μl⁻¹; Sigma Genosys)</td>
<td>1.00 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 u.μl⁻¹; Promega)</td>
<td>0.50 μl</td>
</tr>
<tr>
<td>DNA sample</td>
<td>5.00 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50.00 μl</strong></td>
</tr>
</tbody>
</table>

• Place the tubes in a PCR temperature cycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) and run the following programme:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>60 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>72 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>94 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>60 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>72 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

• Add 5 μl loading buffer⁴ to each sample and mix thoroughly.

• Run 11 μl of each sample on a 2% agarose gel in TBE buffer (National Diagnostics, Hull, UK) containing 10 μg.ml⁻¹ ethidium bromide (Sigma) at 120 mV for approximately 1 h.

• Visualise and photograph under ultraviolet transillumination.

---

¹ NeoF: 5'-GCAGCGATCGCTTCTATC-3'
² NK1-F: 5'-CTGTGGGACTCTAATCTCTCC-3'
³ NK1-R: 5'-ACAGCTGTCAATGGAGTAGATAC-3'
⁴ Loading buffer:
  0.25 % bromophenol blue (Sigma)
  0.25 % xylene cyanol FF (Sigma)
  30 % glycerol (BDH)
Appendix

Protocol 2.4.1. Immunohistochemistry with chromogenic detection

All reactions are carried out with gentle agitation of sections on a tissue rocker at room temperature, except where indicated.

- Rinse sections in 0.1 M PB.
- Quench endogenous peroxidase activity by incubating sections in 0.6 % H$_2$O$_2$ in 0.1 M PB for 30 min.
- Block non-specific binding by incubating sections in Normal Goat Serum (NGS) solution for 2 h.
- Incubate sections in primary antibody in NGS solution overnight or for 2 - 3 d at 4 °C.
- Wash sections in 0.1 M PB three times for 10 min each.
- Incubate sections in biotinylated anti-rabbit IgG raised in goat (1:500 in NGS solution; Vector Laboratories) for 1 h.
- After 30 min, make up avidin-peroxidase solution (0.5 % Reagent A + 0.5 % Reagent B in 0.1 M PB; Vectastain Elite ABC Kit [Standard]; Vector Laboratories) and stir for 1 h before use.
- Wash sections three times for 10 min in 0.1 M PB.
- Incubate sections in avidin-peroxidase solution for 2 h.
- Wash sections twice in 0.1 M PB for 10 min each, then twice in 0.1 M Tris buffer for 10 min each.

---

1 The serum used must be that of the animal in which the secondary antibody was raised.

2 The secondary antibody must be raised against the animal in which the primary antibody was raised.
Appendix

- Make up DAB solution:
  
  Distilled water  
  70.7 ml  
  1 M HCl (BDH)  
  1.4 ml  
  DAB stock solution\(^1\)  
  2.4 ml  
  0.6 M Tris buffer  
  23.6 ml  
  1 M NaOH (BDH)  
  1.9 ml  
  Ammonium nickel sulphate (optional; Sigma)  
  189 mg  

  Total  
  100.0 ml

- Incubate sections in DAB solution for 3 min
- Make up three \(\text{H}_2\text{O}_2\) solutions: 0.015 %, 0.030 % and 0.075 %.
- Add \(\text{H}_2\text{O}_2\) to sections in DAB solution gradually (100 \(\mu\)l at a time), starting with the lowest concentration and working up to the highest. Continue adding 0.075 % solution until sections are dark grey in colour (brown when Ni is omitted), and the antigen can be seen under the microscope.
- Stop the reaction by washing the sections twice in 0.1 M PB for 5 min each.
- Rinse sections briefly in 0.01 M PB.
- Mount sections onto gelatine-coated slides (see page 169) and leave to air dry.
- Dehydrate sections in ethanol solutions of increasing strength (distilled water, 70 % ethanol \(\times 2\), 95 % ethanol \(\times 2\), 100 % ethanol \(\times 2\); 2 min in each).
- Clear in Histoclear (2 min \(\times 2\); National Diagnostics).
- Coverslip slides using DPX mounting medium (BDH).

\(^1\) DAB stock solution:
  
  0.2 M HCl (BDH)
  20 mg.ml\(^1\) DAB (Sigma)
Protocol 2.4.2. Immunohistochemistry with TSA and fluorescent detection using FITC

All reactions are carried out with gentle agitation of sections on a tissue rocker at room temperature, except where indicated.

- Rinse sections in 0.1 M PB.
- Quench endogenous peroxidase activity by transferring sections to 0.6 % \( H_2O_2 \) solution in 0.1 M PB for 30 min.
- Block non-specific binding by incubating sections in NGS solution for 2 h.
- Incubate sections in primary antibody in NGS solution overnight or for 2-3 d at 4 °C.
- Wash sections in 0.1 M PB three times for 10 min each.
- Incubate sections in biotinylated anti-rabbit IgG raised in goat\(^2\) (1:400 in NGS solution; Vector Laboratories) for 90 min.
- Make up avidin-peroxidase solution (0.4 % Reagent A + 0.4 % Reagent B in 0.1 M PB; Vectastain Elite ABC Kit [Standard]; Vector) and stir for 30 min before use.
- Wash sections three times for 10 min in 0.1 M PB.
- Incubate sections in avidin-peroxidase solution for 30 min.
- Wash sections three times for 10 min in 0.1 M PB.
- Incubate sections in biotinylated tyramide (1:75 in kit diluent; TSA\(^TM\) Biotin System; NEN Life Science Products, Boston, USA) for 7 min.
- Wash sections three times for 10 min in 0.1 M PB.
- Incubate sections in the dark in Fluorescein Avidin D (1:600 in 0.1 M PB; Vector Laboratories) for 2 h.
- Wash sections in the dark three times for 10 min in 0.1 M PB.
- Rinse sections briefly in the dark in 0.01 M PB.
- Mount sections onto Superfrost Plus Microscope Slides (BDH) and leave to dry in the dark.

\(^1\) The serum used must be that of the animal in which the secondary antibody was raised.
\(^2\) The secondary antibody must be raised against the animal in which the primary antibody was raised.
• Coverslip with Citifluor (Citifluor, London, UK) or Prolong® Antifade Kit (Molecular Probes, Leiden, The Netherlands).
Protocol 2.4.3. Immunohistochemistry with fluorescent detection

All reactions are carried out with gentle agitation of sections on a tissue rocker at room temperature, except where indicated.

- Rinse sections in 0.1 M PB.
- Block non-specific binding by incubating sections in Normal Serum solution\(^1\) for 1 h.
- Incubate sections in primary antibody in Normal Serum solution overnight or for 2-3 d at 4 °C.
- Wash sections in 0.1 M PB three times for 10 min each.

For directly-conjugated secondary antibodies:

- Incubate sections in the dark in secondary antibody\(^2\) (1:200 in Normal Serum solution) for 3 h.
- Wash sections in the dark in 0.1 M PB for at least 1 h, changing the solution at least twice.
- Rinse sections in the dark in 0.01 M PB.
- Mount sections onto Superfrost Plus Microscope Slides (BDH) and leave to dry in the dark.
- Coverslip with Citifluor (Citifluor) or Prolong® Antifade Kit (Molecular Probes).

Or, for FITC fluorescence (avidin-biotin method):

- Incubate sections in biotinylated anti-rabbit IgG raised in goat\(^2\) (1:200 in NGS solution; Vector Laboratories) for 2 h.
- Wash sections three times for 10 min in 0.1 M PB.

---

\(^1\) The serum used must be that of the animal in which the secondary antibody was raised. NGS solution is given in section 2.2.1. Alternative sera used are:
- Normal Donkey Serum (Jackson Immunoresearch Laboratories, West Grove, USA);
- Normal Rabbit Serum (Vector Laboratories).

\(^2\) The secondary antibody must be raised against the animal in which the primary antibody was raised. Secondary antibodies used are:
- Fluorescein anti-goat IgG (H + L) raised in rabbit (Vector Laboratories);
- Fluorescein anti-rabbit IgG (H + L) raised in goat (Vector Laboratories);
- Texas Red-X goat anti-mouse IgG (H + L; Molecular Probes).
• Incubate sections in the dark in Fluorescein Avidin D (1:200 in 0.1 M PB; Vector Laboratories) for 1 h.
• Wash sections in the dark in 0.1 M PB for 1 h, changing the solution at least twice.
• Rinse sections in the dark briefly in 0.01 M PB.
• Mount sections onto Superfrost Plus Microscope Slides (BDH) and leave to dry in the dark.
• Coverslip with Citifluor (Citifluor) or Prolong® Antifade Kit (Molecular Probes).
Lack of self-administration and behavioural sensitisation to morphine, but not cocaine, in mice lacking NKI receptors

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Abstract

Mice lacking the NK1 receptor, the preferred receptor for substance P, demonstrate normal analgesic responses to morphine on the hot plate assay, but have been predicted, on the basis of conditioned place preference studies, to be insensitive to the rewarding properties of opiates. In this study, self-administration and the development and maintenance of locomotor sensitisation of both morphine and cocaine were investigated in mice that lacked the NK1 gene (NK1 knockout mice, NK1-/-). Both wildtype and NK1-/- mice learned an operant lever-press response to obtain food. When intravenous infusions of morphine (0.2 mg/kg/infusion) were substituted for the food reward, the wildtype mice initially reduced rates of lever pressing, but then increased them on the rewarded lever to obtain approx. 10 infusions per 90 min session; in contrast, NK1-/- mice continued to operate both the rewarded, and non-rewarded levers at low rates. Additionally, NK1-/- mice failed, following repeated administration, to sensitise to the locomotor stimulant effects of morphine (15 mg/kg, i.p.). These deficits were specific to opiates, since NK1-/- mice responding for food or cocaine self-administration (0.65 mg/kg/infusion) did not differ from wildtypes, and they showed normal behavioural sensitisation to repeated cocaine administration (10 mg/kg, i.p.). These results demonstrate that NK1 receptors are critical for the reinforcing properties of morphine, and for adaptive responses elicited by repeated opiate administration. It is postulated that substance P and the NK1 receptor may be necessary for the development of opiate, but not cocaine addiction.

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Keywords: Opiate; Drug addiction; Drug abuse; Substance P

1. Introduction

 Substance P and its preferred receptor, the NK1 receptor, are highly expressed in many areas of the brain involved in affective behaviours, including the hypothalamus, amygdala and nucleus accumbens (Nakaya et al., 1994), and antagonists of the NK1 receptor have been shown to possess anxiolytic and antidepressant properties in both preclinical and clinical studies (Ballard et al., 2001; Papp et al., 2000; Rupniak et al., 2000; Rupniak and Kramer, 1999). NK1 receptor antagonists may thus offer a novel pharmacological route for the treatment of emotional disorders. Since NK1 antagonists show limited activity in rodent species, the availability of mice lacking functional NK1 receptors, generated by homologous recombination (De Felipe et al., 1998), has proven important in understanding the role of these receptors in rodent models. Previous behavioural work has shown that these mice have altered amplification and intensity coding of nociceptive inputs, impaired stress-induced analgesia and a reduction in aggressive responses to territorial challenge (De Felipe et al., 1998). Furthermore, in the maternal separation model of anxiety, mouse pups lacking NK1 receptors emitted fewer ultrasonic vocalisations than their wild-type littermates (Rupniak et al., 2000), suggesting a role of the NK1 receptor in the control of anxiety-related behaviours and the adaptive responses to stress.

Recently Murtra et al. (2000b) provided evidence that
NK1 knockout mice may be insensitive to opiates in models of drug abuse. Firstly, the null mutant mice failed to develop a preference for an environment paired with morphine treatment, using the conditioned place preference (CPP) paradigm, suggesting an impairment of the rewarding effects of the drug. The null mutant mice also failed to show an acute locomotor stimulant effect of the drug. These effects appeared to be specific to opiates, since CPP to food (a natural reward) and to the psychostimulant cocaine, were unaffected by the genetic manipulation. In addition to a loss of the rewarding response to morphine, the mice did not show many of the physical withdrawal signs associated with cessation of morphine treatment, and did not demonstrate a conditioned place aversion to naloxone, suggesting impairment of both physical and motivational aspects of opiate withdrawal. Despite the loss of the rewarding effects of morphine, De Felipe et al. (1998) found that the angesic properties of morphine were not impaired in NK1 knockout mice in the hot-plate test, indicating that the substance P/NK1 system is not critically involved in opiate analgesia. These results therefore suggest that the NK1 receptor plays a critical and specific role in the motivational, but not angesic, properties of opiates, whereas it is not critical for the motivational properties of cocaine.

In the present experiments, we have investigated further the consequences of deleting NK1 receptors in models of drug abuse. A more direct test of the reinforcing properties of drugs than the CPP paradigm, is whether animals will learn an operant response to self-administer the drug. We therefore tested the ability of NK1 knockout, and wildtype mice to learn to operate a lever in order to obtain intravenous infusions of either morphine or cocaine. The repeated administration of drugs of abuse such as opiates and psychostimulants causes a number of adaptive responses within the brain, which may eventually contribute to the development of addiction (Berke and Hyman, 2000; Nestler, 2001). Locomotor sensitisation, consisting of a progressive and persistent enhancement of a drug's psychomotor properties with repeated, intermittent exposure, is a typical behavioural response to repeated treatment with abused drugs, including opiates and psychostimulants. Such behavioural sensitisation depends on facilitation of glutamatergic and dopaminergic transmission within the basal forebrain, and has been suggested to be of importance in the motivational aspects of drug addiction, having been related to drug-seeking behaviour, as well as craving and relapse to drug abuse (De Vries et al., 1998; Kalivas, 1995; Lu et al., 2002; Trujillo, 2000; Vanderschuren et al., 1999; Vanderschuren and Kalivas, 2000; Wolf, 1998). Since the involvement of NK1 receptors in these processes has not been addressed directly, we have also assessed the effects of chronic morphine and chronic cocaine on locomotor activity in NK1 knockout mice.

2. Methods

2.1. Animals

Adult (>7 weeks) male NK1 knockout (NK1<−/−) and wildtype (WT) mice were used in these experiments. For the morphine self-administration experiment and the locomotor sensitisation experiments, the mice were originally derived from C57BL/6 blastocysts implanted with genetically manipulated 129sv stem cells (De Felipe et al., 1998), which were crossed onto an MF1 background. NK1<−/− mice with this genetic background show similar behavioural responses to morphine to those used by Murtra et al. (2000b; Gadd, unpublished observations). For the cocaine self-administration experiment, mice were on a C57BL/6×129sv background.

Mice were housed 2–5 per cage on a 12 h light/dark cycle (lights on at 8 a.m.), at a temperature of 21±1 °C and 50% humidity. Food and water were available ad libitum, except as specified below. Mice for these experiments were bred from homozygous WT and knockout lines. The genotype of all mice used was verified by PCR of tail-tip DNA. All animal procedures were carried out under the UK Animals (Scientific Procedures) Act 1986.

2.2. Self-administration experiment: morphine or cocaine self-administration in NK1<−/− mice

2.2.1. Animals

Body weight at the start of the experiment was approx. 35 g (30 g during the operant experiment, due to food restriction). For the morphine experiment, a total of seven WT and six NK1<−/− mice were used at the start of the experiment. One NK1<−/− animal died in surgery, whilst two WT and one NK1<−/− mouse did not complete the 11 days of self-administration due to either blocked catheters or loss of the head mount. Data are shown from a total of five WT and four NK1<−/− mice. For the cocaine experiment, a total of eight WT and eight NK1<−/− mice were used at the start of the experiment. Two NK1<−/− animals failed to reach criterion in the overnight food shaping session. Two WT animals died during or shortly after surgery, whilst one WT and one NK1<−/− mouse did not complete the 11 days of self-administration due to either a blocked catheter or loss of the head mount. Therefore, data shown are from a total of five WT and five NK1<−/− mice.

2.2.2. Operant food shaping

One week before the start of operant training, the mice were food restricted to reduce body weights to 85% of
the free-feeding weight. Mice were trained to press a lever for a milk reinforcer in one 16 h session which included the dark phase, in mouse operant chambers (model ENV-307; MedAssociates, Georgia, VT, USA) constructed of clear perspex (18x18x15 cm), and contained in sound and light attenuating cubicles. Fans mounted in the sound attenuating cubicles provided masking noise. Each operant chamber possessed a single house light located on the wall opposite the levers. The front wall was fitted with a liquid dipper (model ENV-202A), located between two ultrasensitive mouse levers (model ENV-310A). Activation of one of the levers (the active lever) raised the liquid dipper for 5 s, giving access to a 0.01 ml cup containing a 30% solution of condensed milk (Fazelle’s, York, UK). Activation of the other lever was recorded but had no programmed consequences. At the start of the first session, free reinforcers were delivered every 2 min, until the animal began lever pressing. The response requirement to obtain reinforcers was then progressively increased; a single activation of the lever was required for the first 20 reinforcers (FR1), after which two lever presses were required for the next 10 (FR2), and four lever presses for at least the next 30 reinforcers (FR4), with a least 75% of lever presses on the active lever. To reduce lever bias, the active and inactive levers were switched, and the training schedule set back to FR1. Such reversals (cycles) were repeated throughout the training sessions. Animals which reached criterion (a minimum of 100 earned reinforcers, and two completed cycles), progressed to the main part of the study, and were assigned either to morphine or cocaine intravenous drug self-administration procedure.

2.2.3. Surgical procedure

Animals were given access to food ad libitum for a minimum of 48 h before surgery. Animals were then anaesthetised with a ketamine/xylazine mixture (induction dose: 260 mg/kg ketamine, 2 mg/kg xylazine; Sigma, Poole, UK). Additional anaesthetic was administered as necessary to maintain anaesthesia. A silastic catheter (o.d.=0.636 mm; i.d.=0.305 mm; length = 35 mm; Altec Products, Hampshire, UK) was inserted into the right external jugular vein, under a dissecting microscope, advanced toward the atrium, and secured by sutures and glue, following the procedure described by Rocha et al. (1997). The distal end of the catheter was attached to a 15 mm length of 23 g hypodermic needle tubing using silicone sealant, and was guided subcutaneously behind the ear, and secured to an exposed region of the skull, using acrylic cement (Geristore Dual Cure, Grafton International, Staffordshire, UK). The open end of the hypodermic tubing was sealed with a silicone cap. The dead space of the catheter assembly was approx. 5 μl. Mice were allowed to recover from surgery for 48 h before self-administration experiments began. The animals were again food restricted. Catheters were flushed daily with 0.02 ml heparin in physiological saline (30 U/ml), following self-administration sessions.

2.2.4. Self-administration procedure

Animals were placed in the operant chambers (with the liquid dipper disabled) for 90 min daily sessions during which activation of the active lever (randomly assigned) resulted in a drug infusion (0.65 ml/min) on an FR2 schedule. Each session began with a priming infusion of drug. Each infusion lasted 1.8 s, and was followed by a 10 s time out period, signalled by extinguishing the houselight, during which further activation of the active lever was recorded, but had no programmed consequences. The time out period was incorporated to prevent rapid successive infusions leading to overdose. Responding on the inactive lever had no programmed consequences throughout the session. Each drug infusion delivered approx. 0.2 mg/kg morphine or 0.65 mg/kg cocaine. For the morphine self-administration study, animals were run for a total of 11 daily sessions, whilst for the cocaine self-administration study only seven sessions were run (Fig. 1).

2.2.5. Drugs

Morphine sulphate and cocaine hydrochloride were purchased from MacFarlan Smith (Edinburgh, UK). They were dissolved in sterile 0.9% saline and were administered i.v. through the indwelling catheter as described above. Morphine concentration was 0.3 mg/ml and cocaine concentration was 1 mg/ml (all concentrations were corrected for base).

2.3. Locomotor sensitisation for morphine or cocaine in NK1<sup>-/-</sup> mice

2.3.1. Animals

Thirty NK1<sup>-/-</sup> (33.7±3.68 g at the start of the experiment) and 30 WT mice (36.0±3.96 g) were used. The groups of mice were divided into three groups of

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**Fig. 1** Schematic diagram of experimental procedures.
ten per genotype. Each group was tested for behavioural sensitisation to morphine, cocaine or saline.

2.3.2. Locomotor sensitisation protocol

The sensitisation procedure was modified from that of Martin et al. (2000). Sensitisation to the locomotor responses elicited by chronic morphine or cocaine was assessed in black Perspex boxes (internal dimensions: 9 cm [w]x20 cm [l]x11 cm [h]). Mice were placed individually in the boxes and recorded for 15 min. A video camera attached to a microcomputer running EthoVision 2.2 (Noldus Information Technology, Wageningen, The Netherlands) was used to monitor the position of the mice at a frequency of 5 Hz and locomotor activity was assessed by calculation of each mouse’s mean speed in mm/s over the 15 min recording session. All the activity tests were carried out between 9.30 a.m. and 11.00 a.m.

The animals were habituated to the test environment on days 1, 2 and 3, in order to obtain a stable baseline. On day 4, chronic treatment with morphine (15 mg/kg), cocaine (10 mg/kg) or saline was begun for a period of 15 days. The mice received two injections per day: the morning injections were performed between 9.20 a.m. and 10.50 a.m., and the evening injections between 6.30 p.m. and 7.30 p.m.

The acute locomotor effects of morphine or cocaine were measured on day 4, 10 min after the first injection of morphine, cocaine or saline. Thereafter, during chronic drug treatment, locomotor activity was measured every 3 days (on days 7, 10, 13, 16 and 19), 10 min after the morning injection.

At the end of the chronic drug administration on day 19, the animals did not receive any treatment from days 20 to 25. On day 26, they received a challenge dose of morphine (15 mg/kg), cocaine (10 mg/kg) or saline, and locomotor activity was measured again (Fig. 1).

Due to power failure, no recordings of locomotor activity in any morphine-treated WT mice were made on day 16.

2.3.3. Drugs

Morphine sulphate and cocaine hydrochloride were purchased from Sigma (Poole, UK). They were dissolved in sterile 0.9% saline and administered intraperitoneally (i.p.) in a volume of 6.67 ml/kg. Doses were corrected for the base.

2.4. Statistical analysis

2.4.1. Overnight operant training

The total number of reinforcers and the total number of responses on the training session were analysed using 1-way analysis of variance (ANOVA) with genotype as the between subject factor.

2.4.2. Drug self-administration

The number of reinforcers obtained each day was analysed using a 2-way repeated measures ANOVA with the genotype (WT vs. NK1−/−) as the between subject factor and the day as the within subject factor. Responses on the active and inactive levers were analysed using a 3-way repeated measures ANOVA with the genotype as the between subject factor and the day and the lever (active vs. inactive) as the within subject factors. A significant interaction was followed by post hoc analysis (2- and 1-way ANOVAs for each session). For the morphine self-administration study, the data were analysed over all 11 daily sessions and, following a significant main effect of day, also over sessions 1 to 6 and 7 to 11 separately. These latter periods represent acquisition and maintenance. Acquisition refers to the first six sessions, during which time the animals are learning the drug self-administration procedure; whilst maintenance refers to the period of stable performance comprising sessions 7 to 11. As the animals acquired the self-administration task more rapidly with cocaine, only seven self-administration sessions were performed. Therefore data were analysed over sessions 1 to 3 (acquisition) and sessions 4 to 7 (maintenance).

2.4.3. Locomotor sensitisation

Data were analysed using 3-way ANOVAs with treatment and genotype as between subject factors, and day as a within subject factor. Subsequent 2-way ANOVAs were calculated for each testing day with treatment and genotype as between subject factors, followed by Tukey post hoc comparisons. The data were checked for violation of the assumptions of the ANOVA test procedure (Normality of the error and homogeneity of the variance). Where these assumptions were not upheld, the data were transformed using square root, logarithmic, inverse or Box–Cox transformation as appropriate. p=0.05 was considered statistically significant.

3. Results

3.1. Self-administration experiment: morphine self-administration in NK1−/− mice

3.1.1. Overnight operant training

NK1−/− mice acquired responding for the food reward faster than the WT animals. The NK1−/− mice had a higher response rate, and therefore obtained more reinforcers, than the WT’s on the overnight training session [Fig. 2(a) and (b)]. When the number of lever presses obtained was analysed using a 1-way repeated measures ANOVA, there was a significant main effect of genotype (F₁, r=7.951, p<0.05). A similar pattern of effects was observed when the total number of reinforcers was analysed (F₁, r=12.209, p<0.01).
Fig. 2. Morphine and cocaine self-administration in NK1−/− mice. (a, b) Operant food shaping: In the morphine experiment, the NK1−/− emitted more lever presses, \( p<0.05 \); a) and obtained more reinforcers \( p<0.01 \); b) than their wildtype controls. In the cocaine experiment, NK1−/− and WT mice did not differ in the number of reinforcers obtained \( p>0.1 \) or in the number of lever presses emitted \( p>0.1 \) during the overnight operant session. (c, d) Morphine self-administration (approx 0.2 mg/kg/infusion). Data represent self-administration sessions 7 to 11, where performance was stable. NK1−/− mice \( n=4 \) failed to self-administer morphine at a dose that supported self-administration in WT mice \( n=5 \) (reinforcers: main effect of genotype: \( F_{1,7}=11.000, p<0.05 \)). WT animals had a significantly higher output on the active lever when compared with either their own performance on the inactive lever or when compared with responding by the NK1−/− mice on either lever. (e, f) Cocaine self-administration (approx. 0.65 mg/kg/infusion). Data represent self-administration on sessions 4 to 7, where performance was stable. NK1−/− mice \( n=5 \) self-administered the same number of reinforcers as WT mice \( n=5 \) and both genotypes showed higher rates of responding on the active lever when compared with the inactive lever \( p<0.005 \).

### 3.1.2. Morphine self-administration

Fig. 2(d) shows that the NK1−/− mice self-administered fewer reinforcers than the WT animals. This was significant when analysed both over all the sessions, and when sessions 7 to 11 were analysed separately. Initially the NK1−/− and the WT mice self-administered the same number of morphine reinforcers, an effect that may represent extinction responding for the condensed milk. However, from session 7, the number of morphine reinforcers taken was higher in the WT mice than in the NK1−/− mice.

There were significant main effects of genotype and of days over the 11 sessions (genotype: \( F_{1,7}=11.000, p<0.05 \); days: \( F_{10,70}=2.681, p<0.01 \)) and over sessions 7 to 11 (genotype: \( F_{1,7}=10.510, p<0.05 \); days: \( F_{1,7}=3.784, p<0.05 \)) but no significant genotype by day interaction \( p>0.05 \). When data were analysed over sessions 1 to 6 there were no significant effects \( p>0.05 \) throughout.

The NK1−/− mice also responded on the active lever less than the WT control animals [Fig. 2(c)]. There were no significant differences between the genotypes over the first six sessions, but in the later sessions the WT animals had a significantly higher output on the active lever when compared with either their own performance on the inactive lever or when compared with responding by the NK1−/− mice on either lever.

When the number of responses on each lever was analysed over all 11 sessions, there was a significant main effect of genotype and day (genotype: \( F_{1,7}=6.275, p<0.05 \); days: \( F_{10,70}=4.399, p<0.01 \)), a significant day by lever interaction \( F_{10,70}=2.450, p<0.05 \) and a mar-
original lever by genotype interaction (days: $F_1, 7 = 5.546, p = 0.051$). When these data were further analysed over sessions 7 to 11 there were significant main effects of genotype and lever (genotype: $F_1, 7 = 12.231, p < 0.01$; lever: $F_1, 7 = 7.076, p < 0.05$) and a significant genotype by lever interaction ($F_1, 7 = 5.832, p < 0.05$). Post hoc analysis showed that there was a significant genotype by lever interaction on day 10 and 11 (day 10: $F_1, 7 = 8.702, p < 0.05$; day 11: $F_1, 7 = 7.773, p < 0.05$) and that on these 2 days the WT mice had a significantly higher number of responses on the active lever when compared with the inactive lever (day 10: $F_1, 7 = 19.189, p < 0.05$; day 11: $F_1, 7 = 72.053, p < 0.01$). This effect was not seen in the NK1 receptors mice (day 10: $F_1, 7 = 0.005, p = 0.950$; day 11: $F_1, 7 = 0.095, p = 0.778$).

There was no main effect of day and no significant 2-way or 3-way interactions involving day ($p > 0.05$). Analysis of the number of responses over the first six sessions revealed a main effect of day ($F_3, 24 = 6.176, p < 0.05$), due to the animals showing a high level of lever pressing on the first day, but no other significant effects.

3.2. Self-administration experiment: cocaine self-administration in NK1-/- mice

3.2.1. Overnight operant training

Fig. 2(a) and (b) show the data on food reinforced responding for mice in the cocaine self-administration experiment. There were no significant differences between the two genotypes when the number of reinforcers or the number of lever presses during the 16 h overnight session were analysed (reinforcers: $F_1, 13 = 0.808, p > 0.5$; lever presses: $F_1, 13 = 0.012, p > 0.5$).

3.2.2. Cocaine self-administration

During the maintenance phase of cocaine self-administration, there were no differences between the NK1 receptors and WT mice when the number of reinforcers or the number of responses on the active or inactive lever were analysed [Fig. 2(c) and (d)].

For the number of reinforcers obtained there was no significant main effect of genotype ($F_1, 8 = 1.367, p > 0.1$) or session ($F_3, 24 = 0.257, p > 0.5$) and no genotype by session interaction ($F_3, 24 = 0.258, p > 0.5$). This pattern of effects was also true for the number of responses on the active lever (genotype: $F_1, 8 = 2.153, p > 0.1$; session: $F_3, 24 = 0.282, p > 0.5$; genotype x session: $F_3, 24 = 0.290, p > 0.5$) and on the inactive lever (genotype: $F_1, 8 = 0.047, p > 0.5$; session: $F_3, 24 = 2.408, p > 0.05$; genotype x session: $F_3, 24 = 0.347, p > 0.5$).

3.3. Locomotor sensitisation to morphine

On days 1, 2 and 3, mice were exposed to the locomotor activity boxes for 15 min in order to habituate them to the test environment. The locomotor activity of NK1 receptors and WT mice did not differ on any of the three days ($p > 0.6$). Subsequent chronic administration of morphine (15 mg/kg, i.p.) caused an increase in locomotor activity in WT mice, with NK1 receptors mice moving at similar speeds to those receiving chronic injections of vehicle [Fig. 3(A)]. Three-way ANOVA with genotype (G), drug treatment (T) and day (D) as factors revealed significant main effects of genotype ($F_{1, 26} = 11.2, p = 0.002$) and day ($F_{2, 26} = 8.72, p < 0.001$), and significant G×T ($F_{1, 26} = 10.3, p = 0.003$), G×D ($F_{2, 26} = 4.76, p < 0.001$), T×D ($F_{2, 26} = 9.85, p < 0.001$) and G×T×D ($F_{4, 26} = 2.49, p = 0.031$) interactions.

Subsequent 2-way ANOVAs were used to analyse

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**Fig. 3.** Locomotor sensitisation to morphine or cocaine in NK1 knockout mice. (A) Locomotor responses to morphine. Mean±standard error speeds moved by NK1 receptors and WT mice during habituation to the activity boxes (days 1-3), chronic morphine (15 mg/kg, i.p.) or saline administration (days 4-19; two injections per day), and after a challenge dose of morphine (15 mg/kg, i.p.) or saline on day 26. (B) Locomotor responses to cocaine. Mean±standard error speeds moved by NK1 receptors and WT mice during habituation to the activity boxes (days 1-3), chronic cocaine (10 mg/kg, i.p.) or saline administration (days 4-19; two injections per day), and after a challenge dose of cocaine (10 mg/kg, i.p.) or saline on day 26. *p < 0.05 vs. NK1 receptors; **p < 0.01 vs. NK1 receptors; ***p < 0.001 vs. NK1 receptors; *p < 0.05 vs. saline, **p < 0.01 vs. saline; ***p < 0.001 vs. saline (Tukey post hoc comparisons).
drug and genotype effects on individual days of the experiment. On day 4, acute morphine administration caused a decrease in the locomotor activity of NK1\(^{-/-}\) mice, but had no effect on the locomotion of WT mice. Two-way ANOVA revealed a significant genotype by drug interaction \((F_{1,36}=6.14, p=0.018)\), with post hoc Tukey comparisons indicating that NK1\(^{-/-}\) mice treated with morphine moved significantly more slowly than both WT mice \((p=0.027)\) and saline-treated NK1\(^{-/-}\) mice \((p=0.049; \text{Fig. 3(A)})\).

On day 7, there was a highly significant main effect of genotype \((F_{1,28}=9.79, p=0.004)\), whilst on days 10 \((F_{1,33}=4.60, p=0.039)\) and 13 \((F_{1,33}=16.8, p<0.001)\) there was a significant interaction between genotype and drug, indicating a differential effect of morphine on locomotor activity in the two genotypes on these two days. On day 19, there were significant main effects of treatment \((F_{1,33}=8.75, p=0.006)\) and genotype \((F_{1,33}=5.69, p=0.023)\), but the interaction term did not reach statistical significance. Tukey post hoc comparisons revealed significant differences between the speeds moved by WT and NK1\(^{-/-}\) mice on all 4 days \((p<0.04; \text{Fig. 3(A)})\). In addition, there was a highly significant difference between the speeds of WT mice treated with morphine and saline on day 13 \((p<0.001; \text{Fig. 3(A)})\). There were no significant differences in the locomotor activity induced by saline and morphine in NK1\(^{-/-}\) mice on days 7, 10, 13 or 19.

Taken together, these data indicate that there was a significant locomotor sensitisation to morphine in WT mice, but that this did not occur in NK1\(^{-/-}\) mice. In NK1\(^{-/-}\) mice, acute morphine administration was followed by a slight reduction in locomotor behaviour, although locomotor activity recovered to levels of vehicle-treated mice by the fourth day of chronic morphine administration.

One week after finishing chronic morphine treatment, the mice were given a challenge dose of morphine \((15 \text{ mg/kg, i.p.) or saline before locomotor activity was measured, in order to assess the maintenance of sensitisation to morphine's locomotor effects. Locomotor sensitisation was maintained in morphine-treated WT mice, but morphine- and saline-treated NK1\(^{-/-}\) mice demonstrated similar speeds of locomotion. Two-way ANOVA demonstrated highly significant main effects of treatment \((F_{1,33}=11.5, p=0.002)\) and genotype \((F_{1,33}=21.8, p<0.001)\) on day 26, and a significant interaction between these factors \((F_{1,33}=7.4, p=0.010)\). Post hoc Tukey comparisons revealed that this interaction was due to highly significant differences between the locomotor activity of morphine-treated WT mice and both morphine-treated NK1\(^{-/-}\) mice \((p<0.001)\) and saline-treated WT mice \((p<0.001; \text{Fig. 3(A)})\).

3.4. Locomotor sensitisation to cocaine

On days 1, 2 and 3, mice were exposed to the locomotor activity boxes in order to habituate them to the test environment. The locomotor activity of NK1\(^{-/-}\) and WT mice did not differ on any of the 3 days \((p>0.07)\). Subsequent chronic administration of cocaine \((10 \text{ mg/kg, i.p.) caused an increase in locomotor activity in both WT and NK1\(^{-/-}\) mice after 3 days of drug treatment [Fig. 3(B)]. Three-way ANOVA with genotype \((G)\), drug treatment \((T)\) and day \((D)\) as factors revealed significant main effects of treatment \((F_{1,36}=10.9, p=0.002)\) and day \((F_{9,315}=8.66, p<0.001)\), and significant TxD \((F_{9,315}=18.4, p<0.001)\) and GxTxD interactions \((F_{9,315}=2.44, p=0.011)\).

Subsequent two-way ANOVAs were used to analyse drug and genotype effects on individual days of the experiment. On day 4, acute cocaine administration did not cause any significant changes in locomotor activity in WT or NK1\(^{-/-}\) mice. However, on days 7, 10, 13, 16 and 19, there were significant main effects of treatment \((p<0.02)\), but no significant main effects of genotype \((p>0.1)\) or interactions between treatment and genotype \((p>0.2)\). This suggests that on these recording days, cocaine induced an increase in locomotor activity which was independent of genotype. Chronic cocaine therefore elicits locomotor sensitisation in both NK1\(^{-/-}\) and WT mice.

One week after finishing chronic cocaine treatment, the mice were given a challenge dose of cocaine \((10 \text{ mg/kg, i.p.) or saline before locomotor activity was measured, in order to assess the maintenance of sensitisation to cocaine's locomotor effects. Both NK1\(^{-/-}\) and WT mice maintained their speeds of day 19, with NK1\(^{-/-}\) mice demonstrating a slight increase in locomotor activity. Two-way ANOVA revealed a highly significant main effect of treatment \((F_{1,36}=23.7; p<0.001)\), and a significant interaction between treatment and genotype \((F_{1,33}=4.47, p=0.041)\). Post hoc Tukey comparisons revealed a highly significant difference in locomotor activity between cocaine- and saline-treated NK1\(^{-/-}\) mice \((p<0.001)\), but no significant differences between cocaine-treated NK1\(^{-/-}\) and WT mice \((p=0.187)\), or between cocaine- and saline-treated WT mice \((p=0.226; \text{Fig. 3(B)})\).

4. Discussion

In the present experiments, we have investigated the consequences of deleting the NK1 receptor, the preferred receptor for substance P, on two behaviours thought to model properties of drugs of abuse. The results strongly support a role for NK1 receptors in mediating the rewarding properties of opiates.

In the first set of experiments, NK1 receptor knockout
mice failed to acquire an operant response to obtain intravenous administration of morphine, although wild-type mice readily acquired the response. The impairments in the NK1−/− mice were specific to morphine since the knockouts showed no impairment in responding for food (indeed, they acquired the lever-press response more readily, and obtained more food reinforcers than wildtypes), and did not differ from wildtypes in self-administration of cocaine. Non-specific effects of the mutation on performance of the self-administration response, or effects on reward mechanisms in general can thus be excluded. A possible confound in the present experiments is that the morphine self-administration experiments were carried out in C57BL/6x129sv mice crossed onto an MF1 background, whereas these mice were unavailable for the cocaine self-administration experiment, which was carried out with mice bred on a C57BL/6x129sv background. The difference in background strain may have contributed to differences between the morphine and cocaine self-administration experiments in the effects of genotype on responding for food. In the morphine experiment (MF1 background), the knockout mice showed facilitated responding for food, whereas in the cocaine experiment (C57BLx129sv), no genotype differences were observed. Nevertheless, the wildtype mice of both background strains readily learned the morphine self-administration response (number of morphine reinforcers obtained on day 7 of self-administration procedure: C57BL/6x129sv on MF1 background: 6.0±1.1; C57BL/6x129sv: 17.3±4.1). The findings from this experiment thus parallel those seen in place conditioning experiments, in which NK1 knockout mice, in contrast to wildtypes, failed to acquire a preference for a distinct environment paired with morphine, but showed normal place preference conditioning to cocaine (Murtra et al., 2000b), and suggest strongly that opiate reward requires intact functioning of Substance P-ergic transmission.

Study of the development and maintenance of behavioural sensitisation to morphine and cocaine gave rise to compatible findings. Repeated administration of opiates or psychostimulant drugs tends to cause a gradual enhancement of both the stimulant and rewarding properties of that drug. This response is believed to be due to a facilitation of dopaminergic and glutamatergic transmission within the forebrain, especially in the connections between the ventral tegmental area (VTA), nucleus accumbens (NAcc) and prefrontal cortex (Vanderschuren and Kalivas, 2000). These changes have been shown to be correlated with increases in motivational aspects of drugs of abuse which occur during the development of addiction, such as drug-seeking behaviour, craving and reinstatement of compulsive drug-seeking behaviour (De Vries et al., 1998; Lu et al., 2002; Vanderschuren et al., 1999; Wolf, 1998).

In this study, acute administration of morphine caused a slight reduction in the locomotor activity of NK1 knockout mice, whereas wildtype mice did not show any such alterations relative to vehicle controls. However, chronic morphine treatment caused a dramatic increase in the speed of locomotion of wildtype mice, while the locomotor activity of NK1 knockout mice only recovered to the level of saline-treated wildtype animals. Following a week's abstinence, the maintenance of sensitisation was tested with a challenge dose of morphine. Sensitisation was maintained in wildtype mice, whereas NK1 knockout animals continued to behave in a similar manner to vehicle controls. These data strongly indicate that the activation of NK1 receptors is necessary for the normal development of locomotor sensitisation to morphine. Cocaine, on the other hand, had similar effects in both genotypes. Although acute administration of cocaine did not affect locomotor activity in either genotype, subsequent chronic administration of cocaine caused a similar locomotor sensitisation in both genotypes, which was maintained over a one-week drug-free period. These results suggest that NK1 receptors are crucial not only for the acute reinforcing effects of morphine, but also for the adaptive responses to chronic morphine administration which may underlie the development of sensitisation.

The present study, as well as that of Murtra et al. (2000b), investigated the rewarding properties of only single doses of morphine. It is thus not clear whether the deficits in morphine reward observed in the NK1 knockout mice might be overcome if the animals were given access to higher morphine doses. Certainly, the reduced analgesic response of NK1 knockout mice to morphine in the tail-flick test is overcome at higher morphine doses (De Felipe et al., 1998).

In the present study, the acute administration of morphine did not affect the locomotor activity of wildtype mice, but it did cause reduced locomotion in NK1 knockout animals. At similar doses to that used in this study, Murtra et al. (2000b) saw a stimulant effect of morphine administration in wildtype mice, whereas NK1 mutants did not differ from saline controls. The reasons for this discrepancy could lie either in the experimental procedure, or in the genetic background of the mice used. In the present study, the mice were observed in small boxes to which they had been habituated for three days prior to drug administration, but Murtra et al. (2000b) tested their mice in an open field to which they had not been habituated. The small activity boxes used in the present experiment may not have favoured larger, ambulatory movements, whereas the lack of habituation in Murtra et al. (2000b) may have confounded the effects of drug administration with stress. More likely, however, the difference in genetic background of the mice used in the two experiments may explain the difference (Gerlai, 1996). Whereas Murtra et al. (2000b) were able to elicit a strong CPP to morphine at a dose of 3 mg/kg in
produce CPP in mice of the MF1 strain, suggesting a lower sensitivity to the rewarding effects of morphine as compared to C57BL/6x129sv mice, at least 7.5 mg/kg is necessary to observe a reduction in locomotion in NKl knockout mice at 15 mg/kg (Gadd, unpublished observations). As such, the observed decrease in locomotion in NKl knockout mice at 15 mg/kg may correspond to the similar behavioural pattern observed at a lower dose of morphine by Murtra et al. (2000b).

The exact mechanisms by which NKl receptors contribute to the affective properties of opiates remain unclear. NKl receptors are expressed in many areas of the brain associated with opiate reward and addiction processes, such as the nucleus accumbens and amygdala subnuclei (Nakaya et al., 1994). However, there are few areas where the expression of the NKl receptor and the µ-opioid receptor overlap. One exception is the locus coeruleus, the major supplier of noradrenergic input to the forebrain, which has been implicated in the development of opiate dependence, as well as stress and arousal (Nestler et al., 1993, 1994, 1999; Nestler and Aghajanian, 1997). Since both µ-opioid and NKl receptors are localised on these cells’ somata (Moyle et al., 1997; Santarelli et al., 2001), it is possible that the alterations in noradrenaline release within the forebrain in NKl knockout mice could affect the motivational responses to opiate administration: noradrenaline has been implicated in the motivational properties of opiates, including sensitisation to their locomotor effects (Airio and Ahtee, 1997), and the locus coeruleus has historically been implicated in reward mechanisms (Crow et al., 1972; Ritter and Stein, 1972; Herberg et al., 1975).

Although the noradrenergic system is one plausible candidate for the behavioural changes observed in NKl knockout mice, other systems could be involved. The dopaminergic input from the VTA to the accumbens is critical for the expression of sensitisation to both psychostimulants and opioids (Vanderschuren and Kalivas, 2000), and administration of the Substance P N-terminal fragment, SP17, which acts at NKl receptors, into the VTA mediates accumbens turnover of dopamine during morphine withdrawal (Zhou and Nyberg, 2002). A lack of NKl receptors in the VTA or on the cholinergic interneurons in the accumbens in the NKl knockout mouse could perturb dopaminergic transmission between these nuclei and bring about changes in behaviour as observed in this experiment. Although it has been reported that microinjections of Substance P into the ventral pallidum increase locomotor activity (Kalivas et al., 1995), and may support place preference conditioning (Hasenroth et al., 1992, 1998), and elevate dopamine levels in the accumbens (Boix et al., 1995), it is unlikely that this system accounts for the results presented here, since intracranial injections into the ventral pallidum of cocaine (Gong et al., 1996), but not of morphine (Olmstead and Franklin, 1997), support place preference conditioning. An additional candidate is the 5-hydroxytryptamine (5HT) system. 5HT transmission is known to be enhanced in the dorsal raphe nucleus following pharmacological or genetic disruption of the NK1 receptor through 5HT1A receptor desensitisation (Froger et al., 2001; Haddjeri and Blier, 2001; Santarelli et al., 2001). Although this is known to have no effects on the basal concentrations of extracellular 5HT in the forebrain, sensitisation to morphine can be blocked by pre-treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Sills and Fletcher, 1997), suggesting a possible link between NK1 receptor disruption and the loss of the adaptive response to morphine.

However, whereas morphine reward, withdrawal and addiction behaviours are disrupted in NK1 knockout mice, all cocaine-related behaviours studied are unaffected. It is therefore difficult to reconcile the behavioural differences we have observed solely with global alterations in monoamine function. Although, in contrast to psychostimulants, opiates additionally achieve reinforcing effects through actions in dorsal hippocampus, central grey and lateral hypothalamus, Murtra et al’s. (2000a) observation that CPP to amphetamine is also impaired in mice lacking NKl receptors, suggests that the dissociation of the NK1 receptor’s effects does not fall simply between psychostimulants and opiates. Only a few studies have reported dissociations between the behavioural effects of morphine and amphetamine and those of cocaine. Firstly, ibotenic acid lesions of the rat tegmental pedunculopontine nucleus abolish CPP to both morphine and amphetamine but leave cocaine CPP intact, at least in non-dependent animals (Bechara and van der Kooy, 1989; Parker and van der Kooy, 1995). Conversely, quinolinic acid lesions of the prelimbic area of the medial prefrontal cortex abolish CPP to cocaine, leaving responses to morphine and amphetamine intact (Tzschentke and Schmidt, 1998). Although both of these areas have only weak NK1 receptor immunoreactivity, these findings confirm that separate neural pathways mediate the motivational properties of these groups of drugs. The behavioural observations in NK1 knockout mice suggest that substance P and the NK1 receptor may represent a further aspect of this separation, although further work is required to identify the nature of their participation in reward and addiction behaviours.

It is noteworthy that the results from the NK1 knockout mouse are strikingly similar to those observed in mice lacking CB1 cannabinoid receptors. Like NK1 knockout mice, these mice do not show CPP to morphine, but the response to cocaine is normal (Martin et al., 2000). CB1 knockout mice do not self-administer morphine, in parallel to our findings with NK1 knockout mice, and they do not exhibit many of the somatic responses to morphine withdrawal (Ledent et al., 1999). Furthermore, although CB1 knockout mice display normal acute hyperlocomotor responses to morphine, locomotor sensitisation to morphine is absent, but cocaine...
sensitisation is normal (Martin et al., 2000). The interaction between the opioid and cannabinoid systems is hypothesised to be due to interaction or competition between opioid and cannabinoid receptors at the level of signal transduction cascades. Neurones coexpressing these receptors are found within a number of brain areas associated with motivational behaviours, such as the limbic system and periaqueductal grey (Martin et al., 2000), and it may be of interest that certain behavioural effects of the CB1 antagonist SR 141716A can be antagonised by an NK1 antagonist (Darmani and Pandya, 2000).

The present results clearly demonstrate the functional distinction in the participation of NK1 receptors in opiate and psychostimulant motivational behaviours. The failure of NK1 knockout mice to self-administer morphine, or to sensitise to the locomotor stimulant effects of morphine suggests that manipulation of the substance P and the NK1 receptor may be of use in preventing the development of opiate addiction or reducing craving and relapse. NK1 receptor antagonists may therefore offer a powerful new approach to the management of opiate addiction, in addition to their known effectiveness in alleviating stress, anxiety and depression.

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References


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