Behavioural and molecular responses to amphetamine in the neurokinin-1 receptor knock-out mouse

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I, Julia Slone-Murphy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

The neurokinin-1 receptor knock-out ($NK1R^{-/-}$) mouse is hyperactive and shows deficits in attentional processing, and has recently been put forward as a model of attention deficit hyperactivity disorder (ADHD). Acute amphetamine, a first-line treatment for ADHD and a drug of abuse, paradoxically reduces the hyperactivity of $NK1R^{-/-}$ mice, and the characteristic amphetamine-stimulated increase in striatal dopamine efflux seen in wild-type animals is attenuated in $NK1R^{-/-}$ mice. The research presented in this thesis centres on the behavioural and molecular responses of $NK1R^{-/-}$ mice to amphetamine. Over the course of this work, a fundamental deficit in the striatal cholinergic neuroanatomy of $NK1R^{-/-}$ and wild-type mice was discovered.

While the responses of $NK1R^{-/-}$ mice to amphetamine were normal compared with wild-types, $NK1R^{-/-}$ mice were found to have a reduced number of cholinergic interneurones in the lateral striatum, independent of treatment, and an increased number of cholinergic neurons in the nucleus basalis. In the striatum, the reduction in cell numbers was specific to cholinergic interneurones, which all express the NK1 receptor in wild-type animals and play a key role in regulating striatal dopamine release. The number of striatal parvalbumin-expressing interneurones, which do not express NK1 receptors, did not differ between $NK1R^{-/-}$ and wild-type mice.

The present results demonstrate that global disruption of the NK1 receptor results in significant alterations in central cholinergic system neuroanatomy, which may contribute to the previously observed impaired dopaminergic response to amphetamine. The results presented in this thesis provide support for emerging evidence that deficits in cholinergic transmission play a role in the pathophysiology of ADHD, and could have significant implications for future ADHD research.

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LIST OF ABBREVIATIONS

5-CSRTT	5-choice serial reaction time task
5-HT	5-hydroxytryptamine; serotonin
ABC	Avidin-biotin-peroxidase complex
AChE	Acetylcholinesterase
ADHD	Attention deficit hyperactivity disorder
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AMPH	d-Amphetamine
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
cAMP	Cyclic adenosine monophosphate
CeA	Central nucleus of the amygdala
ChAT	Choline acetyltransferase
CO_2	Carbon dioxide
CPP	Conditioned place preference
CREB	cAMP-response element (CRE)-binding protein
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
DAT	Dopamine transporter
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DRN	Dorsal raphé nuclei
DSM-IV	Diagnostic and statistical manual of mental disorders, 4th revision
EDTA	Ethylenediaminetetraacetic acid
EP	Entopeduncular nucleus
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
GABA	Gamma-amino butyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDP	Guanosine diphosphate
GP	Globus pallidus
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
i.p.	Intraperitoneal / intraperitoneally
ICD-10	International classification of diseases, 10th revision
IP_3	Inositol-3-phosphate
IRES	Internal ribosome entry site
LDEB	Light-dark exploration box
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
MEK	MAPK/ERK kinase 1/2
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neurone
NA	Noradrenaline
NAcc	Nucleus accumbens
NaCl	Sodium chloride
NK1	Neurokinin-1
$NK1R^{-/-}$	NK1 receptor knock-out
NK3	Neurokinin-3
NKA	Neurokinin-A
NKB	Neurokinin-B
p (prefix)	Phosphorylated
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFA	Paraformaldehyde
PKC	Protein kinase C

PLA_2	Phospholipase A_2
PPT-A	Preprotachykinin-A
PVDF	Polyvinylidine difluoride
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPM	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl-sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rat
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SP	Substance P
SSRI	Selective serotonin reuptake inhibitor
TBE	Tris-borate-EDTA (Tris base, boric acid, EDTA)
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tyramide signal amplification
TTBS	Tris-Triton-buffered saline
UP H_2O	Ultrapure water (18 MΩ/cm)
VAChT	Vesicular acetylcholine transporter
VTA	Ventral tegmental area
WKY	Wistar-Kyoto rat

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CHAPTER ONE

1. Introduction

The neuropeptide substance P and its preferred receptor, the neurokinin-1 (NK1) receptor, have had a long and diverse history. Scientific advances over the decades have resulted in several waves of research into substance P and NK1, implicating them in a vast array of physiological roles and dysfunctions, ranging from nociception to emesis through to a number of neurological and psychiatric disorders. The innovation of genomic engineering is one development whose progress over the last 20 years has enabled the manipulation of specific genes in whole animals to become widely utilised in biomedical sciences.

The research presented in this thesis examines behavioural and molecular consequences of the disruption of the gene coding for the NK1 receptor in the mouse, with relevance to disorders such as attention deficit hyperactivity disorder and addiction. This chapter will summarise the history of research into substance P and the NK1 receptor, and will introduce the principals and methods of the use of knock-out mice in biomedical research, before describing studies previously carried out in the NK1 receptor knock-out mouse. As well as providing us with extensive knowledge about the substance P and NK1 receptor (substance P-NK1 receptor) system, such studies have also opened up a number of further questions, some of which will be addressed in this thesis.

1.1. Substance P and the neurokinin-1 receptor

1.1.1. A brief history of research into substance P and the NK1 receptor

The following section will give an overview of the discovery and early research into substance P and the NK1 receptor, while providing basic information about their structure, synthesis and pharmacology.

1.1.1.1. The discovery of substance P

Substance P was discovered by Von Euler and Gaddum (1931), who described the presence of an “unidentified depressor substance” in extracts from horse intestine and brain. At the time, research in their laboratory was centred around determining the actions of acetylcholine, but it was found that the effects of this particular substance – contracting rabbit ileum and causing a fall in blood pressure – were not inhibited by the cholinergic antagonist atropine. Subsequent pharmacological experiments were carried out systematically, in an attempt to identify the active substance (reviewed in Lembeck & Donnerer, 2004). It received its ambiguous name when Gaddum and Schild (1934) precipitated a stable, dry powder from the tissue extract and provisionally labelled it “substance P”. Almost 80 years later, this provisional name still remains.

1.1.1.2. Substance P synthesis

It was not until four decades after its discovery that substance P was finally isolated, sequenced and synthesised (Chang et al., 1971; Studer et al., 1973; Tregear et al., 1971). The full sequence of substance P was found to be H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH^+ (Figure 1.1), which confirmed that it belongs to the tachykinin family, whose members all share the common amino acid sequence: Phe-X-Gly-Leu-Met-NH_2 (where X is Phe or Val (Otsuka & Yoshioka, 1993)). Before the discovery of substance P, tachykinins had been studied only in amphibians and invertebrates. Since then, other mammalian tachykinins including neurokinin A (NKA) and neurokinin B (NKB) have been added to the family and studied extensively (for a review, see Beaujouan et al., 2004).

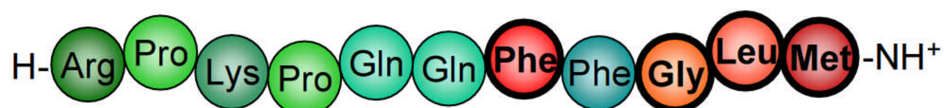

Figure 1.1 Amino acid sequence of substance P.

The part of the sequence that is common to all mammalian tachykinins is highlighted in red and bold (Phe-X-Gly-Leu-Met-NH+).

1.1.1.3. *Discovery of the role of substance P in the central nervous system*

Research into substance P centred on the peripheral nervous system for the first 20 years after its discovery, despite Lembeck (1953) having initially suggested that it may have an excitatory role in the central nervous system. However, the advances in purification of substance P in the 1970s permitted large-scale synthesis and a surge of research. It was then that studies confirmed substance P exists in the dorsal root of bovine spinal cord, at a concentration 10-30 times greater than that in the ventral root (Takahashi et al., 1974), and exerts a powerful excitatory action on frog and rat pup spinal motoneurones at a potency around 200 times higher than glutamate (Konishi & Otsuka, 1974a; Konishi & Otsuka, 1974b; Otsuka et al., 1975). These studies provided evidence that substance P is an important excitatory neurotransmitter in primary afferent neurones.

1.1.1.4. *The NK1 receptor*

The preferred receptor for substance P is the NK1 receptor. This and the two other mammalian tachykinin receptors, NK2 and NK3, were discovered in the 1980s (Buck et al., 1984; Hunter & Maggio, 1984; Iversen et al., 1982; Lee et al., 1982; Watson et al., 1983). Substance P, NKA and NKB can each act as agonists at all three receptors, but they bind with very different affinities, preferring NK1, NK2 and NK3 receptors respectively (Mantyh et al., 1989; Regoli et al., 1989; Saffroy et al., 1988).

The three tachykinin receptors were first cloned from the rat and the cow (Hershey & Krause, 1990; Masu et al., 1987; Shigemoto et al., 1990; Yokota et al., 1989) when they were found to be guanine nucleotide binding protein (G-protein) coupled receptors (Figure 1.3); finally, the human NK1 receptor was cloned in 1991 and found to have 94.5 % homology with the rat receptor (Gerard et al., 1991; Takeda et al., 1991).

1.1.2. Distribution of substance P and the NK1 receptor

Substance P and the NK1 receptor are widely distributed in the body, mirroring their diversity of functions. Immunohistochemical studies have shown generally similar distributions across species, with high levels of expression of both substance P and NK1 receptor in the peripheral and central nervous systems. For the purposes of this thesis, the following description will focus on the distribution in the brain. An example of NK1 receptor expression in the mouse dorsal striatum is shown in Figure 1.2.

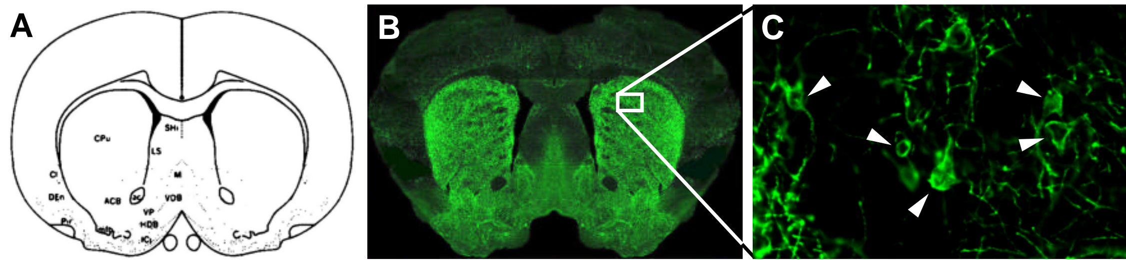

Figure 1.2 NK1 receptor in the striatum.

(A) Striatum as illustrated in the mouse brain atlas (Franklin & Paxinos, 1997). (B-C) NK1 receptor-like immunoreactivity in the mouse striatum (Gadd, 2003).

Both the NK1 receptor and substance P are densely distributed in the caudate putamen and nucleus accumbens (Beach & McGeer, 1984; Caberlotto et al., 2003; Hietala et al., 2005), where substance P is co-expressed with dynorphin in medium spiny GABAergic neurones. These neurones project to the ventral pallidum, substantia nigra and globus pallidus (Anderson & Reiner, 1990; Napier et al., 1995), as well as onto the cholinergic interneurones of the striatum; the NK1 receptor is expressed in the cell bodies and dendrites of 100 % of these cholinergic interneurones (Aubry et al., 1994; Gerfen, 1991) (discussed in more detail in Section 1.5.1 below, and in Chapter 6). Substance P and the NK1 receptor are also expressed in the hypothalamus, amygdala and noradrenergic locus coeruleus (Chen et al., 2000; Dam et al., 1990; Emson et al., 1978; Nakaya et al., 1994). Both are also found in the dorsal raphé nucleus (DRN), but while substance P is consistently found in the serotonergic cells of this nucleus, NK1 receptor distribution varies to some extent between species: in the mouse, it is expressed solely on non-serotonergic cells of the DRN (Froger et al., 2001; Santarelli et al., 2001), while in the rat it is found on some serotonergic and some non-serotonergic cells (mainly GABAergic). Very low levels of substance P and NK1 receptor are found in the cortex and hippocampus (Gadd et al., 2003; Nakaya et al., 1994).

While the brain regions described above exhibit a high correspondence of substance P and NK1 receptor expression, a “mismatch” in other regions is often described (Liu et al., 1994; Nakaya et al., 1994), most notably in the dopaminergic substantia nigra (SN) and ventral tegmental area (VTA), where substance P is abundant but the NK1 receptor is particularly sparse (Cooper et al., 1981; Davies & Dray, 1976; DiFiglia et al., 1982; Gadd et al., 2003; Ghatei et al., 1984; Ljungdahl et al., 1978; Nomura et al., 1987). Other areas which have a high level of

substance P but little NK1 receptor expression are the septum, globus pallidus, medial nucleus of the amygdala, interpeduncular nucleus, lateral habenula and dorsal tegmentum; while the medial habenula, parabrachial nucleus and cerebellum all show dense NK1 receptor expression with little substance P (Danks et al., 1986; Herkenham, 1987; Mantyh et al., 1984; Quirion et al., 1983; Rothman et al., 1984).

Furthermore, while substance P and NK1 receptor are both present in the striatum, electron microscopy reveals that a mismatch occurs between the localisation of NK1 receptors and substance P-containing axon terminals, with only around half of the total substance P-positive terminals synapsing onto NK1 receptor-positive neuronal profiles while the remaining half are on NK1 receptor-negative neuronal profiles; conversely, around half of the non-substance P terminals were found on NK1 receptor-positive neuronal profiles (Li et al., 2000).

Such discrepancies could indicate that as well as acting synaptically, substance P acts extrasynaptically as a paracrine neurotransmitter, diffusing from the site of release to a separate site of action through the extracellular fluid. Alternatively, it implies that selectively localised NK1 receptors allow the widely-expressed substance P to act at specific regions or sub-regions of the brain (Herkenham, 1987; Li et al., 2000). For example, projection neurones in the basal ganglia, known to have an extensive network of collateralised axons (Mailly et al., 2003; Parent et al., 2000), may express substance P at all terminals, but its activity is restricted to the sub-regions where the NK1 receptor is selectively expressed. Different tachykinins may also be present in regions where the NK1 receptor is sparse, such as the substantia nigra pars compacta (SNc) where neurokinin B acts on NK3 receptors as the endogenous ligand (Keegan et al., 1992).

The above description has given an overview of NK1 receptor and substance P distribution in the mammalian brain alone. For comprehensive descriptions of their distribution elsewhere, including in non-mammalian species, see reviews by Quartara and Maggi (1998) and Severini et al (2002).

1.1.3. Biosynthesis of substance P

Substance P is synthesised from preprotachykinin A (PPT-A), a precursor that is encoded by the *ppt-a* or *TAC-1* gene. By alternative splicing, this gene produces four different mRNA transcripts, α-, β-, γ- and δ-PPT-A, which code for substance P, NKA, and two longer forms of

NKA, neuropeptide K and neuropeptide γ (Carter & Krause, 1990; Harmar et al., 1986; Harmar et al., 1990; Krause et al., 1987; Nawa et al., 1984).

1.1.4. NK1R signalling

The NK1R is a guanine-nucleotide binding protein (G-protein)-coupled receptor with seven membrane-spanning domains (Patacchini & Maggi, 1995; Takeda et al., 1991). When substance P binds to the NK1 receptor, a complex is formed, which activates various G-proteins and recruits a number of different second messenger systems (Figure 1.3). Arachidonic acid is mobilised, inducing breakdown of phosphatidyl inositol bisphosphate and increasing intracellular calcium (Khawaja & Rogers, 1996; Mau & Saermark, 1991; Quartara & Maggi, 1997). This series of events leads to an increase in sodium conductance and a decrease in potassium conductance, resulting in a slow excitatory potential (Shen & North, 1992; Stanfield et al., 1985). Gene transcription can also be modulated. Finally, the substance P-NK1 receptor complex is rapidly internalised, where it dissociates in acidified endosomes before the substance P is degraded and the NK1R recycles to the cell membrane (Garland et al., 1996; Grady et al., 1995; Mantyh, 2002).

1.1.5. Physiological roles of substance P and the NK1 receptor

Many years of research have been devoted to substance P and the NK1 receptor, and roles have been revealed for the receptor in a vast array of physiological functions and as a potential therapeutic target for numerous disorders, from pain to anxiety. This section will briefly review the known functions of the substance P-NK1 receptor system that have been revealed by pharmacological and cell ablation studies, focusing on evidence for its role in neuropsychiatric conditions. Section 1.3 below reviews studies in NK1 receptor knock-out mice, which have further elucidated the many roles of the receptor. Table 1.1 below summarises the behavioural paradigms referred to in this chapter.

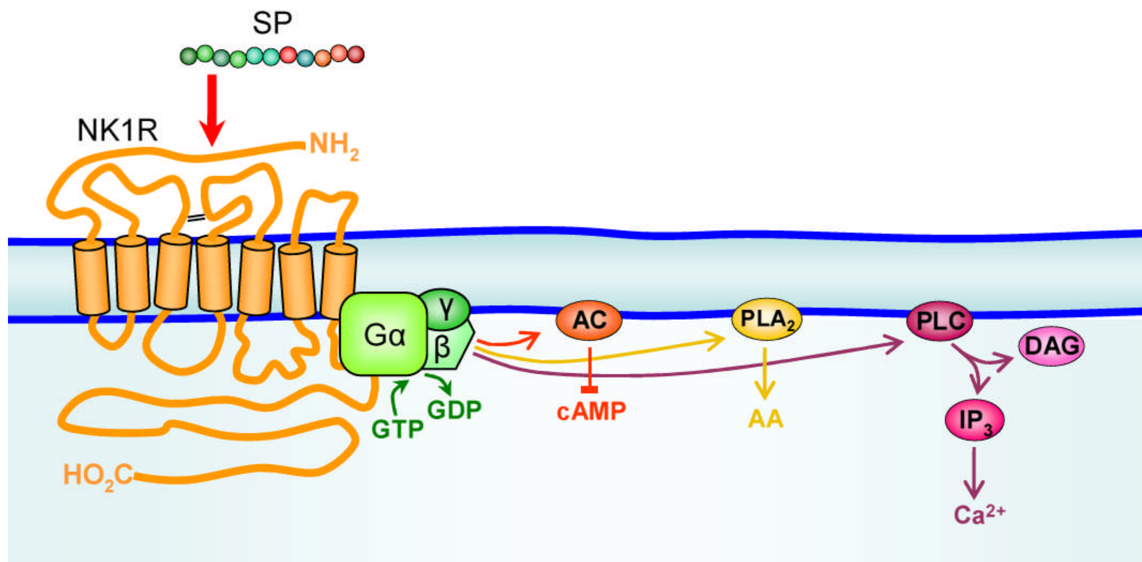

Figure 1.3 Schematic representation of NK1 receptor signalling.

The seven membrane-spanning domains of the NK1 G-protein coupled receptor are shown, with the three second messenger pathways involving phospholipase A_2, phospholipase C and adenylate cyclase. AA, arachidonic acid; AC, adenylate cyclase; Ca^{2+}, intracellular calcium; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; Gα, β and γ, subunits of the heterotrimeric G protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate; IP_3, inositol tris-phosphate; PLA_2, phospholipase A_2.

Behavioural paradigm	***Description***
Tail suspension test	A simple paradigm for use with mice. A mouse is suspended by a lever for six minutes, during which time it will exhibit a period of agitation followed by a period of immobility. Antidepressants lengthen the duration of agitation; the efficacy of putative novel antidepressant drugs can be assessed in this way and compared with established antidepressants. Results from this test tend to be concordant with those from the forced swim test.
Forced swim test	A mouse or rat is placed in a narrow cylinder of water in which it is forced to swim. After a brief period of struggle it will cease attempts to escape and become immobile. As with the tail suspension test, the period of immobility is reduced by antidepressants. Because of the highly stressful nature of this test it can be used to determine the neurochemical, cellular and molecular effects of acute stress.
Elevated plus maze	A “plus”-shaped maze elevated around 50 cm from the ground, with two arms of the maze enclosed by walls, and the remaining two arms open narrow platforms. The animal is free to walk around the whole maze. The more time it spends in the open arms of the maze, the less anxiety behaviour it is considered to exhibit. Anxiolytics increase the time the animal spends in the open arms of the maze.
Separation-induced vocalisation	When pups are separated from their mother they produce a vocalisation response. In some animals (such as guinea pigs) this response is audible to the human ear, while in others (such as mice) it is ultrasonic. The number of vocalisations in a set time period is considered to be a measure of the stress experienced by the animal.
Social stress or resident-intruder test	These are paradigms utilising potent natural stressors that can be used across many species. An animal is placed in the cage of a dominant conspecific animal and allowed to reach social defeat by non-lethal conflict. The test can be acute (one session) or chronic (repeated sessions over a number of days).
Restraint stress	Animals are immobilised for a period of 60 minutes or longer, usually in a small cylindrical holder. Limb movement is restricted in most restraint techniques. Unlike the social stress paradigm, there is no physical injury to the animal.
Conditioned place preference (CPP)	Another model used to investigate the rewarding effects of a drug. A distinct compartment in a chamber is paired with a drug, by repeatedly administering the drug to an animal and immediately placing the animal in that compartment. A second (and sometimes third) compartment is paired with saline or another drug. Once the animal has received a series of injections and associated each compartment with each treatment, it is given free choice of compartments in the chamber, and will spend most time in the compartment paired with a rewarding drug.
Sensitisation	In experimental animals, this is a progressive increase in locomotor activity response to a drug, following previous exposure to the drug. It is considered to be a model of craving and relapse (explained in detail in Chapter 5).

Table 1.1 Behavioural paradigms.

Descriptions of preclinical behavioural paradigms used to model aspects of human neuropsychiatric disorders.

1.1.5.1. Affective disorders

The NK1 receptor is widely expressed in brain regions involved in emotional processing; this characteristic distribution has prompted considerable research into its role in affective disorders.

Depression

A large body of evidence indicates that the NK1 receptor has a role in the control of affect, and dysfunction at this receptor may contribute to affective disorders. Kramer et al (1998) first observed the antidepressant activity induced by NK1 receptor blockade, describing the complete inhibition of isolation-induced vocalisation in guinea pig pups by two different NK1 receptor antagonists, in a similar manner to that seen with established antidepressant and anxiolytic drugs. Several preclinical studies have provided evidence for a monoaminergic mechanism of action for antidepressant activity by demonstrating an interaction between the substance P-NK1 receptor system and the classical monoaminergic neurotransmitters thought to underlie affective disorders (Gobbi & Blier, 2005). For example, the selective NK1 receptor antagonists CP-96,345 and WIN 51,708 modulate the activity of noradrenergic and serotonergic neurones, directly increasing the firing of serotonergic neurones in the rat dorsal raphé nucleus (Haddjeri & Blier, 2001) and also attenuating the inhibitory action of the α_2 adrenoceptor agonist clonidine on the firing activity of both serotonergic and noradrenergic neurones (Haddjeri & Blier, 2000). Furthermore, another NK1 receptor antagonist, GR-205,171, increases the firing rate of dopaminergic cells in the parabrachial nucleus of the ventral tegmental area, and produces a corresponding elevation in dopamine efflux in the frontal cortex (Lejeune et al., 2002).

NK1 receptor antagonists also appeared to be effective in clinical trials with depressed patients. Kramer et al (1998) reported that the NK1 receptor antagonist aprepitant (MK869) was clinically effective in reducing depressive symptoms in patients with major depressive disorder, to a degree comparable to the established antidepressant paroxetine, a selective serotonin reuptake inhibitor (SSRI). This finding was later replicated using a different NK1 receptor antagonist, L-759,274, which also showed well-tolerated antidepressant activity compared with placebo in a group of depressed patients (Kramer et al., 2004).

Results from clinical and preclinical studies such as those described above made NK1 receptor antagonists seem promising as effective antidepressants. However, a subsequent large-scale

investigation using aprepitant in over 2500 patients with major depressive disorder showed that while paroxetine was consistently seen to perform better than placebo in improving depression scores, aprepitant was ineffective, despite providing continuously high levels of NK1 receptor blockade (Keller et al., 2006). Research is ongoing to develop an NK1 receptor antagonist with consistent clinical antidepressant efficacy (Quartara et al., 2009).

Anxiety

The link between the NK1 receptor and adaptation to stressful stimuli has been widely studied. Stress is known to elevate the release of substance P in a number of different brain regions, for example immobilisation and forced swim stressors increase substance P efflux in the medial amygdala and lateral septum (Ebner & Singewald, 2006). Administration of substance P or other NK1 receptor agonists, both locally and systemically, has repeatedly been found to produce NK1 receptor-dependent anxiogenic effects in animals exposed to a number of different paradigms (Bassi et al., 2007a; Bassi et al., 2007b; De Araujo et al., 1999; Gavioli et al., 1999; Gavioli et al., 2002; Hasenohrl et al., 1998b; Nikolaus et al., 2000). Similarly, pharmacological blockade of NK1 receptors has an anxiolytic effect, first demonstrated by Culman et al. (1997), who described how administration of an NK1 receptor antagonist to rats, prior to injection of formalin, inhibited the cardiovascular and behavioural responses characteristically seen following this painful and stressful stimulus. NK1 receptor antagonists also reduced stress-induced neonatal vocalisations in mice and guinea pigs (Rupniak et al., 2000), prolonged social interaction time in gerbils (Cheeta et al., 2001) and reduce stress-induced increases in dopamine and noradrenaline in the medial prefrontal cortex in rats and gerbils (Renoldi & Invernizzi, 2006) and dopamine metabolism in the rat prefrontal cortex (Hutson et al., 2004). However, an increase in anxiety behaviour in the elevated plus maze has been observed following ablation of NK1 receptor expressing neurones in the amygdala (Gadd et al., 2003). It is likely that this discrepancy relates to the nature of the loss of NK1 receptor function; while cell ablation removes the entire neurone, resulting in a loss of co-expressed receptors and interneuronal connections, pharmacological manipulation results in effects specifically related to the receptor in question (Gadd et al., 2003).

1.1.5.2. Reward and addiction

The expression pattern of the NK1 receptor also spans those areas concerned with reward and motivation, and several studies have demonstrated its role in mediating reward responses. In

addition to having anxiogenic properties, substance P can also be rewarding when administered at certain doses or in specific brain regions; so while at high doses substance P is aversive and anxiogenic, at lower doses it is rewarding, producing conditioned place preference both when administered systemically and when microinjected into brain regions including the basal forebrain, amygdala, medial septum and ventromedial caudate putamen (Hasenohrl et al., 1998a; Hasenohrl et al., 1991; Kertes et al., 2009a; Kertes et al., 2009b; Krappmann et al., 1994; Oitzl et al., 1990). Correspondingly, ablation of NK1 receptor expressing neurones in the amygdala produced reductions in morphine reward behaviour (Gadd et al., 2003). In the striatum, NK1 receptors are widely expressed on cholinergic interneurones (see Section 1.5.1). Gadd et al (2003) found that ablation of NK1 receptor-expressing cells in the nucleus accumbens and dorsomedial striatum had no effect on morphine reward behaviour; however, in a series of studies by Hikida and co-workers, ablation of cholinergic neurones in the nucleus accumbens was found to enhance conditioned place preference to morphine and cocaine, as well as conditioned place aversion to morphine withdrawal (Hikida et al., 2001; Hikida et al., 2003; Kitabatake et al., 2003). A difference in the regional specificity of the ablation may be the explanation for the discrepancy between these two sets of studies, as the mice in the studies of Hikida et al. (2001, 2003) had specific ablation of cells in the nucleus accumbens, while mice included in the studies by Gadd et al. (2003) were found to have ablation of cells in the dorsomedial striatum in addition to the nucleus accumbens, potentially affecting the conditioned place preference results from this group.

Neurotransmitter systems shown to mediate the rewarding properties of substance P include the endogenous opioid system, demonstrated by the opioid antagonist naloxone preventing the development of conditioned place preference to substance P (Hasenohrl et al., 1991), and the dopaminergic system, suggested by the increase in extracellular dopamine in the nucleus accumbens that parallel systemic substance P administration (Huston et al., 1993).

The link between alcohol and the substance P-NK1 receptor system has received much attention in recent years (George et al., 2008). Polymorphisms at the *TACR1* gene (the human gene encoding the NK1 receptor) have been revealed to have a significant association with alcohol dependence in humans (Seneviratne et al., 2009), while preclinical studies have shown that NK1 receptor antagonists reduce voluntary alcohol consumption and alcohol reward in mice (Thorsell et al., 2010).

Substance P has been shown to mediate the increased dopamine release following psychostimulants including amphetamine and cocaine, demonstrated by infusion of an NK1 receptor antagonist into the striatum before a systemic injection of cocaine (Loonam et al., 2003). Similarly, a dramatic reduction in amphetamine-induced dopamine release is seen in $NK1R^{-/-}$ mice (Yan et al., 2010). This is discussed further in Sections 1.3 and 1.4 below, and the role of the NK1 receptor in the long-term responses to addictive drugs, with particular reference to amphetamine, will be considered further in Chapters 5 and 6.

1.1.5.3. Other roles

The substance P-NK1 receptor system has been implicated in numerous other disorders and functions, including learning and memory (Hasenohrl et al., 2000), emesis (Diemunsch et al., 2009), and cell growth with implications in wound healing and in anti-tumour activity (Munoz & Rosso, 2010; Reed et al., 2008). The involvement of the NK1 receptor in pain and nociception is one of its most widely studied roles. Substance P is synthesised in primary afferent nociceptors and released into NK1 receptor-expressing neurones of the dorsal horn of the spinal cord following prolonged or intense noxious stimulation (De Koninck & Henry, 1991). In animal models of chronic and inflammatory pain, ablation of NK1 receptor-expressing spinal neurones produces a long-term attenuation of thermal hyperalgesia and mechanical allodynia (Nichols et al., 1999), and intrathecal injection of substance P produces hyperalgesia (Choi et al., 2005; Moochhala & Sawynok, 1984) providing sound evidence that substance P and the NK1 receptor play a role in mediating responses to nociceptive stimuli. Research into NK1 receptor antagonists as analgesics appeared promising initially, showing efficacy in rat and guinea pig models of neuropathic pain (Cumberbatch et al., 1998; Walpole et al., 1998), but once again results from clinical trials proved disappointing in a variety of pain states (Hill, 2000).

1.1.6. Possible reasons for the failure of NK1 receptor antagonists in clinical trials

One of the major obstacles to overcome when working with NK1 receptor antagonists is that there is considerable species variation between NK1 receptors. Although the rat and mouse NK1 receptors are about 95 % homologous with the human receptor (Engberg et al., 2007; Saria, 1999), the apparently small difference in NK1 receptor sequences between species has been found to have considerable effects on antagonist binding (Fong et al., 1992). This not

only means that antagonists that have promising effects in the rat or mouse do not bind successfully in humans, but also results in considerable difficulty in evaluating the efficacy of high-affinity human NK1 receptor antagonists, as they have limited affinity for receptors in the rat and mouse (Rupniak et al., 2001).

1.1.7. Clinical successes

An article appeared in *Science* as long as 12 years ago, "commending" the pharmaceutical industry for its persistence in researching NK1 receptor antagonists as a repeatedly failing target for seemingly countless conditions (Wahlestedt, 1998). Nevertheless, research continued, and since then the NK1 receptor antagonist aprepitant has been licensed for use in the prevention of nausea and vomiting associated with cancer chemotherapy (Martin et al., 2003), and recently LY686017, another antagonist at the NK1 receptor, has shown promise in the clinic by reducing craving in hospitalised alcoholics (George et al., 2008).

1.2. Knock-out mice

Genetic engineering techniques have become widely available and invaluable in biomedical research. Targeted gene deletion or disruption, referred to as genetic "knock-out", is a commonly used example of such techniques, in which a specific gene in an organism is disrupted, resulting in the loss of functional expression of the protein encoded by that gene. The phenotype of the genetically modified organism can be compared against that of the wild-type organism to determine the specific functions of the protein in question. This section will explain the principles of genetic knock-out, before reviewing the known behavioural and neurochemical characteristics of the NK1 receptor knock-out mice used in this thesis.

1.2.1. Methodological principles of a single gene knock-out

The process of gene targeting using embryonic stem cells consists of two stages, illustrated in Figure 1.4. In the first stage, a targeting vector, or cassette, is constructed. This typically consists of a reporter gene, a selectable marker (usually an antibiotic resistance gene), and regions at either end that are homologous to the gene to be targeted. The second stage involves inserting the cassette into cultured embryonic stem cells from a donor mouse. The cells with the correct insertion are selected from the culture by utilising the selectable marker in the insertion cassette, and the stem cells are injected into an embryo from a host mouse,

and implanted into a surrogate mouse. The surrogate mouse will produce a litter of chimeric mice, made up of a mixture of cells from the host embryo and from the genetically modified cells. The chimeras are bred, giving rise to mice whose genomes are completely derived from that of the selected cells.

1.2.2. Importance of genetic background

The influence of genetic background in studies using knock-out mice should not be underestimated. There are outward strain differences in many behaviours including locomotor activity, learning and memory tasks, aggression and parental behaviours (Crawley et al., 1997). Genetic background can have profound effects on results obtained from different mouse strains with the same genetic modification. Studies in NK1 receptor knock-out mice have reported apparently contradictory results related to differences in genetic background. This is illustrated by McCutcheon et al. (2008), who reported that NK1 receptor knock-out mice on a mixed C57BL/6 x 129/Sv background exhibited greater novelty-induced hyperlocomotion, increased hippocampal neurogenesis and significantly elevated glucocorticoid receptor immunoreactivity than wild-type mice, while NK1 receptor knock-out mice on a congenic C57BL/6 background were no different from wild-type mice. It is therefore important to interpret with caution studies from knock-out animals derived from a single background strain, ideally using more than one strain for the knock-out in order to avoid false or incomplete findings.

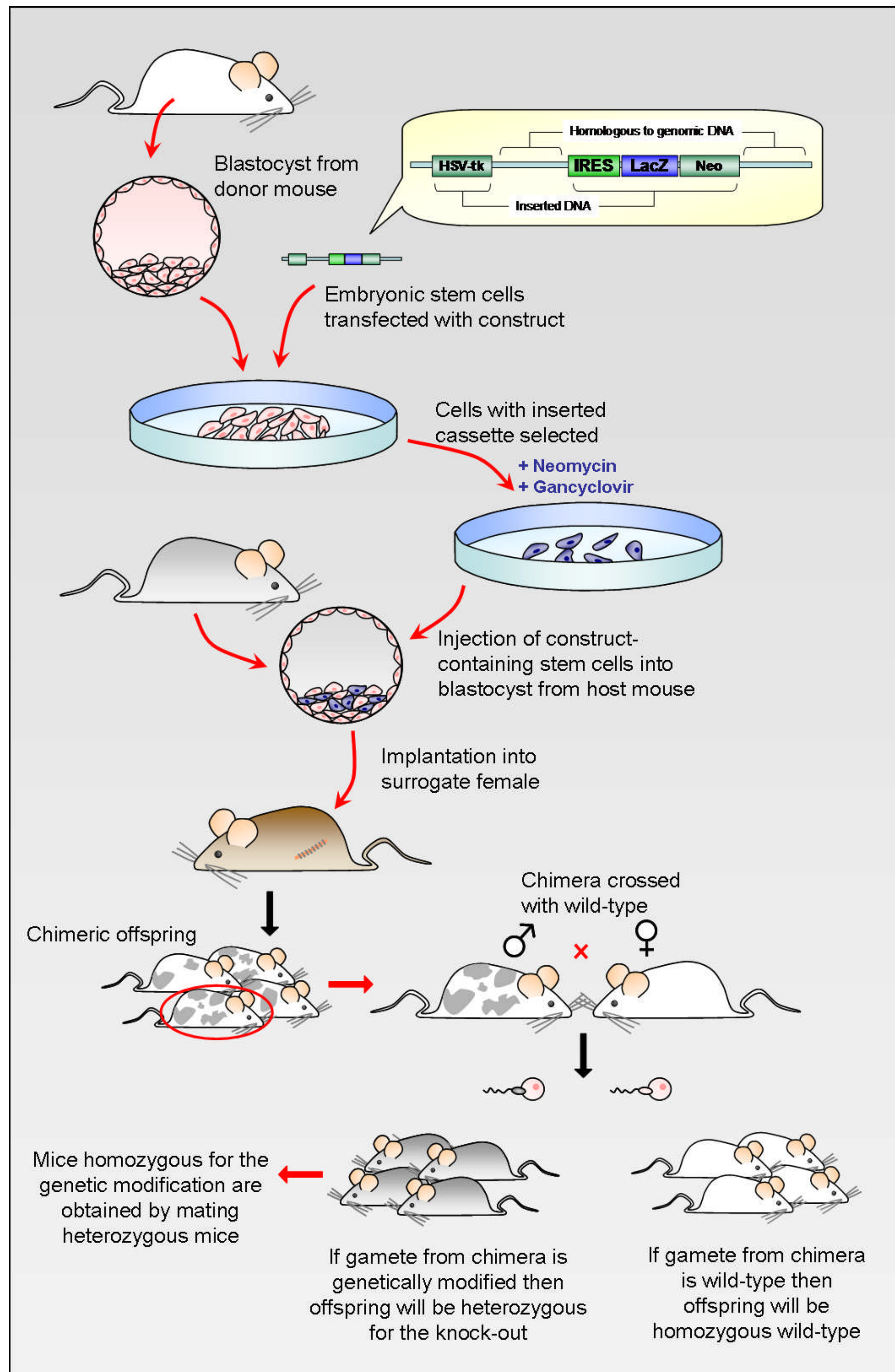

Figure 1.4 Creation of a genetically modified mouse.

Illustration of the basic procedure used in the creation of a genetically modified mouse, using homologous recombination. See text for details. Adapted from an illustration by Annika Rohl for the Nobel Committee for Physiology or Medicine.

1.3. NK1 receptor knock-out mice

The NK1R$^{-/-}$ mouse was created in 1998 (De Felipe et al., 1998). Full descriptions of the methods used in the creation of the mouse, as well as details regarding colony maintenance and breeding, are given in Chapter 2. The NK1R$^{-/-}$ mouse was initially developed with the purpose of being a model for affective disorders, but has proved to be a valuable tool for the investigation of the many roles of the NK1 receptor. Experiments using NK1R$^{-/-}$ mice have provided further evidence in support of the studies using cell ablation and pharmacological tools described in the previous section that the NK1 receptor plays a key role in mediating a range of different behaviours and physiological functions, and highlights the receptor as a target for treatment of a variety of disorders.

The following sections will give an overview of the behavioural and neurochemical phenotype of the NK1R$^{-/-}$ mouse, before describing its specific use as a model for attention deficit hyperactivity disorder (ADHD).

1.3.1. Behavioural phenotype of the NK1R$^{-/-}$ mouse

1.3.1.1. Hyperactivity

The most apparent characteristic of NK1R$^{-/-}$ mice is that they are hyperactive, particularly in a novel environment, and it was this feature which first led them to be investigated as a model for ADHD. This is discussed further in Section 1.4 below.

1.3.1.2. Behaviour related to affective disorders

NK1R$^{-/-}$ mice were originally developed to study depression; several studies have demonstrated that the behaviour of NK1R$^{-/-}$ mice resembles that of wild-type mice maintained on antidepressants. In studies relating to anxiety and depression, NK1R$^{-/-}$ mice have been shown to have a lower attack score and increased latency to attack in the resident-intruder test (Rupniak et al., 2001), while in the forced swim test NK1R$^{-/-}$ mice continue swimming for a longer duration than their wild-type counterparts (De Felipe et al., 1998; Santarelli et al., 2001), supporting pharmacological studies that suggest a role for the NK1 receptor in the control of aggression and affect. Furthermore, in the tail suspension test, NK1R$^{-/-}$ mice exhibited prolonged struggle behaviour compared to wild-types, although this was not replicated with antagonists either in the mouse or in the gerbil, suggesting that this is a

developmental alteration resulting from the genetic disruption of the NK1 receptor (Rupniak et al., 2001). Immunohistochemical studies have also shown that $NK1R^{-/-}$ mice undergo increased hippocampal neurogenesis, the same effect as is seen with mice maintained chronically on antidepressants (McCutcheon et al., 2008; Morcuende et al., 2003). Glucocorticoid receptors are involved in terminating the release of the stress hormone corticosterone; as mentioned in the preceding section, $NK1R^{-/-}$ mice have been shown to have an elevated level of glucocorticoid receptor immunoreactivity, although this was shown only in mice on a mixed C57BL/6 x 129/Sv background and not in those on a congenic C57BL/6 background (McCutcheon et al., 2008). Further supporting the role of NK1 receptors in stress responses, $NK1R^{-/-}$ mice display the same level of reduction in stress-induced neonatal vocalisation as mice given antidepressants (Rupniak et al., 2000), and have markedly reduced anxiety-related behaviours in the elevated plus maze, novelty-suppressed feeding and maternal separation (Santarelli et al., 2001), providing robust evidence that the receptor is critical to the development of adaptive responses to stressful stimuli.

1.3.1.3. Altered reward responses

In reward paradigms, self-administration and behavioural sensitisation to morphine do not develop in $NK1R^{-/-}$ mice (Gadd et al., 2003; Murtra et al., 2000; Ripley et al., 2002), and they have a reduced voluntary intake of alcohol (George et al., 2008). However, conditioned place preference and sensitisation develop normally to cocaine and to food (a natural reward) in $NK1R^{-/-}$ mice (Gadd et al., 2003; Murtra et al., 2000; Ripley et al., 2002). Recently, $NK1R^{-/-}$ mice have been found to develop normal conditioned place preference to amphetamine also.

1.3.1.4. Reduced nociception

The role of substance P in pain and the modulation of nociceptive signalling has long been widely accepted, so as expected, $NK1R^{-/-}$ mice were found to have reduced responses to nociceptive stimuli, similar to mice given analgesics. $NK1R^{-/-}$ mice do not develop capsaicin-induced hyperalgesia either viscerally or in the paw (Laird et al., 2000; Laird et al., 2001), and noxiously-evoked descending inhibition is absent in these mice (Bester et al., 2001). However, $NK1R^{-/-}$ mice do not develop stress-induced analgesia (De Felipe et al., 1998). The fact that substance P can generate both antinociceptive and pronociceptive responses that effectively cancel each other out may explain why systemically administered NK1 antagonists are ineffective as analgesics.

1.3.2. Neurochemical phenotype

1.3.2.1. Noradrenaline

$NK1R^{-/-}$ mice have abnormal noradrenaline transmission, with a two- to four-fold increase in basal noradrenaline efflux observed in the frontal cortex compared with wild-type mice (Fisher et al., 2007; Herpfer et al., 2005). It appears that this is due to the increased release of noradrenaline, rather than a decrease in clearance, as noradrenaline transporter expression and function was observed to be similar in wild-type and $NK1R^{-/-}$ mice (Fisher et al., 2007; Herpfer et al., 2005). Feedback control of noradrenaline release by postsynaptic α_2-adrenoceptors is not affected in $NK1R^{-/-}$ mice, as local administration of an α_2-adrenoceptor antagonist (RX-821002) into the prefrontal cortex increased noradrenaline efflux to a similar degree in both wild-type and $NK1R^{-/-}$ mice (Yan et al., 2009); and the density of these receptors, both at the terminals and in the locus coeruleus, is also similar in both genotypes (Fisher et al., 2007). However, a study looking at α_{2A}-adrenoceptor function by $[^{35}S]$-$GTP_{\gamma S}$ binding to activated α_{2A}-adrenoceptors revealed that $NK1R^{-/-}$ mice show only 30 % of the binding seen in wild-type mice in the locus coeruleus, suggesting that there is a functional desensitisation of somatodendritic α_{2A}-adrenoceptors. Noradrenaline neurones projecting from the locus coeruleus are tonically active, so a desensitisation of somatodendritic α_{2A}-adrenoceptors would disinhibit noradrenergic cell firing and increase release at terminals in the frontal cortex (Yan et al., 2009).

1.3.2.2. Serotonin

Substance P and the NK1 receptor are both expressed in the serotonergic DRN. In $NK1R^{-/-}$ mice, spontaneous firing of 5-HT neurones in the DRN is increased compared with wild-type mice, a finding that has been replicated using NK1 receptor antagonists in wild-type mice (Santarelli et al., 2001). Genetic disruption or pharmacological blockade of the NK1 receptor also results in desensitisation of 5-HT_{1A} autoreceptors, mirroring the effect seen after chronic treatment with selective serotonin reuptake inhibitor (SSRI) antidepressants (Froger et al., 2001; Santarelli et al., 2001). Furthermore, administration of the SSRI paroxetine produces a greater increase in cortical serotonin efflux in freely-moving $NK1R^{-/-}$ mice than in wild-types (Froger et al., 2001), suggesting that endogenous substance P in wild-type animals normally limits the cortical efflux of serotonin following SSRI administration.

1.3.2.3. Dopamine

While the other monoaminergic systems have been widely studied in NK1R$^{-/-}$ mice, the dopaminergic system is less well documented in these animals. Studies suggesting that the dopaminergic system is affected in NK1R$^{-/-}$ mice have until recently been indirect, relating to the lack of reward responses to alcohol and morphine in NK1R$^{-/-}$ mice (George et al., 2008; Murtra et al., 2000), thought to be mediated by dopamine. However, two striking findings have emerged recently. In NK1R$^{-/-}$ mice, basal dopamine efflux in the prefrontal cortex is less than 50 % of that seen in wild-types (Yan et al., 2010). Furthermore, NK1R$^{-/-}$ mice exhibit a remarkable deficit in amphetamine-induced striatal dopamine release (Yan et al., 2010). As well as implicating the substance P-NK1 receptor system in addictive disorders, evidence such as this has great relevance to attention deficit hyperactivity disorder, as psychostimulant medication is the most common first-line treatment for the disorder. The following section addresses ADHD in more detail.

1.4. A model for attention deficit hyperactivity disorder

ADHD is the most commonly diagnosed childhood psychiatric disorder, affecting over 7 % of children when even the most stringent diagnostic criteria are used (Wender, 2002), and frequently persists into adulthood. It is a highly heritable disorder (Fisher et al., 2002) that can result in significant impairment socially, academically and occupationally. This section will describe the clinical features of ADHD, give an overview of the currently available models of ADHD that are commonly used, and illustrate the similarities between the disorder and the phenotype of the NK1R$^{-/-}$ mouse.

1.4.1. ADHD in the clinic

1.4.1.1. Symptoms and treatment

ADHD typically presents with three core behavioural symptoms: inattentiveness, impulsivity and hyperactivity. While these symptoms alone can be highly disruptive, people with ADHD are also at increased risk of comorbid and later life disorders including addiction, eating disorders, anxiety and depression (Biederman et al., 2010). The most widely used treatments are psychostimulants, most commonly methylphenidate (Ritalin) or a mixture of amphetamine salts (Adderall); over 90 % of children diagnosed with ADHD are treated in this way.

Psychostimulants such as methylphenidate and amphetamine act on central dopaminergic and noradrenergic systems, and so improve inhibitory control and cognition both in people with ADHD as well as in healthy subjects. However, while in healthy people psychostimulants have a characteristic effect of increasing activity, the hyperactivity exhibited by people with ADHD is paradoxically reversed upon administration of such drugs.

1.4.1.2. Subtypes

There are three subtypes of ADHD, defined by a combination of the three major symptoms. These are the inattentive subtype, the hyperactive-impulsive subtype, and the combined subtype. The hyperactive-impulsive and combined subtypes are more common in boys, while girls tend to present more often with the inattentive subtype (Weiss et al., 2003). Patients with the inattentive subtype are also more likely to be older than those with the subtypes involving hyperactivity, as hyperactive and disruptive behaviours decline with age while problems with executive function persist (Biederman et al., 2000). Although ADHD is more commonly diagnosed in boys than girls, there is a risk that the disorder is under-diagnosed in girls, as while children with the hyperactive-impulsive or combined subtypes are disruptive in class and demand attention from teachers and parents, those with the inattentive subtype tend to be quiet, resulting in referral bias unfavourable to girls (Pinkhardt et al., 2009).

1.4.1.3. Adult ADHD

Although typically thought of as a childhood disorder, ADHD persists into adulthood in up to two-thirds of people diagnosed in childhood (Wender et al., 2001), and a recent meta-analysis based on strict diagnostic criteria estimated the prevalence of adult ADHD to be 2.5 % (Simon et al., 2009). Similarly, more cases are being described where ADHD is not diagnosed until adulthood. However, currently a diagnosis cannot be made unless symptoms can be traced back to childhood, which, combined with the strict diagnostic criteria for such a heterogeneous disorder, may lead to an underestimation of the prevalence in adults (Simon et al., 2009). In adulthood, symptoms are often less overt than in childhood, manifesting as inattentiveness and impulsivity rather than hyperactivity (Biederman et al., 2000). Although disruptive as a childhood disorder, adult ADHD can be equally debilitating. For example, the inability to concentrate in meetings at work, or even in day-to-day conversations, as well as impulsive traits leading to financial problems or drug abuse, can lead to a poor quality of life.

1.4.1.4. Neurology

Clinical research in ADHD has centred on frontostriatal brain networks, comprising the lateral prefrontal cortex, dorsal anterior cingulate cortex and striatum. This network has been shown to control behaviours in which a dysfunction makes up the core symptoms of ADHD, including impulse suppression, distraction inhibition, and cued switching between tasks (Vaidya & Stollstorff, 2008). Neuroimaging studies consistently show that children with ADHD have smaller volumes in the dorsolateral prefrontal cortex, caudate nucleus and corpus callosum compared to control subjects (Seidman et al., 2005).

Abnormal noradrenaline and dopamine transmission in these regions is thought to be involved in the pathophysiology of ADHD. The dopamine hypothesis of ADHD is well established, although while dopamine was previously believed to be central to the pathogenesis of ADHD, it is now accepted that such a multifaceted disorder involves more than a single neurotransmitter system. Among the evidence for dopamine's involvement in the disorder is the fact that psychostimulants such as amphetamine and methylphenidate, the most common first-line treatments for ADHD (Wilens, 2003), enhance extracellular dopamine, suggesting that an underlying dopamine deficit is corrected by these drugs. Impairment in dopaminergic receptor function has been hypothesised to underlie ADHD; the dopamine D1 receptor subtype is implicated in the regulation of motor and cognitive processes (Arnsten & Li, 2005; Granon et al., 2000; Heijtz et al., 2007) and polymorphisms in the D4 receptor have been associated with ADHD (Langley et al., 2004). A particularly long-standing theory is that striatal dopamine transporters (DAT) are overexpressed or overactive in people with ADHD, given that they are blocked by psychostimulants, although studies looking at DAT binding and expression in people with ADHD have yielded inconsistent results (Spencer et al., 2007; Spencer et al., 2005; Volkow et al., 2007a).

A number of models of ADHD indicate that an inappropriately increased release of noradrenaline at cortical terminal fields is caused by hypofunctional α_2-autoreceptors (Russell et al., 2005). In addition, the locus coeruleus noradrenergic (LC-NA) system is thought to be involved in the control of attention via tonic and phasic activity of LC-NA neurones (Aston-Jones & Cohen, 2005). When attentional performance in a task is optimal, LC-NA neurones exhibit low baseline (tonic) activity and are activated phasically and selectively following task-relevant stimuli, while a high baseline activation is associated with poor task performance and

a high number of false-alarm errors (inattentiveness and impulsivity) (Aston-Jones & Cohen, 2005).

While the dopaminergic theory of ADHD still predominates in research into the disorder, the cholinergic system is becoming increasingly recognised as a potential target for therapeutic intervention of ADHD. A higher rate of cigarette smoking is found in adolescents and adults with ADHD than in the general population, with 42 % of patients with ADHD currently smoking compared with 28.1 % in the general population (Pomerleau et al., 1995). Observations such as this prompted investigation into cholinergic agents as a treatment for ADHD. Wilens et al (1999) reported that a three-week treatment with ABT-418, a nicotinic agonist, reduced ADHD symptoms in 40 % of patients, compared with an improvement of 13 % in those who received placebo. Similarly, acute nicotine administration has been shown to improve performance on a number of cognitive domains including behavioural inhibition, delay aversion and recognition memory (Potter & Newhouse, 2008), indicating that central cholinergic systems are involved in the aetiology of the disorder.

1.4.1.5. Comorbidity with substance abuse

In addition to cigarette smoking, it is well documented that ADHD is highly comorbid with substance abuse (reviewed in Wilson & Levin, 2005), including alcoholism, with up to 50 % of adults with persistent ADHD having comorbid substance abuse disorders, and similarly, 25 % of people in substance abuse treatment centres also having ADHD (Wilson & Levin, 2005). The cause of this association has long been the subject of considerable debate, as it is unknown as yet whether the widespread treatment of ADHD with psychostimulants, particularly in childhood or adolescence, affects the risk of developing later substance use disorders (Elkins et al., 2007). There is ongoing debate as to whether psychostimulant treatment of ADHD is a cause of the high comorbidity with substance abuse, or whether the treatment prevents later drug abuse. This issue is discussed further in Chapter 5. ADHD is also comorbid with conduct disorder and affective disorders.

1.4.1.6. Controversy

The diagnosis of any psychiatric disorder is open to controversy, due to a lack of biomarkers and the resulting subjective process of diagnosis. The heterogeneous nature of ADHD in particular has attracted widespread media coverage over the last two decades, and as public awareness of the disorder grows, so do debates over the accuracy of diagnosis and necessity

of pharmacological treatment (Parens & Johnston, 2009; Wender, 2002). Treatment with stimulants is increasing rapidly in North America and across Europe; in Germany there has been a 47-fold increase in the use of stimulant medication for ADHD in the last decade (Parens & Johnston, 2009). Diagnosis rates vary between countries depending on the diagnostic criteria used, for example, the International Classification of Diseases (ICD-10), used across Europe, lists more stringent criteria than the Diagnostic and Statistical Manual of mental disorders (DSM-IV), used in the USA, resulting in a higher reported prevalence of the disorder in the USA than in Europe. Indeed, a study of 759 children diagnosed with ADHD using the DSM-IV criteria found that 75 % of these children would not have been diagnosed using ICD-10 criteria (Santosh et al., 2005). Such discrepancies indicate that considerable further research is required.

1.4.2. Currently used animal models of ADHD

A good animal model of ADHD must meet three validation criteria: it must represent the behavioural characteristics of the human disorder (face validity); conform to a theoretical rationale for the pathophysiology of the disorder (construct validity); and be able to predict unknown aspects of the disorder, relating to behaviour, genetics, neurobiology or treatment (predictive validity) (Sagvolden et al., 2005b). With a disorder such as ADHD, while face validity (such as hyperactivity) and predictive validity (such as response to psychostimulants) can be tested relatively easily, the construct validity of a model is difficult to assess, as the aetiology of ADHD is as yet unknown and is the subject of debate. A wealth of models, both genetic and pharmacological, have been proposed for ADHD. Many of the models have distinctly separate neurological defects yet all result in some of the behavioural characteristics of the disorder, highlighting the heterogeneity both of the symptoms and of the possible neurological aetiology of the disorder. Below is an overview of two of the most widely used models.

1.4.2.1. Dopamine transporter (DAT) knock-out mouse

The DAT knock-out mouse model of ADHD is one of few that meets all three validity criteria. The model conforms to the hyperdopaminergic theory of ADHD, the animals are hyperactive, and their hyperactivity is reduced with both amphetamine and methylphenidate (reviewed in Gainetdinov, 2008). The disadvantage of this model is that while hyperactivity is reduced with psychostimulants, this is likely due to their action on other monoamine transporters, namely the noradrenaline and the serotonin transporters, as the principle site of action for these drugs

(i.e. the DAT) has been disrupted. Serotonin reuptake inhibitors, however, are of no therapeutic value to people with ADHD, and can in fact stimulate their motor activity (Gainetdinov & Caron, 2001; Sagvolden et al., 2005b).

1.4.2.2. Spontaneously hypertensive rat

The spontaneously hypertensive rat (SHR) is one of the most commonly used models for ADHD. Created in the 1960s (Okamoto & Aoki, 1963) from the Wistar-Kyoto rat (WKY), its behavioural phenotype has been extensively characterised, and mirrors several aspects of ADHD. As well as being hyperactive and impulsive, the SHR is deficient in tests of sustained attention, and has response re-engagement deficits, comparable to children tested with a similar behavioural schedule (Sagvolden, 2000). The DAT is also hypofunctional in the SHR. However a disadvantage of this model is that reports of its behavioural response to psychostimulants are mixed. While a reduction in hyperactivity with psychostimulants in SHR has been reported (Ueno et al., 2002), this was in a stroke-prone sub-strain, and other reports have in fact demonstrated an increase in locomotor activity with both methylphenidate and amphetamine (Van Den Buuse & De Jong, 1989; Wultz et al., 1990), bringing into question its predictive validity.

1.4.2.3. Other models

The table below gives an overview of some of the other animal models currently used to study ADHD, and summarises the degree to which each satisfies the validity criteria for animal models. The $NK1R^{-/-}$ mouse is included in this table and is described further in the subsequent sections.

Model	Face validity			Construct	Predictive	
	Hyper-activity	Attention deficits	Impulsivity	Dopamine hypothesis	AMPH	MPH
DAT knock-out mouse	✓	NT	✓	✓	✓	✓
DAT knock-down mouse	✓	NT	✓	✓	✓	NT
Spontaneously hypertensive rat	✓	✓	✓	✓	✓	✗
Neonatal DA lesioning	✓	✓	NT	✓	✓	✓
Neonatal hypoxia	✓	NT	NT	✗	✓	NT
Prenatal BrdU	✓	NT	NT	NT	NT	✗
Cerebellar stunting in rats	✓	NT	NT	NT	✗	✗
Hippocampal irradiation	✓	✓	NT	NT	✓	NT
Naples high excitability rat	✓	✓	NT	✓	NT	NT
Hyperactive wheel-running mouse	✓	NT	NT	NT	NT	✓
Coloboma mouse	✓	NT	NT	NT	✓	✗
NK1R$^{-/-}$ mouse	✓	Testing	✓	✓	✓	✓

Table 1.2 ADHD models.

Overview of currently used animal models of ADHD and their compliance with the three sets of validity criteria. AMPH, amphetamine; BrdU, Bromodeoxyuridine; DAT, dopamine transporter; MPH, methylphenidate; NT, not tested. Adapted from van der Kooij & Glennon (2007).

1.4.3. NK1R$^{-/-}$ mice as a model for ADHD

The distinctive behavioural characteristics of the NK1R$^{-/-}$ mouse have led to it being put forward as a model for ADHD (Yan et al., 2009). Meeting all three validity criteria in multiple ways, it is an exciting development and promises to be valuable in the study of this disorder.

1.4.3.1. Face validity

Hyperactivity

During initial experiments with NK1R$^{-/-}$ mice as a model to study affective disorders, it was noted that NK1R$^{-/-}$ mice are hyperactive, particularly in the light (novel) zone of a light-dark exploration box (Fisher et al., 2007; Herpfer et al., 2005). This has since been supported by experimental data in wild-type mice administered either of the NK1 receptor antagonists

RP67580 or L733060, while the antagonists have no effect on the locomotor activity of NK1R$^{-/-}$ mice (Yan et al., 2010).

Impulsivity

NK1R$^{-/-}$ mice return to the light zone of a light dark exploration box more readily than wild-type mice, and transition between the light and dark zones more frequently than wild-type mice, which could indicate increased impulsivity (Fisher et al., 2007). However, the light-dark exploration box is a widely used measure of anxiety-like behaviours (Crawley, 1985) and increased light-dark zone shuttling could indicate reduced anxiety in NK1R$^{-/-}$ mice, in agreement with previous pharmacological and genetic studies (see sections 1.1.5.1 and 1.3.1.2). Similarly, it could simply be a factor of the hyperactivity observed in NK1R$^{-/-}$ mice. Furthermore, there are a number of varieties of impulsivity which lead to different kinds of impulsive behaviour, such as motor or cognitive impulsivity, response inhibition deficits or increased behavioural switching (Evenden, 1999). People with ADHD often exhibit many of these impulsive behaviours, and increased transitioning between the light and dark zones of the exploration box only reflects a subset of these impulsitivies. Work is ongoing to determine whether NK1R$^{-/-}$ mice exhibit impulsivity or attention deficits in the advanced stages of the 5-choice serial reaction time task (5-CSRTT).

1.4.3.2. Construct validity

Dopamine

While basal dopamine efflux is similar in NK1R$^{-/-}$ and wild-type mice, the increase in striatal dopamine efflux normally seen in response to amphetamine is abolished in NK1R$^{-/-}$ mice. This is consistent with findings from a positron emission tomography (PET) study in adults with untreated ADHD, which demonstrated that patients with ADHD exhibited a blunted caudate dopamine response to methylphenidate compared with controls (Volkow et al., 2007b).

Noradrenaline

Basal noradrenergic efflux in the prefrontal cortex of NK1R$^{-/-}$ mice is augmented compared with wild-types, and locus coeruleus α_2-adrenoceptors are correspondingly hypofunctional. The “adaptive gain” theory (Aston-Jones & Cohen, 2005) proposes that an excess of tonic activity (consistent with that seen in NK1R$^{-/-}$ mice) and a deficit in task-related phasic firing of noradrenaline cells in the locus coeruleus (yet to be examined in NK1R$^{-/-}$ mice) provides a

complex and specific role for noradrenaline in the control of certain behaviours, including those related to ADHD.

1.4.3.3. Predictive validity

Paradoxical response to amphetamine

When NK1R$^{-/-}$ mice receive the psychostimulants amphetamine or methylphenidate, a paradoxical reduction in activity is observed, returning the locomotor activity level to that of saline-treated wild-type mice (Figure 1.5). Similarly, the activity of wild-type mice treated with RP 67580 or L-733060 is also reduced with amphetamine (Yan et al., 2010).

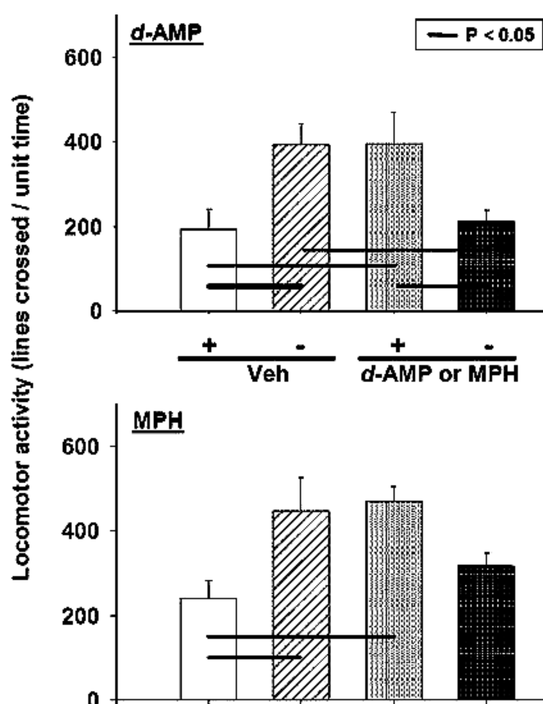

Figure 1.5 Previously observed effects of amphetamine and methylphenidate on locomotor activity of NK1R$^{-/-}$ mice.

Locomotor activity of NK1R$^{-/-}$ mice in the bright zone of the light-dark exploration box, demonstrating their hyperactivity and paradoxical response to amphetamine (d-AMP) or methylphenidate (MPH), compared with wild-type mice (reproduced from Yan et al., 2010).

Prediction of a genetic association

The similarities observed between NK1R$^{-/-}$ mice and the human symptoms of ADHD prompted a genetic linkage investigation on the human *TACR1* gene in ADHD patients. In a case-control study of 450 people with ADHD and 600 screened controls, four single nucleotide polymorphisms at the *TACR1* gene were found to be strongly associated with ADHD (rs3771829, p=0.0006; rs3771833, p=0.00008; rs3771856, p=0.0106 and rs1701137, p=0.0089).

1.5. Principal brain regions examined in this thesis

1.5.1. Striatum

The striatum is a key processing region of the mammalian brain, integrating diverse signals relating to motor control, emotion and cognition. It is the largest component of the basal ganglia, a group of nuclei that also contains the globus pallidus, subthalamic nucleus and substantia nigra (pars compacta and pars reticulata). Despite the variety of functions mediated by the striatum, morphologically it is a large, homogeneous structure, with the only visible boundaries being those that define the mosaic of patches, or striosomes, inside the extrastriosomal matrix. Although no other visible boundaries exist within the striatum, a number of distinct sub-regions can be distinguished functionally, the most recognised of which are the dorsal striatum (caudate putamen) and ventral striatum (nucleus accumbens shell and core). The research presented in this thesis focuses principally on the dorsal striatum, as it is in this region that the lack of dopamine response to amphetamine was observed in NK1R$^{-/-}$ mice (Yan et al., 2010). The next section will provide an overview of the neuroanatomical and functional organisation of the striatum.

1.5.1.1. Organisation of the striatum

Projections

The striatum receives excitatory glutamatergic input from virtually all parts of the cerebral cortex and thalamic nuclei, in addition to dopaminergic and serotonergic inputs from the substantia nigra pars compacta (SNc) and DRN respectively. The major output nuclei of the basal ganglia are the internal segment of the globus pallidus (in rodents this is known as the entopeduncular nucleus, EP) and the substantia nigra pars reticulata (SNr), which provide inhibitory GABAergic projections to the thalamus and brainstem.

Striatal projection cells are medium sized spiny neurones (MSNs), which release GABA and constitute the vast majority of cells in the striatum. Approximately half of these co-release substance P, principally express the D1 dopamine receptor and constitute the "direct" (excitatory) pathway, connecting the striatum monosynaptically to the output nuclei, thus disinhibiting targets in the thalamus and brainstem. The remaining MSNs of the "indirect" (inhibitory) pathway co-release enkephalin, principally express D2 receptors and inhibit the external segment of the globus pallidus (simply termed the globus pallidus in rodents, GP); this disinhibits the subthalamic nucleus, which provides stimulatory glutamatergic input to the output nuclei (EP/SNr), thus having a net inhibitory effect on the target nuclei outside the basal ganglia (Gerfen, 1992; Surmeier et al., 1996). This circuitry is illustrated schematically in Figure 1.6. While this model has been generally accepted for many years, increasing evidence portrays this as a simplified model, with anatomical studies demonstrating that virtually all striatal neurones contain both D1 and D2 receptors (Aizman et al., 2000) and indicating that in fact three different subtypes of MSN branch to the EP, to both the GP and SNr, or to the GP, EP and SNr via extensive axonal collateralisation (Wu et al., 2000).

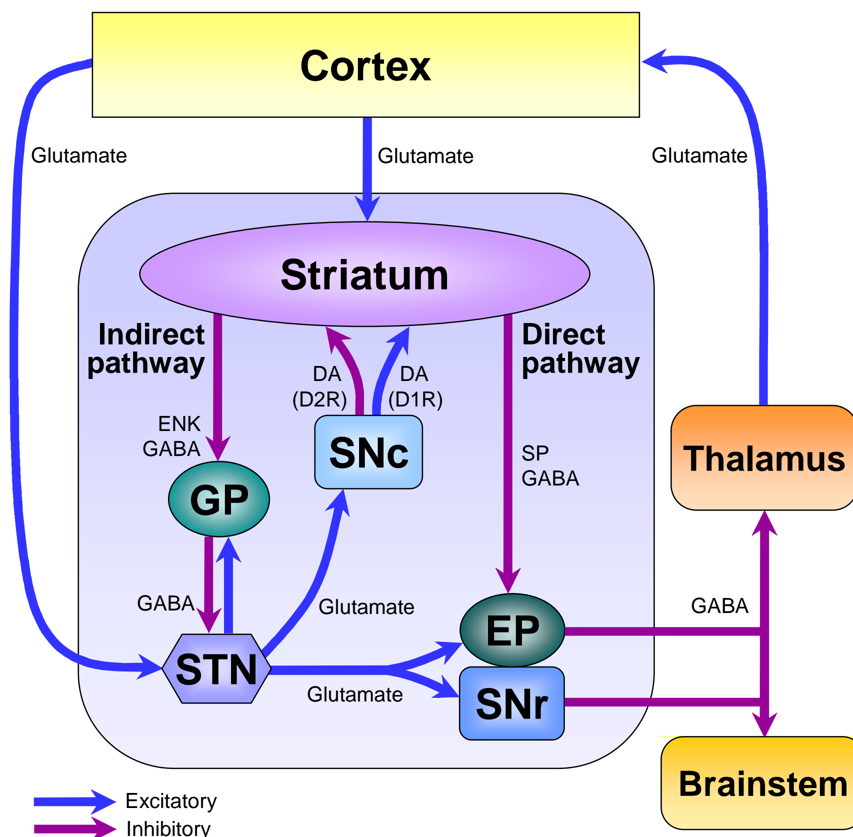

Figure 1.6 Basal ganglia circuitry.

Diagram of the classic model of basal ganglia circuitry, illustrating the direct and indirect medium spiny neurone (MSN) pathways. The striatum receives stimulatory glutamatergic input from the cortex, and its output is inhibitory (GABAergic). The major output nuclei of the basal ganglia are the EP and SNr. Upon stimulation of D1 receptors in the direct pathway, EP/SNr are inhibited, thus disinhibiting targets in the thalamus and brainstem. Conversely, the indirect pathway, thought to express mainly D2 receptors, has a net inhibitory effect on target nuclei, as inhibition of the GP leads to disinhibition of the STN, which excites EP/SNr and in turn inhibits targets in the thalamus and brainstem. ENK, enkephalin; EP, entopeduncular nucleus; GP, globus pallidus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus. Adapted from Lewis et al (2003).

Interneurones

Around 5 % of striatal neurones are interneurones, and although small in number, play a significant role in modulating the activity of MSNs. Interneurones fall into two classes, GABAergic interneurones (of which there are three subtypes: (1) calretinin-containing; (2) parvalbumin-containing; and (3) somatostatin-, neuropeptide Y- and neuronal nitric oxide synthase-containing) and cholinergic interneurones. While the role of the GABAergic interneurones is to provide strong inhibition of MSNs, the cholinergic interneurones modulate the activity of MSNs, particularly in response to saliency or reward (Tepper & Bolam, 2004).

Cholinergic interneurones

A powerful bidirectional association exists between acetylcholine and dopamine in the striatum. Cholinergic interneurones make up approximately 1 % of the total cells (Wilson, 2006) in the striatum. All express the NK1 receptor in rats (Gerfen, 1991) and in wild-type mice (C. Price, unpublished data), and NK1 receptor agonists stimulate striatal acetylcholine release (Anderson et al., 1993; Arenas et al., 1991). Despite being sparsely distributed, the widespread dendritic and axonal arborisations of the cholinergic interneurones enable them to exert a significant effect on dopaminergic control of the striatum, and consequently motor activity (Pisani et al., 2007; Wang et al., 2006). Cholinergic interneurones are tonically active, and their intrastriatal targets include terminals of dopaminergic and glutamatergic neurones in addition to MSNs; overall, they act to increase MSN excitability and responsiveness to glutamatergic signals (Surmeier et al., 2007). Dopamine acting at D1 receptors has an excitatory effect on cholinergic interneurones, promoting release of acetylcholine (Acquas & Di Chiara, 1999). Conversely, a predominantly inhibitory effect on striatal cholinergic neurones is exerted via D2 receptors, which inhibit acetylcholine release by reducing tonic firing of the interneurones and by inhibiting entry of Ca^{2+} required for exocytosis (DeBoer et al., 1996; Maurice et al., 2004).

1.5.2. Nucleus basalis

The nucleus basalis magnocellularis (or nucleus basalis of Meynert in humans) is a cholinergic region in the basal forebrain, and forms part of the magnocellular basal forebain cholinergic system with the substantia innominata and ventral pallidum, often considered together as one structure (Mesulam et al., 1983). It provides the major cholinergic input to the cerebral cortex and amydala, and is central to the modulation of attention and arousal processes (Baxter & Chiba, 1999). A substantial body of evidence has additionally implicated the nucleus basalis in

the mediation of reward; electrophysiological studies in primates have shown that the firing rate of nucleus basalis cells increases in response to rewarding stimuli (Richardson & DeLong, 1988), while microinjection of substance P into the nucleus basalis has been shown to produce conditioned place preference (CPP) (Hasenohrl et al., 1998a; Holzhauer-Oitzl et al., 1988) and promote learning (Hasenohrl et al., 2000), as well as increasing locomotor activity (McBride et al., 1999). The nucleus basalis is innervated by dopaminergic, noradrenergic and serotonergic cells, with all three systems influencing the release of acetylcholine in the cerebral cortex (Smiley et al., 1999). A number of previous studies have shown that the NK1 receptor is expressed on almost all cholinergic neurones of the basal forebrain in rats (Chen et al., 2001; Gerfen, 1991), suggesting that cholinergic neurones in the basal forebrain are directly modulated by tachykinins through the NK1 receptor. Together, this evidence points to the nucleus basalis as an important brain region for the study of attentional and locomotor disorders and of the substance P-NK1 receptor system.

1.6. Amphetamine

The drug used throughout the experiments described in this thesis is amphetamine. As well as being one of the most common first-line treatments for ADHD (Adderall), it is also a drug that is widely abused and highly addictive, with an estimated 16 to 51 million people consuming amphetamine-type stimulants worldwide, far more than those using cocaine and heroin (UNODC, 2009). Its mechanism of action is principally dopaminergic, however it can also directly affect noradrenergic and serotonergic systems. The mode of action of amphetamine depends on the dose administered. At low doses, it acts at the presynaptic terminal, blocking dopamine reuptake by binding to the DAT, thus prolonging the action of dopamine at the synapse, and it also causes increased release of dopamine from the terminal. This direct releasing effect is unique to amphetamine in the psychostimulant drug class, as cocaine and methylphenidate do not have this effect (Wall et al., 1995). At high doses amphetamine can also bind to monoamine oxidase within the dopaminergic terminal, preventing the breakdown of dopamine and resulting in an increased amount of free dopamine. The specific isomer of amphetamine used in the experiments presented here is the dextrorotary stereoisomer, d-amphetamine.

1.7. Aims of thesis

The experiments presented in this thesis aim to extend the investigation of the $NK1R^{-/-}$ mouse phenotype, in particular its responses to systemic administration of amphetamine, against the background discussed in this chapter. Specifically, while previous research has shown that $NK1R^{-/-}$ mice have altered neurochemical and behavioural responses to acute amphetamine, the response to repeated amphetamine has not been investigated in these animals. The research presented here investigates the behavioural and molecular responses of $NK1R^{-/-}$ mice to acute and repeated amphetamine. Results are discussed in the context of the $NK1R^{-/-}$ mouse as a model for ADHD, as well as considering their broader implications in relation to amphetamine as a drug of abuse.

2. General methods and materials

This chapter describes the methods and materials that are common to all chapters in this thesis. In addition, each subsequent chapter has a summary of general methods and details of any methods specific to that chapter.

2.1. Mice

Mice were aged between 6 and 9 weeks at the start of all experiments described in this thesis. All procedures were carried out in accordance with UK Home Office regulations.

2.1.1. NK1 receptor knock-out (NK1R$^{-/-}$) mice

NK1R$^{-/-}$ mice were created by De Felipe et al (1998). A 129/SvJ x C57BL/6J mouse line was generated by homologous recombination in embryonic stem cells, and subsequent mating. The 129/SvJ x C57BL/6J mouse line was then backcrossed once onto an outbred MF1 strain (Harlan OLAC, Bicester, UK) in order to reintroduce a mixed background, resulting in an animal with a genomic make-up of 25 % 129/SvJ, 25 % C57BL/6J and 50 % MF1. Targeted disruption of the NK1 receptor gene was carried out by the insertion of a cassette consisting of an internal ribosome entry site (IRES), to permit the initiation of translation in the middle of an mRNA sequence; the reporter gene *lacZ*, coding for beta-galactosidase; and a neomycin resistance gene for positive selection. The cassette was inserted at a unique Stu I restriction site, causing disruption of the gene at exon 1 (Figure 2.1).

2.1.2. Verification of genotype

The genotype of all animals in the experiments described in this thesis was verified using one of the following two methods.

2.1.2.1. Polymerase chain reaction (PCR)

PCR was used to verify the genotype of animals from tail-tip DNA. Where possible, the tissue was collected once the animal had been terminally anaesthetised. On awake animals, the tail tip was anaesthetised with ethyl chloride spray before a maximum of 5 mm was cut with a sharp scalpel blade. The tissue was lysed in 750 µl tail buffer (50 mM Tris, 100 mM EDTA, 100 mM NaCl and 1 % SDS in 18 MΩ/cm ultrapure water) and 0.6 mg/ml proteinase K (Sigma-Aldrich, Poole, Dorset, UK) in a water bath at 55 °C overnight. Three primers were used in the DNA amplification process: NK1-R, NK1-F and Neo-F. Full PCR protocol details are provided in the Appendix.

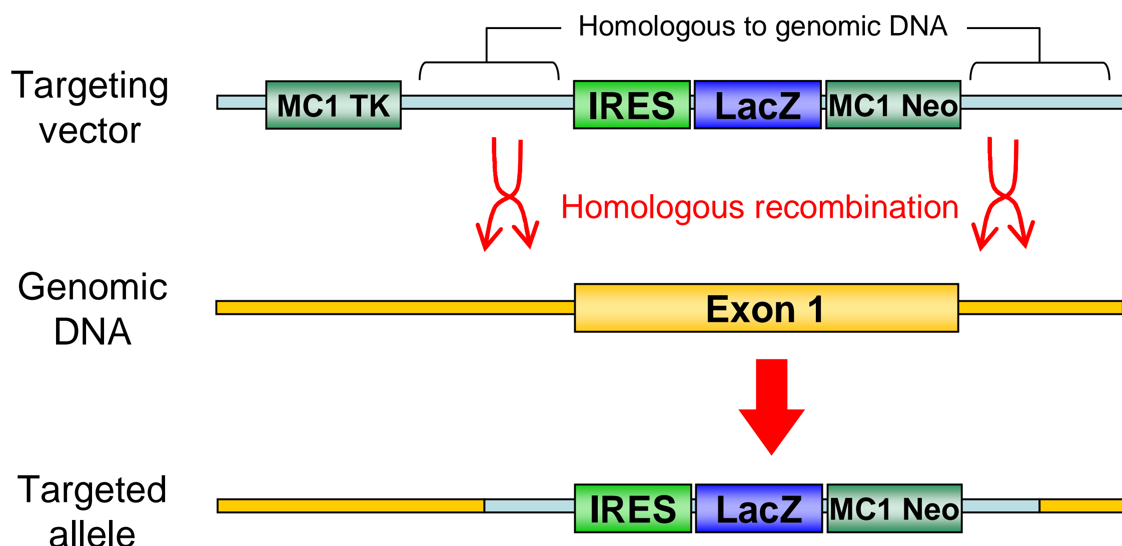

Figure 2.1 Targeted disruption of the NK1 receptor.

Diagram of the targeting (replacement) vector, the region of the wild-type NK1 receptor locus containing exon 1, and the predicted structure of the targeted NK1 receptor gene. IRES, internal ribosome entry site; lacZ, coding sequence for beta-galactosidase; MC1, promoter; Neo, neomycin resistance gene; TK, herpes simplex virus thymidine kinase gene. Adapted from De Felipe et al (1998) and McCutcheon (2006).

After amplification, 15 µl of each sample (as well as wild-type, $NK1R^{-/-}$ and UP H_2O controls) were loaded onto a 2 % Tris-borate-EDTA and agarose (TBE-agarose) gel, electrophoresed in TBE buffer (National Diagnostics, Hull, UK) with ethidium bromide (1 µl per ml 10 x TBE) and photographed on an ultraviolet transilluminator plate (UVP Ltd, Cambridge, UK). Genotypes of

the samples were determined by comparing against amplified DNA from known homozygous wild-type and NK1R$^{-/-}$ controls.

For homozygous wild-type animals, a single band is seen at 350 bases, corresponding to the section of exon 1 that includes the NK1 receptor gene and is amplified by the primers NK1-F and NK1-R. In homozygous NK1R$^{-/-}$ animals, a band is seen at 260 bases, corresponding to a section amplified by the primers NK1-R and Neo-F, which includes part of the inserted cassette. An example of a PCR gel is shown in Figure 2.2.

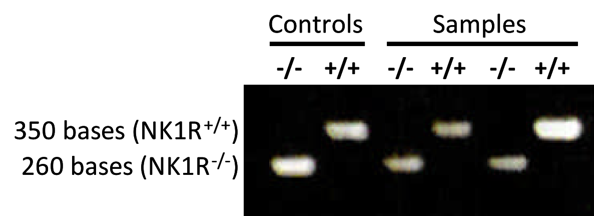

Figure 2.2 Genotyping of NK1R$^{-/-}$ and wild-type mice using the polymerase chain reaction (PCR).

Example of a developed PCR gel showing bands representing wild-type and NK1R$^{-/-}$ genotypes.

2.1.2.2. Immunohistochemistry

Where immunohistochemistry was carried out in brain sections to label specific antigens of interest, the NK1 receptor was also labelled concurrently in a separate set of sections, to confirm the genotype of the animals. Details of immunohistochemistry methods are given in Section 2.6. Where positive staining for NK1 receptors could be seen, this indicated that the tissue was from a wild-type mouse, while if no NK1 receptor staining was present, this confirmed the animal was NK1R$^{-/-}$. Figure 2.3 shows a typical image of the striatum from a wild-type and a NK1R$^{-/-}$ mouse.

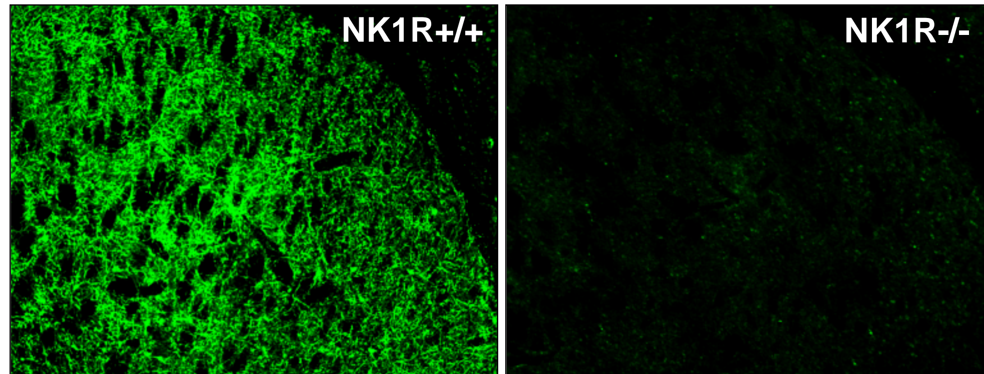

Figure 2.3 Genotyping of NK1R$^{-/-}$ and wild-type mice using immunohistochemistry.
Representative images of striatal sections from wild-type and NK1R$^{-/-}$ mice. The NK1 receptor is clearly labelled throughout the striatum in the wild-type but absent in the NK1R$^{-/-}$, providing confirmation of the genotype of the experimental animals.

2.1.3. Housing

Mice were housed in groups of 2 to 5 in the Biological Services Unit, UCL, London. The housing room was climate-controlled (temperature 19-23 °C, humidity 55 % ± 10 %) with a 12-hour light-dark cycle (lights on at 8 a.m.) and food and water freely available. Individually-housed mice were not used in experiments as prolonged periods of isolation may affect neurochemical and behavioural responses.

2.1.4. Colony maintenance

It is recognised that the method by which the animals were bred is a principal limitation throughout this thesis. Due to various constraints, all animals used in the experiments presented in this thesis were bred from separate homozygous NK1R$^{-/-}$ and wild-type colonies for more than 10 generations. Undesirably, keeping the colonies separate in this way allows for genetic drift and spontaneous mutations. For this reason the results in this thesis must be interpreted with caution as, while the effects observed may genuinely be the effects of the disruption of the NK1 receptor, it is also possible that the two colonies have developed phenotypic line differences over time that are unrelated to the disruption of the NK1 receptor. Ideally the wild-type and NK1R$^{-/-}$ mice used should be littermates, as behavioural differences in mouse colonies separated even for a single generation may rather reflect different levels of care provided to the offspring by mothers of different genotypes. In order to ensure reliable breeding, mating pairs were kept between two and eight months old and culled after their

sixth litter, and when new breeding pairs were required they were set up from the second or third litters from existing breeding pairs.

2.1.5. Culling

Mice were culled by CO_2 asphyxiation or injection of sodium pentobarbital (Euthatal, 200 mg/ml; Merial, Harlow, UK) followed by perfusion (Section 2.6.1). Where mice were culled by CO_2 asphyxiation, the animal was placed in a chamber and CO_2 flow was slowly increased until the mouse was unconscious. Cervical dislocation was performed to ensure euthanasia. Where animals were killed by pentobarbital injection, 0.2 ml of pentobarbital was injected intraperitoneally (i.p.) to induce deep anaesthesia, and perfusion was carried out.

2.2. Amphetamine

d-Amphetamine sulphate (Sigma, Poole, Dorset, UK) was dissolved in 0.9 % sterile sodium chloride and administered i.p. in a volume of 6.67 ml per kg body weight in all experiments. Doses are expressed as the salt. Control treatments consisted of an equivalent volume of saline.

2.3. Experimental design

Wild-type and NK1R$^{-/-}$ mice were allocated saline or amphetamine treatment groups, and assigned a pseudo-random order. The order assigned was particularly important where experiments had to be split into “sets” over a number of days/weeks (for example sensitisation, where a maximum of 8 animals could undertake the experiment at one time) so that each group would be represented by at least one animal in every set. All stages of experiments were carried out in the order assigned at the start of the experiment, including (where relevant) injections, behavioural tests, perfusion, immunohistochemistry and PCR. The exception was in the case of Western blots, where samples were loaded into the gel in order of treatment/genotype groups, rather than the pre-arranged order, for the purposes of presentation.

All behavioural experiments were performed between 11:00 and 15:00 h in order to avoid circadian fluctuations, and tracking was carried out by computer software. All cell counting was conducted blind to treatment or genotype.

2.4. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistics were carried out using PASW Statistics version 18 for Windows (SPSS Inc, Chicago, Illinois, USA). $P<0.05$ was considered statistically significant. Specific statistical methods for individual experiments are detailed in the relevant chapters.

2.5. Locomotor activity

Full methods for locomotor activity measurement are detailed in Chapter 5. Briefly, four locomotor activity experiments were carried out, one measuring the response to acute amphetamine or saline, and three recording the activity over several days of repeated amphetamine or saline injections to induce amphetamine sensitisation. Activity was measured in one of two sets of automated locomotor activity equipment, small rectangular chambers or circular corridors (Figure 2.4). Activity of naïve mice was measured at the start of each experiment, before the animals had received any injections. Locomotor activity following acute amphetamine or saline was assessed on the first day of each sensitisation experiment (i.e. the first time the animals had received an injection), as well as in the acute experiment. Following sensitisation experiments, animals were killed by perfusion or CO_2 asphyxiation, their brains removed and the tissue used for the immunohistochemistry or Western blot experiments described in Chapter 6.

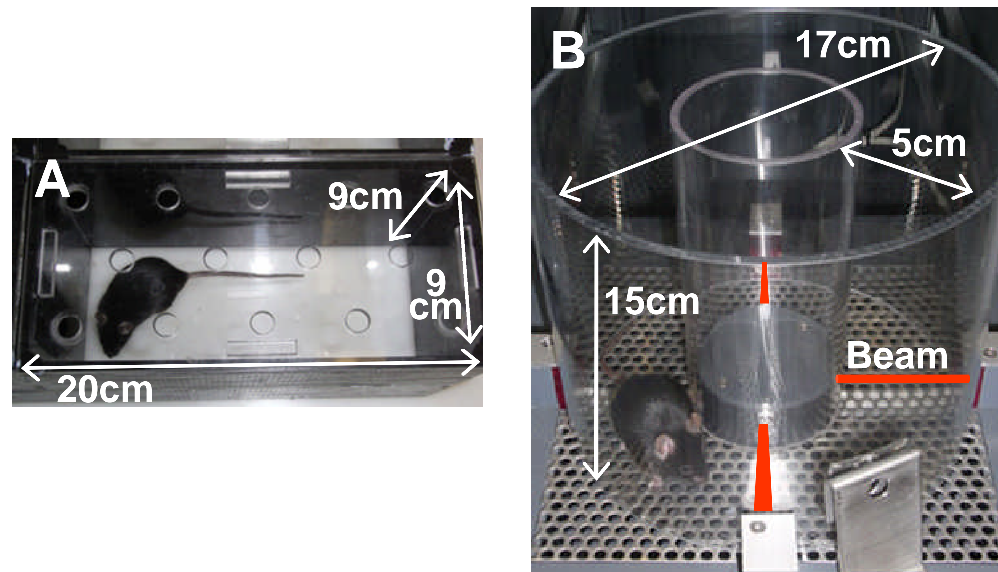

Figure 2.4 Locomotor activity chambers.

Individual activity chambers used to assess locomotor activity of mice. Full experimental details of locomotor activity experiments are described in Chapter 5.

2.6. Immunohistochemistry

Immunohistochemistry enables the visualisation of a protein of interest by exploiting the principle of antibodies binding specifically to antigens in a biological tissue. Antibodies raised against the protein of interest (the antigen) will bind specifically to the protein, and are labelled for identification, for example by conjugation with a fluorophore. The experiments presented in this thesis employ the avidin-biotin system, which utilises the strong non-covalent bond which forms between the proteins avidin and biotin. The avidin-biotin system enables a vast range of biotin-tagged (biotinylated) antibodies to be labelled with an avidin-conjugated fluorophore (such as FITC) or enzyme (such as peroxidase).

2.6.1. Tissue preparation

Appropriate preparation of the tissue is of paramount importance in immunohistochemistry. Fixation with paraformaldehyde forms cross-links between basic amino acid residues of proteins, thus stabilising the proteins and ensuring that the cell morphology and tissue architecture is preserved as far as possible.

2.6.1.1. Perfusion

Mice were terminally anaesthetised by an i.p. injection of 6.67 ml/kg of sodium pentobarbital (Euthatal, 200 mg/ml; Merial, Harlow, UK). Upon respiratory cessation, the animals were perfused intracardially with 2.5 ml ice-cold heparinised saline, followed by about 100 ml of ice-cold 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at a rate of 3.5 RPM (13.9 ml/min) using the Watson-Marlow 520S peristaltic pump (Watson-Marlow, Falmouth, UK).

2.6.1.2. Post-fixation and cryoprotection

Brains were removed and post-fixed in 4 % PFA at 4 °C for 2-4 h or overnight, depending on the immunohistochemistry protocol to be used. After post-fixing, the brains were cryoprotected in 30 % sucrose (in 0.1 M PB containing 0.02 % sodium azide, NaN_3) for at least 24 h, before 40 µm sections were cut with a freezing microtome.

2.6.1.3. Sectioning

Sections (40 µm) were cut coronally in serial sets of four (for the nucleus basalis), or six (for the striatum), so that every fourth or sixth section (i.e. sections 160 or 240 µm apart, respectively) was used for analysis. Cut sections were collected in 5 % sucrose (in 0.1 M PB containing 0.02 % NaN_3), and stored at 4 °C until use. Where phosphorylated antigens such as pERK or pMEF2 were to be labelled, sections were not cut until a maximum of one day before immunohistochemistry, to ensure freshness of tissue and good staining of the antigen. Before beginning the immunohistochemistry protocol, sections were rinsed once in 0.1 M PB to wash away sucrose and NaN_3.

All immunohistochemistry was performed on free-floating sections. All antibodies were prepared in Tris-Triton buffered saline (TTBS); washes consisted of three 10-minute rinses in 0.1 M PB, and all incubations were carried out on a rocking platform either at room temperature (RT) overnight, or at 4 °C for 3 days. Detailed immunohistochemistry protocols are described in the Appendix.

2.6.1.4. Blocking

In order to minimise non-specific binding in the immunohistochemical reaction, sections were incubated in blocking agent for 1 h at the start of the procedure. The blocking agent was made

up of 0.1 M PB containing 0.03 % Triton X-100 (BDH Laboratory Supplies, Poole, UK) and 0.03 % normal (non-immune) serum from the host species of the secondary antibody (Vector Labs, Burlingame, CA, USA). Sections were removed from the blocking solution, but not washed, before incubation with the primary antibody.

2.6.2. Antibodies

Primary antibodies used were as follows: rabbit anti-NK1R polyclonal antibody (Eurogentec, Southampton, Hampshire, UK); rat anti-dopamine transporter (DAT) N-terminus monoclonal antibody (Millipore, Watford, UK); rabbit anti-phospho-p44/42 (Thr202/Tyr204) MAP kinase (pERK1/2) monoclonal antibody (Cell Signaling Technology, Beverley, MA, USA); goat anti-ChAT polyclonal antibody (Chemicon); rabbit anti-parvalbumin antibody (Swant, Bellinzona, Switzerland); rabbit anti-pMEF2A (phospho-Ser408) polyclonal antibody (GenWay Biotech Inc, San Diego, CA, USA); rabbit anti-pCREB (phosphor-Ser133) polyclonal antibody (Millipore).

Secondary antibodies used were as follows: biotinylated anti-rabbit IgG, made in goat; biotinylated anti-rat IgG, made in goat; biotinylated anti-goat IgG, made in horse; and FITC avidin (all four from Vector Labs); Alexa-594-conjugated anti-goat IgG, made in chicken; and Alexa-594-conjugated anti-rabbit IgG, made in chicken (both from Molecular Probes, Invitrogen, Oregon).

The concentrations of the antibodies used, and the conditions (temperature and length of time) in which they are applied, are listed in Table 2.1 (single immunohistochemistry) and Table 2.2 (double immunohistochemistry).

2.6.3. Chromogenic immunohistochemistry (DAB)

Chromogenic staining uses 3,3'-diaminobenzidine tetrahydrochloride (DAB), which is oxidised to an insoluble brown polymer in the presence of the enzyme horseradish peroxidase (HRP). This can be used with the avidin-biotin system to visualise the HRP-avidin complexes around the primary antibody in the tissue.

To eliminate endogenous peroxidase activity, 0.02 % hydrogen peroxide (H_2O_2; VWR International Ltd., Leicestershire, UK) was added to the blocking agent at the start of the procedure. Following incubation in the primary antibody, sections were washed in PB, then incubated in biotinylated secondary antibody at 1:500 dilution, for 2 h at RT, and washed again. Avidin-biotin-peroxidase complex (ABC, Vectastain kit, Vector Labs) was made 30

minutes prior to addition to the sections, to allow the complex to form, by adding 1 µl Reagent A (avidin) and 1 µl Reagent B (biotinylated HRP) per ml TTBS. The sections were then incubated in ABC for 1 h, washed, and finally the labelled cells were visualised using the DAB Vectastain kit (Vector Labs).

Protocol	*Duration of post-fix*	*Primary antibody*		*Secondary antibody*	
		Antibody	*Dilutions & conditions*	*Antibody*	*Visualisation*
NK1R	Variable	Rabbit α-NK1R	1:1000 Overnight, RT	Biotinylated goat α-rabbit	FITC
DAT	2 h	Rat α-DAT	1:6000 3 days, 4°C	Biotinylated goat α-rat	FITC
pERK	2-4 h	Rabbit α-p44/42 MAP kinase	1:200 Overnight, RT	Biotinylated goat α-rabbit	DAB + Nickel
PV	3-4 h	Rabbit α-PV	1:5000 Overnight, RT	Biotinylated goat α-rabbit	FITC

Table 2.1 Antibodies used in single antigen labelling immunohistochemistry.

Summary table of antibodies used in single immunohistochemistry experiments described in this thesis. DAB, diaminobenzidine (chromogenic stain); DAT, dopamine transporter; FITC, fluorescein thiocyanate (fluorophore); pERK, phosphorylated extracellular-signal regulated kinase; PV, parvalbumin; RT, room temperature. NK1 receptor immunohistochemistry was used only to verify the genotype of the animals used in experiments, so the duration of post-fix varied depending on that required for the principal antibody of interest. All biotinylated secondary antibodies were used at 1:500 dilution, 2 h, RT; FITC was used at 1:200, 2 h, RT.

Protocol	Duration of post-fix	First stain (TSA)			Second stain (direct)	
		Primary antibody	Dilution & conditions	Secondary antibody	Primary antibody	Secondary antibody
NK1R & ChAT	Overnight	Rabbit α-NK1R	1:10,000 24 h, RT	Biotinylated goat α-rabbit + TSA	Goat α-ChAT 1:100 overnight	Alexa594-conjugated chicken α-goat
pERK & ChAT	2-4 h	Rabbit α-p44/42 MAP kinase	1:200 Overnight, RT	Biotinylated donkey α-rabbit + TSA	Goat α-ChAT 1:100 overnight	Alexa594-conjugated chicken α-goat
pMEF2 & ChAT	3-4 h	Rabbit α-pMEF2	1:1000 3 d, 4 °C	Biotinylated goat α-rabbit + TSA	Goat α-ChAT 1:100 overnight	Alexa594-conjugated chicken α-goat
pCREB & ChAT	3-4 h	Rabbit α-pCREB	1:1000 3 d, 4 °C	Biotinylated goat α-rabbit + TSA	Goat α-ChAT 1:100 overnight	Alexa594-conjugated chicken α-goat

Table 2.2 Antibodies used in double antigen labelling immunohistochemistry.
Summary table of antibodies used in single immunohistochemistry experiments described in this thesis. pMEF2, phosphorylated myocyte-enhancer factor 2; pCREB, phosphorylated cAMP-response element (CRE)-binding protein; TSA, tyramide signal amplification used; other abbreviations as in Table 2.1. All biotinylated secondary antibodies were used at 1:400 dilution, 90 min, RT; followed by TSA reagent, 1:75, 7 min, RT; followed by FITC, 1:600, 2 h, RT. All Alexa594-conjugated antibodies were used at 1:500, 2 h, RT.

2.6.4. Fluorescent immunohistochemistry

Fluorescent immunohistochemistry involves the use of fluorophore labelling. In the experiments presented in this thesis, either FITC-conjugated avidin was applied following incubation of the tissue with primary then biotinylated secondary antibody; or a fluorophore-conjugated secondary antibody was used, Alexa594-conjugated chicken anti-goat IgG.

2.6.4.1. Single antigen labelling

In single staining experiments, following incubation with primary antibody, the sections were washed and placed in biotinylated secondary antibody at 1:500 dilution for 2 h at RT. Following another wash, the sections were then incubated with FITC-avidin at 1:200 dilution for 2 h at RT in the dark (covered with aluminium foil). Details of the antibodies are provided in Table 2.1, and complete protocols are provided in the Appendix.

2.6.4.2. Double antigen labelling

Tyramide signal amplification (TSA; PerkinElmer, MA, USA) was employed in double-staining protocols, where two antigens were labelled in the same tissue. Tyramide is a phenolic compound that is activated by HRP and covalently binds to electron-rich regions of immediately adjacent proteins, thus providing considerable amplification of the signal.

First stain

Sections were incubated in the first primary antibody (see Table 2.2 for details), washed, and placed in biotinylated antibody at RT for 90 min at 1:400 dilution. ABC was made up as described in Section 2.6.3 above, except at a higher concentration of 4 µl each of Reagent A and Reagent B per ml TTBS. After washing, sections were placed in ABC for 30 min, washed again, and placed in biotinylated tyramide solution, 13.3 µl tyramide per ml diluent (PerkinElmer), for 7 min. Finally, the sections were washed again before being incubated in FITC-conjugated avidin, 1:600 for 2 h at RT in the dark.

Second stain

The sections were washed after the final incubation of the first stain, and the second primary antibody was applied (Table 2.2). After washing again, the sections were placed directly in a fluorophore-conjugated secondary antibody (Table 2.2) at 1:500 dilution, for 2 h at RT in the dark.

2.6.5. Mounting

All sections were rinsed in 0.01 M PB, mounted onto gelatine-subbed slides and allowed to air-dry. DAB stained sections were dehydrated through a series of alcohols prior to coverslipping with DPX mounting medium (BDH Laboratory Supplies, Poole, UK). Fluorescence-stained sections were coverslipped with Fluoromount aqueous mounting medium (Sigma-Aldrich, Poole, UK).

2.6.6. Quantification and statistical analysis

Sections were viewed using a Leica DMR microscope (Leica Imaging Systems Ltd., Cambridge, UK) with a x 20 objective. Cells expressing immunoreactivity of the antibody of interest in the relevant brain region were quantified in at least two sections per animal. The brain region was defined according to the mouse brain atlas (Franklin & Paxinos, 1997). From these counts, a mean was taken for that animal (total number of cells counted, divided by number of sections analysed), and this mean was used for analysis. Specific quantification methods varied depending on the antibody and brain region analysed, so for clarity, comprehensive details regarding quantification and statistical analysis are provided within the relevant chapters.

2.6.7. Imaging

2.6.7.1. Image capture

A Leica DMR microscope equipped with a CCD camera (C4742-95; Hamamatsu Photonics, Hamamatsu, Japan) was used to photograph the images presented in this thesis. The camera was connected to a Macintosh PowerPC G3 computer running OpenLab software (PerkinElmer) to capture the images.

2.6.7.2. Photoshop

Post-acquisition processing was carried out in Photoshop 7.0. Red and green channels were processed separately, changing only the curve of each channel to adjust the tonal range of the image for clarity and presentation purposes.

2.7. Western blotting

Protein immunoblotting, or Western blotting, involves separation of proteins by their molecular weight using gel electrophoresis, and transfer of the proteins onto a membrane, where they can be detected using antibodies specific to the protein of interest.

2.7.1. Tissue preparation

2.7.1.1. Dissection

Western blots were carried out on striatal tissue from animals that had undergone the sensitisation protocol, to examine changes in protein expression following sensitisation. Four to six hours after the injection on the final day of the protocol, the animals were killed, their brains taken and snap-frozen in liquid nitrogen, and the striatum from both sides dissected using a tissue-punch. Frozen tissue was stored at -80 °C until use.

2.7.1.2. Homogenisation

It was important to keep the tissue on ice (or in a refrigerated homogeniser or centrifuge) throughout the preparation process to avoid protein denaturing. Protease Inhibitor Cocktail (Sigma-Aldrich; containing 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-64, leupeptin and pepstatin A) and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich; containing microcystin LR, cantharidin, (−)-p-bromotetramisole, sodium vanadate, sodium molybdate, sodium tartrate and imidazole) were added to RIPA buffer (containing 1 % NP-40, 20 mM HEPES pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM Na_3VO_4, 5 mM ETDA). Ten µl of each cocktail were added per 1 ml of buffer, and 200 µl of this mixture added to each sample of striatum. Samples were homogenised in a FastPrep Biopulverizer (MP Biomedicals Europe, Illkirch, France) and incubated on ice for 1-2 hours to allow further breakdown of the tissue. Following homogenisation, samples were centrifuged for 15 min at 12,000 RPM and 4 °C, and the supernatant kept.

2.7.1.3. Protein determination

Protein measurement was carried out using the bicinchoninic acid (BCA) kit for protein determination (Sigma-Aldrich). Bicinchoninic acid and 4 % copper sulphate ($CuSO_4.5H_2O$) solution were mixed in a 50:1 ratio, and 200 µl of the mixture were added to protein standards of known concentration (constituting a standard curve) and samples in a 96-well plate. This

was incubated for 30 min at 37 °C, and a colorimeter was used to determine the concentration of protein in the samples.

2.7.2. Protein separation and transfer

Samples were boiled for 5 min to denature the proteins, and cooled on ice before loading into a 12 % gel (Bio-Rad Criterion). Samples containing 20 μg protein were loaded into the gel, and the proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), before being transferred to a PVDF membrane for immunoblotting. Where it was necessary to use more than one gel to accommodate all the samples, a control (wild-type saline) sample was loaded onto each gel to normalise between gels.

2.7.3. Immunoblotting

All immunoblotting was carried out in tubes which were gently rolled throughout the incubation to ensure even coverage of the membrane with antibody, and all washes consisted of 6 x 5 min rinses in 0.1 M phosphate-buffered saline (PBS) containing 0.1 % Tween 20 (PBS/Tween).

2.7.3.1. Antibodies

All antibodies were made up in 4 % skimmed milk, at dilutions specified in Table 2.3. Primary antibodies used were: goat anti-ChAT polyclonal antibody (Millipore); rabbit anti-MEF2A polyclonal antibody and rabbit anti-pMEF2A (phospho-Ser408) polyclonal antibody (both from GenWay); mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Secondary antibodies were HRP-conjugated rabbit anti-goat IgG, HRP-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse (all from Santa Cruz).

2.7.3.2. Immunoblotting for protein of interest

The membrane was blocked for 1 h at RT in 4 % skimmed milk in PBS/Tween, incubated with primary antibody overnight at 4 °C, and washed before being incubated with HRP-conjugated secondary antibody for 45 min at RT. After this, the membrane was washed again, and finally rinsed in PBS (without Tween) prior to developing with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) and photographing using the ChemiDoc Imaging system and Quantity One software (Bio-Rad Laboratories).

2.7.3.3. Normalising with GAPDH

All proteins of interest were normalised against the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in each sample. Following immunoblotting for the protein of interest, the membrane was rinsed in PBS/Tween, incubated with mouse anti-GAPDH, 1:2000, for 90 min at RT, and washed again before being incubated in HRP-conjugated anti-mouse for 45 min at RT. As above, the membrane was rinsed with PBS without Tween before developing and photographing.

Protocol	Primary antibody		Secondary antibody
	Antibody	Dilutions & conditions	
ChAT	Goat α-ChAT	1:100 Overnight, 4°C	α-goat HRP
MEF2	Rabbit α-MEF2A	1:500 Overnight, 4°C	α-rabbit HRP
pMEF2	Rabbit α-pMEF2	1:1000 Overnight, 4°C	α-rabbit HRP
GAPDH	Mouse α-GAPDH	1:2000 90 min, RT	α-mouse HRP

Table 2.3 Antibodies used in Western blots.

Summary table of antibodies used in the Western blot experiments described in this thesis. ChAT, choline acetyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; MEF2, myocyte enhancer factor 2; pMEF2, phosphorylated MEF2; RT, room temperature. All HRP-conjugated secondary antibodies were used at 1:2000 dilution for 45 min at RT.

2.7.4. Analysis

Photographed membranes were quantified by band densitometry analysis, using Image J software (NIH). Two-way ANOVAs were performed on the data, with genotype and drug treatment as between-subject variables.

CHAPTER THREE

3. Basal expression of striatal dopamine transporter

3.1. Introduction

Recent work in our lab has demonstrated that $NK1R^{-/-}$ mice have a profoundly diminished striatal dopamine response to amphetamine, compared to the increase in dopamine efflux characteristically seen in wild-type mice and other species after administration of the drug (Yan et al., 2009; 2010). This brief chapter describes a simple immunohistochemistry experiment carried out to determine whether the level of striatal dopamine transporter (DAT), the principal regulator of the amplitude and duration of action of dopamine at the synapse, is altered in $NK1R^{-/-}$ mice compared with wild-types.

3.1.1. The dopamine transporter

3.1.1.1. <u>Control of dopamine activity</u>

The DAT is a presynaptic membrane-spanning protein which controls the amplitude and duration of dopamine by terminating its presence at the synapse, by binding dopamine in the synaptic cleft and returning it to the neuronal terminal (Chen & Reith, 2000; Giros & Caron, 1993). It belongs to the SLC6 family of Na^+/Cl^--dependent transporters, which also include the noradrenaline transporter and the serotonin transporter. The DAT is located presynaptically at dopaminergic terminals, and is one of the main sites of action for amphetamine and other psychostimulant drugs. Amphetamine elevates extracellular levels of dopamine via the DAT in two ways. It promotes efflux of dopamine from the cell by causing the transporter to act in reverse, thus elevating the amount of dopamine in the synapse (Jones et al., 1998); and it also

regulates the expression of DAT at the cell surface by stimulating endocytosis of the transporter (Robertson et al., 2009). The DAT has as yet not been investigated in $NK1R^{-/-}$ mice despite their altered dopaminergic response to amphetamine.

3.1.1.2. The dopamine transporter and ADHD

DAT is implicated in the pathogenesis of ADHD. The DAT knock-out ($DAT^{-/-}$) mouse is an established model of ADHD, whose hyperactivity arises from the excess of striatal dopamine resulting from the lack of presynaptic dopamine reuptake. In addition, a lower level of striatal DAT mRNA has been documented in juvenile spontaneously hypertensive rats (SHR; another widely used model of ADHD) (Leo et al., 2003). In humans, the gene coding for DAT is *SLC6A3*, and polymorphisms in this gene have been associated with ADHD (Friedel et al., 2007), as well as with the response to the stimulant effects of amphetamine in healthy volunteers (Hamidovic et al.). Furthermore, mice lacking DAT show a reduction in striatal dopamine response following amphetamine (Giros et al., 1996), similarly to that previously observed in $NK1R^{-/-}$ mice (Yan et al., 2010). Given the above, and that DAT is the principal site of action of amphetamine, it was hypothesised that $NK1R^{-/-}$ mice would have reduced striatal expression of DAT. Expression of the transporter in the dorsal striatum was examined in a simple experiment using immunohistochemistry in naïve wild-type and $NK1R^{-/-}$ mice.

3.2. Methods

3.2.1. Tissue preparation and immunohistochemistry

Brains were taken from three naïve $NK1R^{-/-}$ and three naïve wild-type mice killed by perfusion. Forty µm sections were cut in serial sets of six, so that every sixth section (240 µm apart) was used for analysis. Bilateral striatal sections (8-12 per animal) were selected and stained for DAT protein expression using FITC as a fluorescent marker. Full details of immunohistochemistry methods are provided in Chapter 2.

3.2.2. Quantification

DAT expression was quantified by pixel luminosity measurements, measured using MRC600 software via a confocal scanning laser (Bio-Rad 600, Bio-Rad, Hertfordshire, UK) attached to a

Nikon microscope with a x 10 objective. Background luminosity of each section was measured and controlled for by subtracting the background reading (from the cortex where there is little DAT expression) from the luminosity reading of the dorsal striatum. The area in which DAT was quantified is illustrated in Figure 3.1B.

3.3. Results

3.3.1. Dopamine transporter expression is normal in NK1R$^{-/-}$ mice

No difference was found between NK1R$^{-/-}$ and wild-type animals in striatal expression of DAT protein (Figure 3.1), with pixel luminosity measurements being 48.2 ± 10.5 and 52.5 ± 18.4 units for wild-type and NK1R$^{-/-}$ mice respectively.

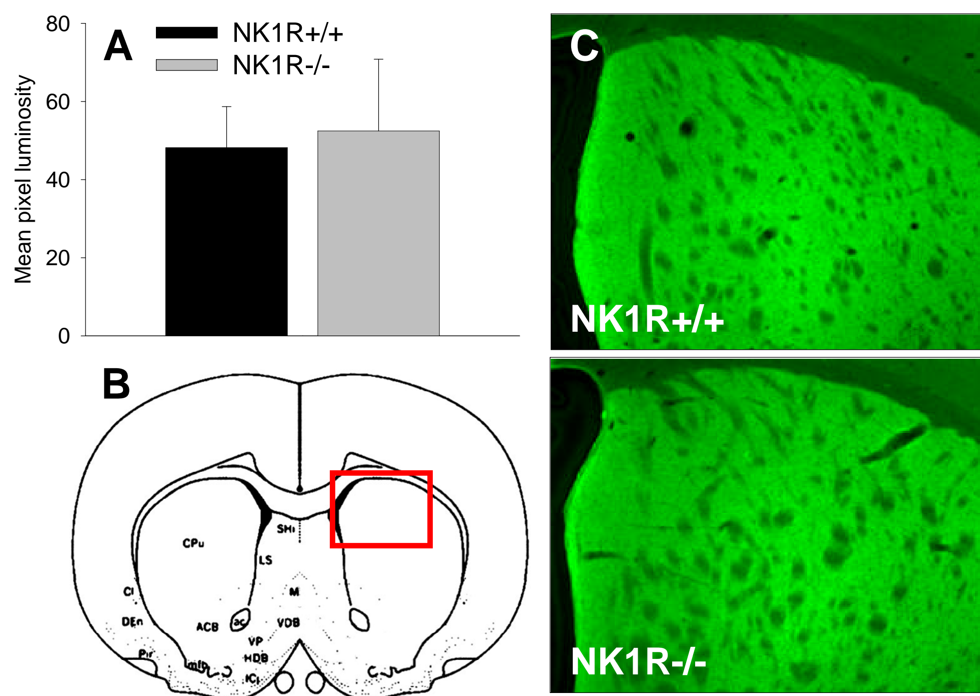

Figure 3.1 Dopamine transporter (DAT) expression in dorsal striatum.

Expression of DAT in dorsal striatum of naïve wild-type and NK1R$^{-/-}$ mice. A, luminosity measurements of DAT expression in wild-type and NK1R$^{-/-}$ mouse dorsal striatum, mean ± SEM, n=3; B, region in which DAT expression was investigated; C, images showing DAT expression in representative sections from wild-type and NK1R$^{-/-}$ mouse dorsal striatum.

3.4. Discussion

Despite the profound impairment in dopamine efflux previously observed in $NK1R^{-/-}$ mice following stimulation with amphetamine, no difference was found between wild-type and $NK1R^{-/-}$ basal expression of DAT, the principal site of action of amphetamine and a key regulator of dopamine activity at terminal fields, using the methods employed in this chapter. However, while the mean pixel luminosity data are similar between wild-type and $NK1R^{-/-}$ striata, there is a large degree of variability within each group. This may arise in part from the small number of animals used per group, and also from the methods used to assess DAT expression. Immunohistochemistry is a multi-stage technique, comprising perfusion, binding of multiple antibodies, visualisation and analysis, and each stage introduces further variability to the study, in addition to interindividual variability between subjects. In light of this, it is recognised that the difference in DAT expression between wild-type and $NK1R^{-/-}$ animals would have to be very large in order to reach statistical significance.

However, the present result supports the previous finding that while amphetamine-stimulated dopamine efflux is almost abolished in $NK1R^{-/-}$ mice, the basal efflux of dopamine is no different from that of wild-type mice (Yan et al., 2010), and indicates that, unlike in $DAT^{-/-}$ mice, the hyperactivity of $NK1R^{-/-}$ mice does not result from altered expression of striatal DAT. It also suggests that the lack of striatal dopamine response to amphetamine is not due to an altered level of DAT, but is rather due to reduced release from dopaminergic terminals; this requires investigation in $NK1R^{-/-}$ mice. The activity and regulation of DAT rather than its global expression may also contribute to the altered regulation of stimulated dopamine efflux and is yet to be investigated in $NK1R^{-/-}$ mice. DAT is a highly dynamic protein and translocates rapidly between endosomal and plasmalemmal membranes, for example, brief exposure to amphetamine induces internalisation of the transporter while cocaine causes up-regulation of cell surface DAT (Zahniser & Sorkin, 2004). DAT trafficking is regulated by intracellular signalling pathways such as extracellular signal regulated kinase (ERK), with inhibition of ERK resulting in reduced dopamine uptake by transporters in striatal synaptosomes as well as down-regulation of cell surface DAT expression in HEK293 cell cultures (Moron et al., 2003).

The next chapter describes the activation of the ERK pathway following administration of a single injection of amphetamine, while the subsequent chapters investigate the behavioural and molecular responses to repeated injections of the drug.

4. Activation of extracellular signal-regulated kinase in the striatum and nucleus basalis following acute amphetamine

4.1. Introduction

Intracellular signalling cascades are the principal mechanisms by which a signal at the cell membrane is communicated to its intracellular targets. The extracellular signal-regulated kinase (ERK) pathway is a key signalling pathway involved in dopamine-regulated locomotor activity, and its expression can be used as a marker of cellular activation, in the same way as the proto-oncogene *c-fos*. In this chapter a series of immunohistochemical experiments is described which determine the activation of ERK in NK1R$^{-/-}$ mice compared with wild-types in a number of brain regions, in response to a single injection of amphetamine. The subsequent chapters address the behavioural and molecular responses of NK1R$^{-/-}$ mice to repeated administration of amphetamine.

4.1.1. The extracellular signal-regulated kinase (ERK) pathway

ERK is a member of a superfamily of mitogen-activated protein kinases (MAPK). Collectively, components of the MAPK family communicate numerous different extracellular stimuli to a wide variety of intracellular targets, resulting in a range of effects. ERK comprises two isoforms, ERK1 and ERK2, and is activated by the concurrent phosphorylation of threonine and tyrosine residues on the kinase, carried out by MAPK/ERK kinase 1/2 (MEK). MEK, in turn, is

phosphorylated by the kinase Raf, which is itself activated by an increase in the active, GTP-bound, small G-protein Ras (Thomas & Huganir, 2004). Once activated, phosphorylated ERK (pERK) translocates into the nucleus to activate a number of transcription factors such as cAMP response element binding protein (CREB).

4.1.2. Striatal ERK and behaviour

Studies in ERK1 knock-out ($ERK1^{-/-}$) mice have revealed a role for ERK in the control of behavioural excitement. $ERK1^{-/-}$ mice have a behavioural phenotype similar to that seen in response to psychostimulants: increased locomotor activity and goal-directed activity compared with wild-types, as well as an increase in pleasure-related activity with potential harmful consequences (Engel et al., 2009). Significantly, these characteristics are also present in people with ADHD (Bruce et al., 2009). Addictive drugs induce a specific pattern of ERK activation in brain regions including the striatum, nucleus accumbens and amygdala (Valjent et al., 2004), and ERK activation in the striatum has been demonstrated to be necessary for the long-term effects of amphetamine and cocaine (such as behavioural sensitisation to subsequent drug exposure) following a single injection of either drug (Valjent et al., 2006). In the striatum, dopamine regulates ERK activation in D1-expressing neurones via modulation by D1 and D3 receptors, with activation mediated by the D1 receptor and inhibited by the D3 receptor (Valjent et al., 2005; Zhang et al., 2004). DAT knock-out ($DAT^{-/-}$) mice, whose hyperactivity is reduced with psychostimulants as it is in $NK1R^{-/-}$ mice, have an elevated level of ERK activation under basal conditions compared with their wild-type littermates; correspondingly, while in wild-type mice ERK activation is increased by administration of psychostimulants, the level of ERK activation in $DAT^{-/-}$ mice is reduced with amphetamine or methylphenidate (Beaulieu et al., 2006).

Increasing evidence indicates that the NK1 receptor mediates dopamine-stimulated locomotor activity. Blockade of NK1 receptors in the substantia nigra or in the striatum prevents D1- or amphetamine-stimulated increases in locomotor activity (Bishop & Walker, 2004; Gonzalez-Nicolini & McGinty, 2002; Krolewski et al., 2005), and activation of striatal NK1 receptors induces striatal dopamine efflux (Krolewski et al., 2005; Tremblay et al., 1992). Furthermore, inhibition of ERK activation by administration of an antagonist of the upstream effector MEK prevents the development of amphetamine-induced hyperactivity in wild-type mice (Beaulieu

et al., 2006). Together, this evidence led to the hypothesis that striatal ERK activation following amphetamine will be reduced in $NK1R^{-/-}$ mice compared with wild-types.

4.1.3. Nucleus basalis

The nucleus basalis receives input from all parts of the striatum, and projects extensively to regions of the cortex involved in attentional processing (Henderson, 1997; Mesulam et al., 1983). As such, the nucleus basalis is central to attention, cortical arousal and cognitive function, in particular in situations that demand increased attentional effort (Robbins et al., 1989; Sarter et al., 2006; Winkler et al., 1995). For example, selective cholinergic lesions in the nucleus basalis of rats produced performance deficits (increased number of ommissions and incorrect responses) in the 5-choice serial reaction time task (5-CSRTT), a measure of visual attention, while no effects were reported on variables related to locomotor activity or motivation (Harati et al., 2008). In the same study, shortening the duration of the stimulus increased the observed deficits, while lengthening the stimulus duration alleviated the deficits.

The NK1 receptor is known to be expressed in almost all cholinergic neurones of the basal forebrain in rats (Chen et al., 2001; Gerfen, 1991), but colocalisation studies in the mouse are lacking. Acetylcholine is synthesised from choline and acetyl-coA by the enzyme choline acetyltransferase (ChAT), commonly used as a marker for cholinergic neurones as it is unique to cells containing acetylcholine. Here, ChAT and NK1 receptor co-expression was assessed in the basal forebrain of wild-type mice.

Dopaminergic, noradrenergic and serotonergic cells innervate the nucleus basalis, influencing the release of acetylcholine in the cerebral cortex (Smiley et al., 1999). As all three systems are stimulated by amphetamine and are disrupted in $NK1R^{-/-}$ mice, it was hypothesised that pERK expression in cholinergic cells of the nucleus basalis following a single injection of amphetamine would also be disrupted in $NK1R^{-/-}$ mice compared with their wild-type counterparts.

4.2. Materials and methods

4.2.1. Habituation

The aim of this experiment was to determine the molecular responses to a single dose of amphetamine, so habituation was important to eliminate as far as possible the non-specific effects of handling or injection. Animals were habituated to saline injections in their home cage for three days prior to the test day, and on the fourth (test) day they were acclimatised to the test room for two hours before injection.

4.2.2. Amphetamine

Amphetamine was used at a high dose of 10 mg/kg i.p. for pERK expression in dorsal striatum and amygdala as it is known to cause a robust ERK response at this dose (Valjent et al., 2005). A second dose of 2.5 mg/kg was added for the nucleus basalis experiment, as this was the dose used by Yan et al. (2010) in the studies in which amphetamine-induced dopamine response is reduced, and at which a reduction in hyperactivity of NK1R$^{-/-}$ mice was observed.

4.2.3. Immunohistochemistry

Fifteen minutes after the test injection was administered, animals were killed by perfusion, their brains taken and postfixed for two hours, placed into 30 % sucrose until sectioning into 40 µm sections, as described in Chapter 2. For the striatum, every sixth section (240 µm apart) was selected, while for the nucleus basalis every fourth section (160 µm apart) was selected as it is a smaller region. Some sections containing the central nucleus of the amygdala were also selected for a preliminary analysis of pERK expression in the amygdala. Fluorescent immunohistochemistry was used to visualise the double-stained (ChAT and pERK) nucleus basalis and neuronal counts were carried out manually, using coded slides with no indication of genotype or treatment, to ensure assessment was conducted blind. MCID imaging software was used to quantify DAB-visualised pERK-positive cells in the striatum. At least 4 sections were counted per animal, with each side taken as a separate count; from these counts, a mean was taken for that animal (total number of cells counted, divided by the number of unilateral sections), and this mean was used for analysis.

4.2.3.1. Automated cell counting

As the striatum contained large numbers of cells expressing chromogenically-labelled pERK, bright-field microscopy coupled with a grain-counting program in MCID software (Interfocus Imaging Ltd, Cambridge, UK) was used for analysis. Cells were counted in sections located around 0.4 to 0.9 mm caudal to bregma. A rectangular area of 0.7 x 1 mm was selected in the dorsal striatum for each animal and the total number of cells within the area was recorded. In order for the software to detect individual cells as accurately as possible, target acceptance criteria were defined manually for each immunohistochemistry experiment. Optical density levels were set to include as many distinct cells and as little background staining as possible, and settings were additionally corrected for the size and shape of cells, to allow for multiple counting in areas with dense staining. Once specified, the settings remained fixed until analysis was complete for the whole experiment.

4.2.3.2. Manual cell counting

Neuronal counts were carried out manually in the nucleus basalis, due to its relatively small size, and in the central nucleus of the amygdala, due to the very dense staining that occured following amphetamine stimulation, which meant that MCID was not able to detect individual cells with sufficient accuracy. The nucleus basalis was defined according to the mouse brain atlas (Franklin & Paxinos, 1997) and every visible cell in the region was counted in at least two sections per animal. Once again all counts were carried out following blinding to genotype and treatment. For the preliminary analysis of ERK activation in the amygdala, the region was defined according to the mouse brain atlas, and every visible cell was counted in one section per animal.

4.3. Results

4.3.1. pERK expression is increased following acute amphetamine in the striatum of wild-type and NK1R$^{-/-}$ mice

Striatal pERK expression was measured using DAB immunohistochemistry in wild-type and NK1R$^{-/-}$ mice following a single injection of saline or amphetamine (10 mg/kg) (Figure 4.1). A total of 3-5 striatal sections were counted per animal, with each side counted separately,

resulting in 6-10 counts per animal. As expected, pERK expression was greater following amphetamine than following saline injection, however no difference between genotypes was observed. The mean (± SEM) number of pERK expressing cells in the dorsal striatal region analysed was 4.3 ± 2.4 and 2.3 ± 1.2 in wild-type and NK1R$^{-/-}$ mice, respectively, following saline, and 98.7 ± 11.9 and 98.9 ± 29.5, respectively, after amphetamine. Two-way analysis of variance (ANOVA) was carried out on neuronal counts, with genotype and treatment as between-subjects factors. A significant main effect of treatment was observed ($p<0.001$), but no effect of genotype ($p=0.932$) and no genotype x treatment interaction ($p=0.932$) were observed.

4.3.2. pERK expression in central nucleus of amygdala in wild-type and NK1R$^{-/-}$ mice following acute amphetamine or saline

The expression of pERK in the central nucleus of the amygdala (CeA) was quantified in a preliminary experiment (Figure 4.2). One section per animal was analysed (with left and right counted separately, resulting in 2 counts per animal). A clear increase in neuropil staining was observed following amphetamine compared with saline in both wild-type and NK1R$^{-/-}$ mice, but statistical analysis was not carried out on cell counts due to the small number of animals used (n=2) which resulted in large within-group variability. Nevertheless, a trend towards an increase in pERK expression following amphetamine was observed, with pERK expression around twice as high as that following saline, both in wild-type (188.3 ± 16.8 after saline compared with 387.8 ± 77.3 after amphetamine) and NK1R$^{-/-}$ mice (343.0 ± 194.0 and 715.5 ± 217.5 after saline and amphetamine, respectively).

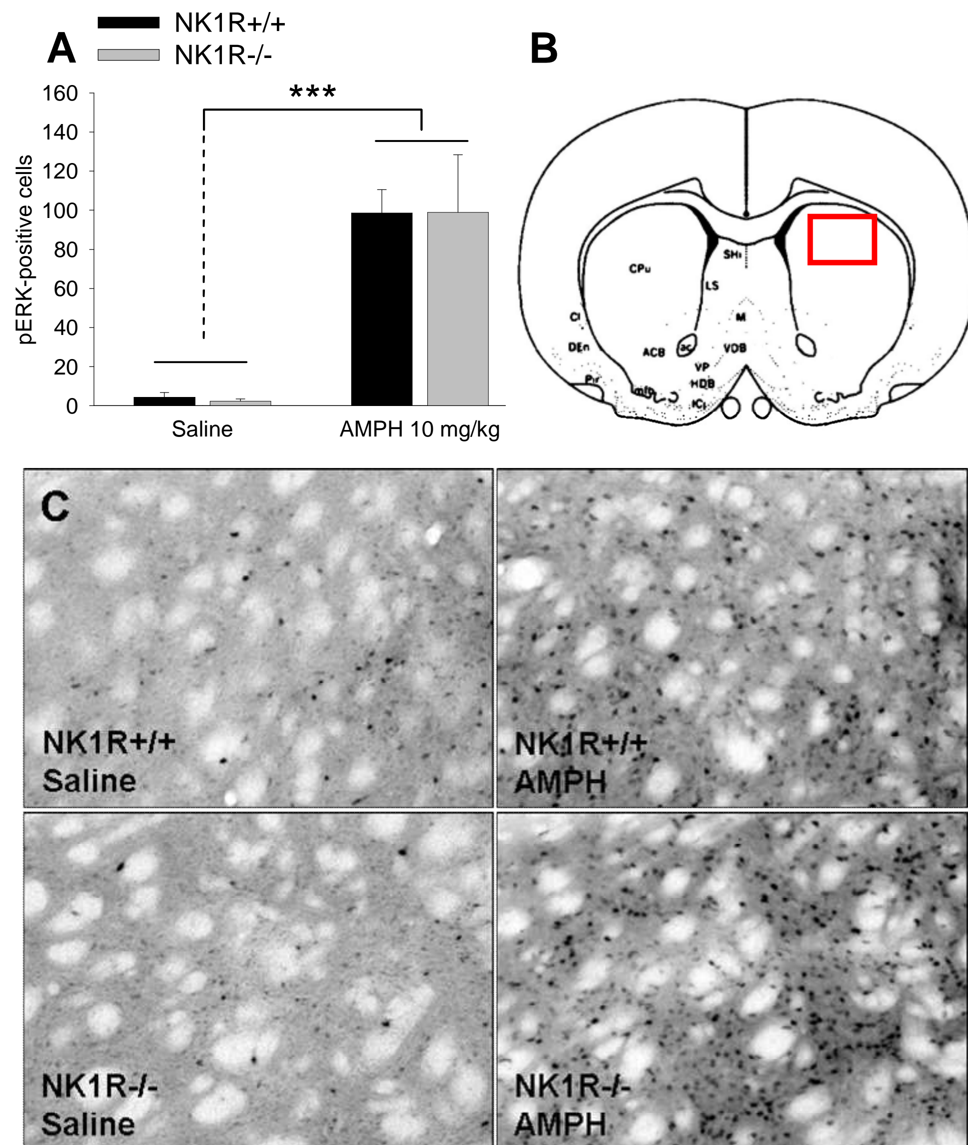

Figure 4.1 pERK expression in striatum.

*Expression of pERK in dorsal striatum of NK1R$^{-/-}$ and wild-type mice following a single i.p. injection of saline or amphetamine (10 mg/kg). A, graph showing expression of pERK in dorsal striatum, quantified by MCID imaging software. B, graphical representation of region quantified. C, representative images of pERK expression in dorsal striatum of NK1R$^{-/-}$ and wild-type mice following saline or amphetamine. ***p<0.001*

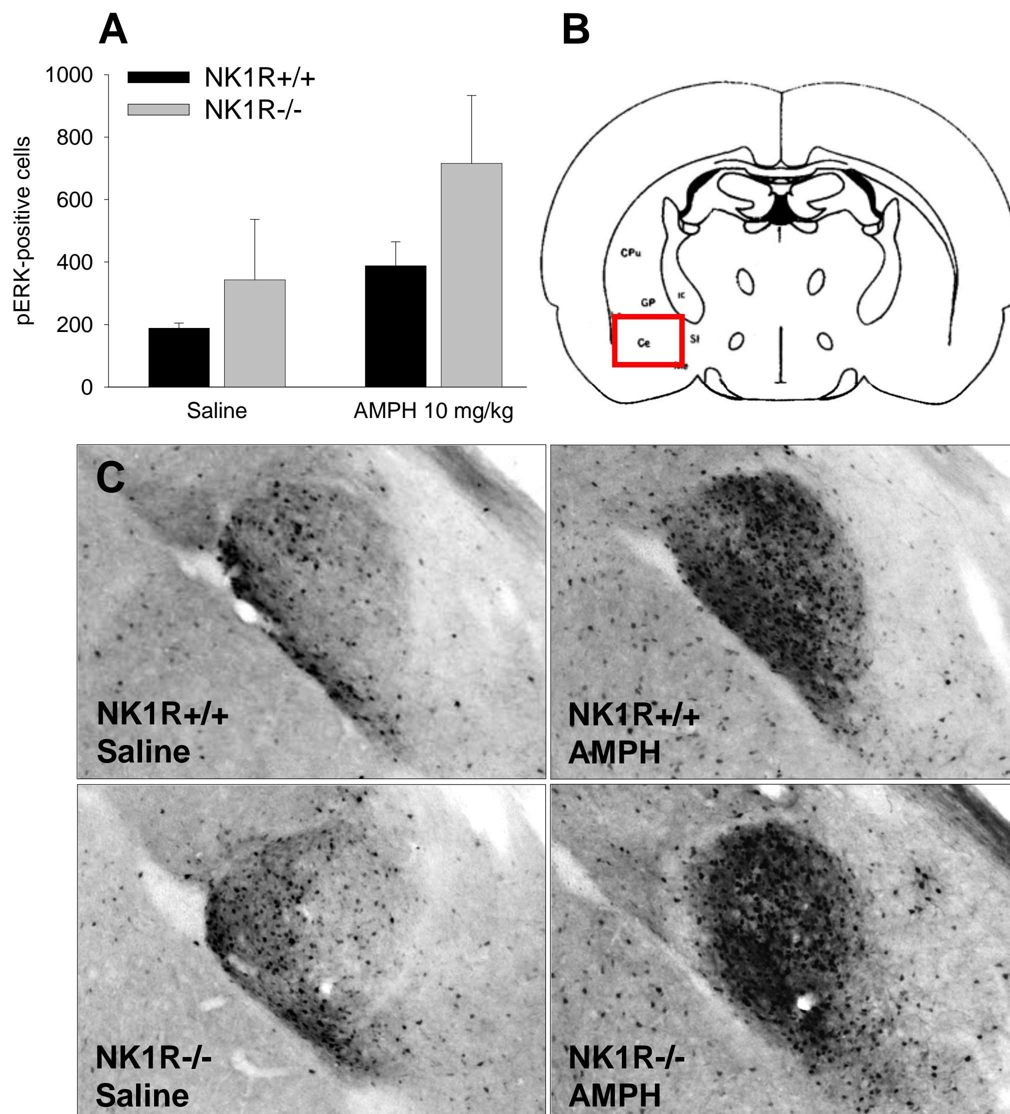

Figure 4.2 pERK expression in amygdala.

Expression of pERK in the central nucleus of the amygdala (CeA) in NK1R$^{-/-}$ and wild-type mice, following a single i.p. injection of saline or amphetamine (10 mg/kg). A, graph showing expression of pERK in CeA, quantified by MCID imaging software. B, graphical representation of region quantified. C, representative images showing pERK expression in CeA of NK1R$^{-/-}$ and wild-type mice following saline or amphetamine (10 mg/kg).

4.3.3. Sparse co-expression of ChAT and NK1 receptor in the basal forebrain

Fluorescence immunohistochemistry was used to assess the co-expression of ChAT and NK1 receptor in the basal forebrain of three wild-type mice (Figure 4.3). There was a sparse distribution of NK1 receptor-positive cell bodies and dendrites throughout the basal forebrain. Neuronal counts of ChAT and NK1 receptor expressing cells were carried out and the mean ± SEM calculated for the three animals. Of 97.2 ± 23.6 cholinergic neurones, only 4.1 ± 1.3 cells (4.3 ± 1.0 %) co-expressed the NK1 receptor (Figure 4.3).

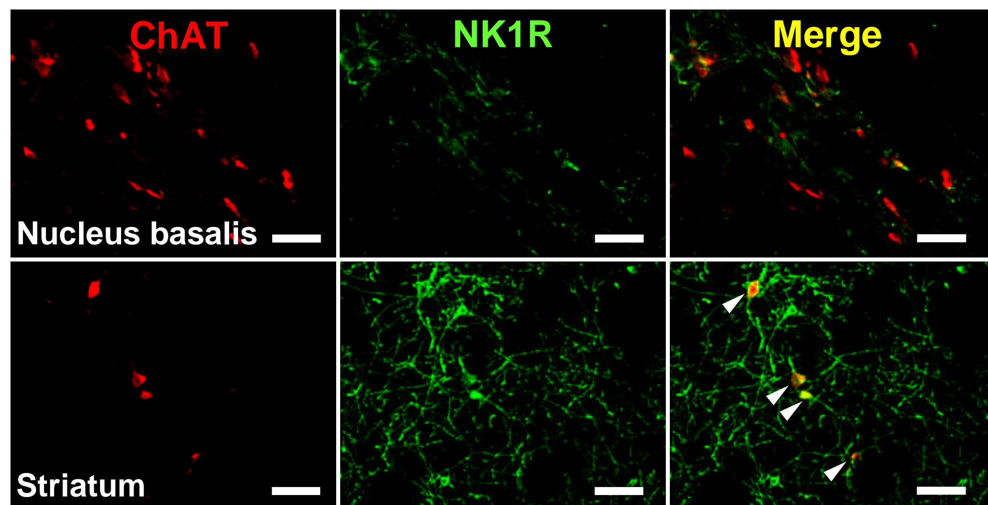

Figure 4.3 ChAT and NK1 receptor immunoreactivity in the nucleus basalis.
Representative images showing ChAT- and NK1 receptor-like immunoreactivity in the nucleus basalis of the basal forebrain of a wild-type mouse. NK1 receptor co-expressing cholinergic neurones were sparse in the nucleus basalis. Co-expression (arrowheads) in the striatum of the same animal is shown for comparison. Scale bar, 200 µm.

4.3.4. Amphetamine-stimulated pERK and ChAT expression in nucleus basalis

ERK activation in cholinergic cells of the nucleus basalis was assessed in $NK1R^{-/-}$ and wild-type mice following a single i.p. injection of amphetamine (2.5 or 10 mg/kg) (Figure 4.4 and Figure 4.5). A total of 2-5 sections were counted for each animal (with each side counted separately, resulting in 4-10 counts per animal). Group mean ± SEM data are presented in Table 4.1.

Neuronal counts	*Genotype*	*Saline*	*AMPH 2.5 mg/kg*	*AMPH 10 mg/kg*
Number of ChAT-immunoreactive cells	Wild-type	46.8 ± 3.9	44.3 ± 1.5	47.8 ± 1.8
	$NK1R^{-/-}$	59.6 ± 5.0	32.8 ± 4.5	65.0 ± 1.1
Number of pERK + ChAT double-labelled cells	Wild-type	3.8 ± 1.1	2.5 ± 1.0	11.0 ± 6.7
	$NK1R^{-/-}$	3.6 ± 1.2	1.3 ± 0.8	17.0 ± 4.7
% ChAT cells co-expressing pERK	Wild-type	8.2 ± 2.7	5.6 ± 2.0	23.0 ± 10.9
	$NK1R^{-/-}$	6.0 ± 1.6	4.1 ± 1.5	26.2 ± 8.1

Table 4.1 ChAT and pERK expression in nucleus basalis.
Summary table of mean ± SEM number of ChAT-immunoreactive cells and number of pERK and ChAT double-labelled cells in the nucleus basalis of wild-type and $NK1R^{-/-}$ *mice following saline or amphetamine (2.5 or 10 mg/kg). Also shown is the number of double-labelled cells expressed as a percentage of the total number of ChAT-immunoreactive cells counted. ChAT, choline acetyltransferase; pERK, phosphorylated extracellular signal regulated kinase.*

A two-way multivariate ANOVA with treatment and genotype as independent factors was carried out on data for ChAT-immunoreactive cell numbers and for the number of ChAT and pERK co-expressing cells, expressed as a percentage of the total number of cholinergic neurones counted.

A significant effect of treatment (p=0.007) but not genotype (p=0.822) was observed for the percent of cholinergic cells that co-expressed pERK. Data were collapsed across genotypes and a subsequent one-way ANOVA with Dunnett's post-hoc analysis was carried out to determine the effect of treatment, and revealed that the percent of ChAT-positive cells that expressed pERK following administration of 10 mg/kg amphetamine was significantly higher than that observed in the saline-treated group (p=0.008), while 2.5 mg/kg amphetamine had no effect compared with saline (p=0.850).

Surprisingly, a significant genotype (p=0.034) and treatment (p<0.001) effect, and a genotype x treatment interaction (p=0.001), were observed in the number of ChAT-expressing neurones. A subsequent one-way ANOVA was carried out on all six treatment-genotype groups (wild-type

saline, wild-type amphetamine 2.5 mg/kg, wild-type amphetamine 10 mg/kg, NK1R$^{-/-}$ saline, NK1R$^{-/-}$ amphetamine 2.5 mg/kg, NK1R$^{-/-}$ amphetamine 10 mg/kg) with Fisher's LSD post-hoc analysis to determine specific genotype and treatment differences. This revealed that the cholinergic cell number in wild-type mice given 2.5 mg/kg or 10 mg/kg amphetamine did not differ from those given saline (p=0.589 and p=0.823 respectively), indicating that cholinergic cell numbers were stable across treatments. However, saline-treated NK1R$^{-/-}$ mice were found to have a significantly higher number of ChAT-immunoreactive cells in the nucleus basalis than saline-treated wild-type mice (p=0.012). Interestingly, cholinergic cell number was significantly reduced in NK1R$^{-/-}$ mice given 2.5 mg/kg amphetamine (p<0.001 compared with NK1R$^{-/-}$ saline), but the number returned to NK1R$^{-/-}$ saline levels after 10 mg/kg amphetamine (p=0.247 compared with NK1R$^{-/-}$ saline).

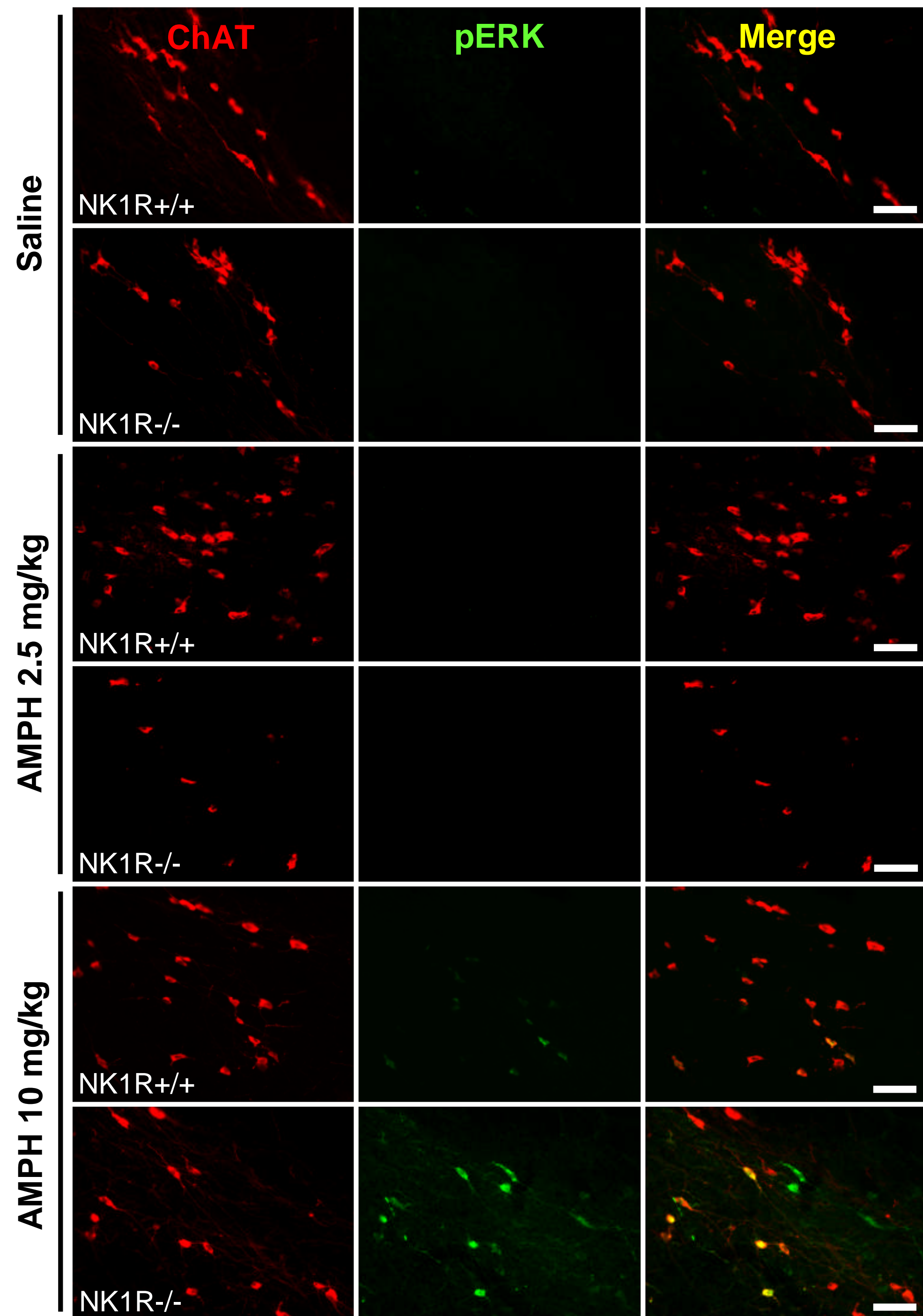

Figure 4.4 ChAT and pERK co-expression in nucleus basalis.

Images of ChAT and pERK expression in nucleus basalis of wild-type and NK1R$^{-/-}$ mice following a single i.p. injection of saline or amphetamine (2.5 or 10 mg/kg). AMPH, amphetamine. Scale bar, 200 µm.

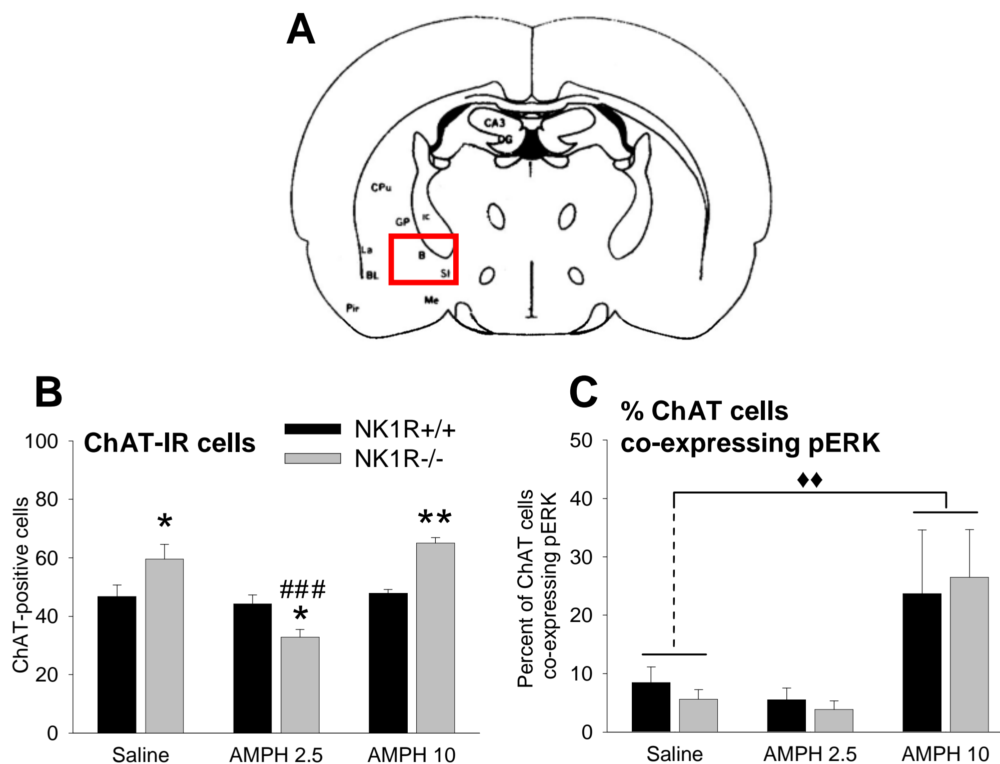

Figure 4.5 pERK and ChAT expression in nucleus basalis.

*pERK and ChAT expression in nucleus basalis in wild-type and NK1R$^{-/-}$ mice following a single injection of saline or amphetamine (2.5 or 10 mg/kg i.p.). A, graphical representation of nucleus basalis localisation; B, total number of counted ChAT-immunoreactive cells in nucleus basalis; C, percent of ChAT-immunoreactive cells that co-expressed pERK in nucleus basalis. n=4 per group. * denotes significant difference from wild-type at each treatment (*p<0.05, **p<0.01); ### denotes significant difference from NK1R$^{-/-}$ saline group (p<0.001); ♦♦ denotes significant difference between AMPH 10 mg/kg group and saline group, independent of genotype (p<0.01).*

4.4. Discussion

4.4.1. Amphetamine stimulates ERK activation in the dorsal striatum in NK1R$^{-/-}$ and wild-type mice

As expected, an increase in pERK expression was observed in the dorsal striatum of wild-type animals treated with amphetamine compared with those that received saline, and in a preliminary experiment in the central nucleus of the amygdala, pERK expression showed a trend to increase in animals that had received amphetamine compared with those that received saline. Surprisingly, the same effect was seen in NK1R$^{-/-}$ mice. Dopamine is known to regulate ERK activation, and the dopamine response following amphetamine is almost abolished in NK1R$^{-/-}$ mice (Yan et al., 2010). Furthermore, amphetamine has previously been shown to reduce the hyperactivity seen in NK1R$^{-/-}$ mice (Yan et al., 2010), and in another model this has been associated with inhibition of ERK activation (Beaulieu et al., 2006). It is possible that the high dose used to elicit a robust response in wild-type mice masked genotype differences that may have been observed at a lower dose, closer to the equivalent used therapeutically. Another explanation is that compensatory mechanisms have developed in NK1R$^{-/-}$ mice to enable ERK activation in the absence of a dopamine response following stimulation. While the principal mechanism of action of amphetamine is to increase striatal dopamine in wild-type animals, the striatum is also densely innervated by serotonergic fibres from the dorsal raphé nucleus (Tork, 1990) and psychostimulants enhance serotonin levels by blocking reuptake and increasing release of serotonin (Ritz et al., 1987). Serotonin has also been shown to mediate ERK activation, with serotonin reuptake inhibitors such as fluoxetine increasing ERK activation following acute administration (Beaulieu et al., 2006; Valjent et al., 2004). Spontaneous firing of serotonin neurones in the dorsal raphé is increased in NK1R$^{-/-}$ mice (Santarelli et al., 2001), and the serotonin reuptake inhibitor paroxetine causes a greater increase in cortical serotonin efflux in these animals than in wild-types (Froger et al., 2001). Striatal serotonin efflux following amphetamine is yet to be measured in these animals, but amphetamine may cause an enhanced increase in striatal serotonin efflux in NK1R$^{-/-}$ mice compared with wild-types, thus permitting the same degree of ERK activation as in wild-types despite the lack of dopaminergic response.

4.4.2. Amphetamine stimulates ERK activation in nucleus basalis of NK1R$^{-/-}$ and wild-type mice

The number of ERK-immunoreactive cholinergic cells in the nucleus basalis was greater in animals that received 10 mg/kg amphetamine than in those that received saline, independent of genotype, indicating that amphetamine treatment stimulates cholinergic cell activity in the nucleus basalis in both wild-type and NK1R$^{-/-}$ mice. As basal forebrain cholinergic neurones project to the cortex, the present result conforms with numerous studies demonstrating that amphetamine causes an increase in cortical acetylcholine efflux (Arnold et al., 2001; Day & Fibiger, 1992; Nelson et al., 2000; Robbins, 2002). While amphetamine-stimulated cortical acetylcholine release has not been examined in NK1R$^{-/-}$ mice, the present result would suggest that a similar response would be seen in NK1R$^{-/-}$ and wild-type mice. With cautious extrapolation to the clinical setting, this would indicate that any enhancement of cognitive function in people with ADHD by treatment with psychostimulants does not result from an enhanced responsiveness to amphetamine of cholinergic cells in the basal forebrain compared to people without ADHD.

4.4.3. Sparse co-localisation of ChAT and NK1 receptor in basal forebrain of wild-type mice

While previous studies have demonstrated that the NK1 receptor is extensively expressed on cholinergic cells of the basal forebrain of rats (Chen et al., 2001; Gerfen, 1991), the results presented here show that the same distribution does not occur in mice, as a very small proportion of cholinergic cells co-expressed the NK1 receptor. This indicates that there are species differences in the NK1 receptor-mediated modulation of basal forebrain cholinergic function by tachykinins, with a direct modulation occurring in rats while an indirect modulation is likely to occur in mice.

4.4.4. NK1R$^{-/-}$ mice express a higher number of ChAT-immunoreactive cells in nucleus basalis after saline than wild-type mice

When ChAT-expressing cell numbers were compared in saline-treated wild-type and NK1R$^{-/-}$ mice, expression was found to be higher in NK1R$^{-/-}$ mice. The increase in ChAT-expressing cell numbers could indicate either that cholinergic cell numbers are increased, or that the amount

of ChAT is increased per cell, thus enabling the enhanced detection of an increased number of cholinergic neurones. An increase in cell number, or an increase in the acetylcholine-synthesising ChAT, in the nucleus basalis may indicate that cortical acetylcholine function is enhanced in $NK1R^{-/-}$ mice, which is yet to be investigated in $NK1R^{-/-}$ mice. Considering the $NK1R^{-/-}$ mouse as a model for ADHD, the results presented here are in accord with research implicating the basal forebrain cholinergic system in attention (Everitt & Robbins, 1997; Sarter & Bruno, 1997) and support mounting evidence that central cholinergic systems are involved in the pathogenesis of ADHD (Potter & Newhouse, 2008; Potter et al., 2006; Wilens & Decker, 2007).

4.4.5. Moderate but not high dose amphetamine reduces cholinergic cell number in $NK1R^{-/-}$ mice

Surprisingly, while cholinergic cell number remained constant in wild-type mice throughout the three treatments, administration of 2.5 mg/kg amphetamine resulted in a significantly lower number of ChAT-immunoreactive cells than that seen after saline in $NK1R^{-/-}$ mice. At 10 mg/kg this difference was lost and ChAT-immunoreactive cell numbers were similar to those following saline. If ChAT is assumed to be a stable marker of cholinergic cells, then the result suggests that an acute moderate, but not high, dose of amphetamine reduces the number of cholinergic cells in the nucleus basalis, possibly by a form of neurotoxicity. The second explanation is that the moderate dose of amphetamine, rather than causing a decrease in the number of cells, induces a reduction in the amount of ChAT per cell. This would result in fewer cholinergic neurones being identified using the methods employed here, and would suggest that the functional state of basal forebrain cholinergic neurones is reduced. The reason for this apparent cholinergic deficit just 15 min after a single injection of 2.5 mg/kg amphetamine is not clear. Basal forebrain cholinergic neurones project to the cortex, and psychostimulant use has been linked with a reduction in ChAT activity in the cortex of human subjects (Kish et al., 1999) but only at very high doses. Regulation of ChAT expression in the nucleus basalis following acute administration of amphetamine has not been previously reported and requires further investigation.

4.5. Conclusions

The key findings presented here suggest that the activation of cholinergic neurons in the nucleus basalis of NK1R$^{-/-}$ mice, as indicated by pERK expression, is no different from that seen in wild-types, but that amphetamine can induce an aberrant regulation of choline acetyltransferase in the basal forebrain of NK1R$^{-/-}$ mice, an effect that has not previously been reported and requires further investigation.

Significantly, despite a striking disruption in striatal dopaminergic response and a paradoxical reduction in locomotor activity following amphetamine, NK1R$^{-/-}$ mice exhibit a normal level of ERK activation in the striatum following acute administration of the drug.

The behavioural effects of repeated amphetamine have not yet been examined in NK1R$^{-/-}$ mice. The next chapter describes a series of experiments which determine whether NK1R$^{-/-}$ mice develop sensitisation to amphetamine to the same degree as wild-type mice.

CHAPTER FIVE

5. Amphetamine-stimulated locomotor activity

5.1. Introduction

Previous research in our lab demonstrated that the hyperactivity observed in NK1R$^{-/-}$ mice is reduced with a single injection of amphetamine (2.5 mg/kg), and that a similar result is obtained in wild-type mice given the NK1 receptor antagonists RP 67580 or L-733060 prior to amphetamine administration (Yan et al., 2010).

Treatment of ADHD with psychostimulant medication often continues for many years. While a number of long-term studies have been, and continue to be, conducted in people with ADHD, to determine the effects of chronic treatment with psychostimulants, such studies take many years, and are hindered by many factors. The use of animal models is therefore valuable in identifying specific behavioural, neurochemical and molecular effects of chronic administration of psychostimulants.

Striatal dopamine has long been accepted as a key mediator of locomotor activity, both under normal conditions and in response to drugs of abuse. Furthermore, the expression of locomotor sensitisation is associated with the increasingly augmented striatal dopamine efflux that occurs with repeated exposure to addictive drugs (Paulson & Robinson, 1995). This chapter describes a series of experiments investigating the behavioural responses of NK1R$^{-/-}$ mice to a repeated administration schedule of amphetamine. Given the paradoxical behavioural response and the lack of striatal dopamine response previously observed in NK1R$^{-/-}$ mice following acute amphetamine, it was hypothesised that responses to repeated amphetamine would be similarly diminished and that NK1R$^{-/-}$ mice would not develop

behavioural sensitisation to the same degree as their wild-type counterparts. The results are discussed in the context of the NK1R$^{-/-}$ mouse as a model for ADHD, and their implications for the role of the NK1 receptor in behavioural responses to amphetamine as a drug of abuse are additionally considered.

5.1.1. The role of dopamine in locomotor activity and reward

5.1.1.1. Motor control

It has long been recognized that the nigrostriatal dopamine pathway, which originates in the substantia nigra and terminates in the dorsal striatum, is involved in motor control. Unilateral lesions of cells in this pathway cause rotational behaviour, and the loss of midbrain dopamine neurones is known as the cause of the primary motor impairment symptoms of Parkinson's disease (reviewed in Moore, 2003).

5.1.1.2. "Liking" and "wanting"

The mesocorticolimbic dopamine system comprises the mesocortical and mesolimbic pathways, which originate in the ventral tegmental area (VTA), and terminate in the frontal cortex and limbic system (including the nucleus accumbens, amygdala and the hypothalamus) respectively. The mesocorticolimbic system is considered to be important in feelings of pleasure and in the reinforcing effects of drugs or natural rewards (Pierce & Kumaresan, 2006). The experience of wanting or craving, also known as the state of incentive salience, is considered to be caused by drug-induced hyperactivity of the mesocorticolimbic dopamine system (Robinson & Berridge, 2008).

5.1.1.3. Drug-induced hyperactivity

While the nigrostriatal and mesocorticolimbic systems were considered separate entities for many years with regard to their roles in locomotor control and reward, it is becoming accepted that there is little defined anatomical or functional distinction between the two, and that both systems play a significant role in reward and locomotor response to drugs (Wise, 2009). What is remarkable is the abundant evidence that although different classes of drugs of abuse (including alcohol, opioids, cannabinoids and psychomotor stimulants) target many different neurotransmitter systems, the neurochemical changes that occur following administration of a

drug of abuse converge on the same dopaminergic pathways; striatal dopamine efflux is increased after acute exposure, and chronic exposure causes a long-term reduction in basal striatal dopamine efflux (Nestler, 2005).

5.1.2. Locomotor sensitisation

A wealth of research has emerged on the subject of sensitisation since the first reports of the phenomenon were published in experimental animals over 70 years ago (Downs & Eddy, 1932), followed by reports of sensitisation in humans in the 1950s (Angrist & Sudilovsky, 1978; Connell, 1958). Locomotor sensitisation is defined as the progressive increase in locomotor activity observed upon repeated administration of a drug (Stewart & Badiani, 1993). The sensitised response persists for a long time, remaining augmented following a period of abstinence of months or even years (Paulson et al., 1991). Behavioural sensitisation is considered to be mediated by a combination of learned associations and long-lasting drug induced neuronal adaptations (Nestler, 2001; Vanderschuren & Kalivas, 2000), and to be related to the craving and relapse components of addiction (Kalivas et al., 1998; Robinson & Berridge, 2008; Stewart & Badiani, 1993).

5.1.2.1. Development of sensitisation

The development of sensitisation undergoes two defined stages: initiation and expression (Cador et al., 1995; Kalivas & Stewart, 1991). During the initiation stage, immediate changes occur at the cellular and molecular level which do not necessarily have behavioural consequences but are critical to the subsequent development of sensitisation, while the expression of sensitisation refers to the longer-term effects of these immediate changes including changes in plasticity. The two stages occur in separate anatomical locations and are also pharmacologically distinct. Dopamine neurones have specific roles in the initiation and expression stages of sensitisation.

Initiation of sensitisation occurs in cell bodies of dopamine neurones in the ventral tegmental area (VTA) and substantia nigra (SN). Acute microinjections of sensitising drugs (such as psychostimulants or opioids) into these regions do not cause any increase in locomotor activity, but repeated administration results in behavioural sensitisation to a subsequent microinjection of the same drug into those regions; similarly, injections into these regions are

also sufficient to induce behavioural sensitisation to a subsequent systemic drug challenge (Perugini & Vezina, 1994; Stewart & Vezina, 1989).

While microinjection of drugs into the VTA produces sensitisation but no acute response, the reverse is seen with administration of drugs into dopamine terminal fields. For example, when psychostimulants or opioids are administered into the nucleus accumbens (NAc), an increase in locomotor activity is observed, but repeated administration into this region does not induce any further increase in behavioural response (Dougherty & Ellinwood, 1981; Henry & White, 1991; Kalivas & Weber, 1988; Stewart & Vezina, 1988; Vezina & Stewart, 1990). Hence it is considered that the expression of sensitisation occurs in dopamine terminal fields in the dorsal and ventral striatum, but that this is dependent upon the initiation of sensitisation occurring in the cell bodies in the VTA and SN.

Expression of the behavioural effects of sensitising drugs is not necessary for the development of sensitisation (Cador et al., 1995). Blocking the behavioural effects of a series of repeated injections of amphetamine using a dopamine D2 receptor antagonist does not block the development of sensitisation, and injecting drugs into the VTA has no behavioural effect, yet sensitises the body to a subsequent systemic injection of the drug (Kalivas & Weber, 1988; Vezina & Stewart, 1990). Furthermore, the sensitising dose may be below the threshold of behavioural stimulation, but when a higher challenge dose is administered, sensitisation is observed (Mattingly et al., 1988).

5.1.2.2. *Cross-sensitisation between different drugs, stress and environmental stimuli*

Drugs in the same class can cross-sensitise to one another, for example repeated administration of cocaine will cause an increased response to a subsequent injection of amphetamine. Systemic or VTA administration of one drug can also cause sensitisation to the subsequent systemic administration of a different drug, even if it is in a different class; amphetamine pre-treatment, for example, cross-sensitises the response to systemic morphine administration (Vezina & Stewart, 1990). This phenomenon also occurs when both drugs are self-administered (Liu et al., 2007). Similarly, repeated stressful stimuli can cross-sensitise a response to a drug. A single or repeated exposure to a stressful stimulus has been shown to

elicit the same elevation in striatal dopamine release upon a subsequent stress or drug challenge as exposure to drugs (de Jong et al., 2005; Kalivas & Stewart, 1991), and food-induced behavioural sensitisation has also been shown to cross-sensitise to cocaine and morphine (Le Merrer & Stephens, 2006; Mendez et al., 2009).

Behavioural sensitisation to a drug can be readily influenced by external stimuli that coincide with the administration of the drug. If the sensitisation protocol is carried out in the home cage, sensitisation is attenuated, compared to carrying out the protocol in a distinct testing environment (Badiani et al., 1995). Similarly, a greater degree of sensitisation is attained when the sensitising injections and the challenge injection are administered in the same environment, than when the challenge injection is administered in a novel environment (Mattingly & Gotsick, 1989). Correspondingly, the increase seen in extracellular dopamine efflux in the nucleus accumbens following a cocaine challenge after a chronic administration schedule is greater when cocaine is consistently administered in a distinctive environment than when the challenge injection is administered in a different environment to the pre-treatment injections (Duvauchelle et al., 2000). Similarly, sensitisation is considered to be drug state-dependent, meaning that if an animal receives repeated administrations of a combination of drugs, no sensitisation response will be observed when a challenge is given of a single drug in the combination. For example, mice given two injections, of amphetamine followed immediately by the benzodiazepine chlordiazepoxide (CDP), daily for 8 days displayed sensitisation on Day 8, but when challenged with amphetamine alone on Day 9 displayed no evidence of sensitisation (Stephens et al., 2000). Furthermore, striatal dopamine increases in cocaine abusers are associated with craving, but only when paired with cocaine cues such as videos of subjects taking cocaine (Volkow et al., 2008). Cue-conditioning is so powerful that a drug-like response in animals can occur to a “challenge” injection of saline after a series of sensitising injections (Badiani et al., 1995). However, sensitisation induced by injections of drugs into the VTA is independent of environmental context (Vezina & Stewart, 1990), and when the development of an association between environment and injection is prevented, sensitisation can still occur (Stewart & Badiani, 1993).

5.1.3. ADHD and drug use

ADHD is normally treated with psychostimulant drugs, most commonly methylphenidate (Ritalin) or a mixture of amphetamine salts (Adderall), which have the potential to be abused. Additionally, ADHD is highly comorbid with drug abuse. There is ongoing debate as to whether treatment with drugs with abuse potential leads to later-life drug abuse, or whether treating the symptoms of ADHD such as impulsivity, which is thought to predispose subjects to drug addiction (Belin et al., 2008), attenuates the predisposition and diminishes the risk of future drug abuse.

5.1.3.1. *Therapeutic drug use*

Amphetamine and methylphenidate act on central dopaminergic and noradrenergic systems, improving inhibitory control and cognition both in people with ADHD as well as in healthy subjects. Indeed, a growing problem exists amongst schools and universities in the UK and USA in which drugs obtained on prescription for ADHD are being traded for their cognition enhancing effects in healthy students (Greely et al., 2008; Sahakian & Morein-Zamir, 2007). However, while in healthy subjects the characteristic response to psychostimulant drugs is an increase in locomotor activity, the symptom of hyperactivity exhibited by people with ADHD (and in some animal models), is paradoxically attenuated upon administration of psychostimulant drugs. The mechanism of action that results in psychostimulant drugs reverting a hyperactive locomotor state to that of normal activity is as yet not well understood, but may be related to their action at the DAT. The increase in resting-state extracellular dopamine elicited by blockade of DATs causes presynaptic D2 autoreceptors to inhibit the stimulated release of further dopamine, possibly resulting in a lower degree of activation of postsynaptic dopamine receptors, in turn leading to a reduction in locomotor activity (Isaac & Berridge, 2003; Seeman & Madras, 1998).

5.1.3.2. *Substance abuse*

One of the core symptoms of ADHD is impulsivity, which has been shown to predict compulsive drug use (Belin et al., 2008; Everitt et al., 2008), and ADHD is indeed highly comorbid with substance abuse and addictive disorders, with up to 45 % of people with ADHD also having a substance use disorder (Biederman, 2003; Mannuzza et al., 2003). People in the general population are often exposed to addictive substances (for example in schools and

colleges as mentioned above), but only a relatively small proportion develop addictions. There is concern as to whether the widespread psychostimulant treatment of children with ADHD is a cause of the high comorbidity with substance abuse in later life, particularly as long-term studies of addiction have shown that a young age at first exposure to a drug of abuse is correlated with an increased risk for later addiction (Grant & Dawson, 1997). However a considerable body of evidence in children and adults with ADHD is emerging supporting the theory that psychostimulant treatment in fact protects against later substance use disorders and cigarette smoking (Biederman et al., 2008; Wilens et al., 2008).

5.1.4. Substance P and the NK1 receptor in reward and sensitisation

The highest concentration of substance P is found in the VTA and SN, brain regions critical to the development of sensitisation, as discussed above. Furthermore, the NK1 receptor is heavily expressed in the dorsal and ventral striatum, which play a key role in the expression of sensitisation. In rats, substance P produces conditioned place preference (CPP) when injected systemically or into the basal forebrain, and when self-administered acutely into the ventromedial caudate-putamen (Hasenohrl et al., 1998b; Huston & Hasenohrl, 1995; Huston et al., 1993; Nikolaus et al., 1999).

Early work in our NK1R$^{-/-}$ mice demonstrated that they display a blunted response to morphine in three behavioural models of drug addiction: CPP, self-administration and sensitisation (Murtra et al., 2000; Ripley et al., 2002). Conditioned place aversion to the opiate receptor antagonist naloxone and some symptoms of opiate withdrawal are also attenuated in NK1R$^{-/-}$ mice (Murtra et al., 2000), and NK1 receptor-expressing neurons in the amygdala have been shown to mediate morphine-induced CPP (Gadd et al., 2003). The analgesic properties of morphine, however, remain unaffected (De Felipe et al., 1998), implying that the NK1 receptor plays a specific role in the motivational properties of opiates, rather than simply modulating all responses to µ-opioid agonists. Recent evidence has also linked the NK1 receptor to alcohol dependence, with promising clinical results from NK1 receptor antagonists (George et al., 2008; Heilig et al., 2010; Seneviratne et al., 2009).

Interestingly, NK1R$^{-/-}$ mice have normal responses to cocaine in all the above tests, and additionally develop CPP normally to amphetamine and food (a natural reward), indicating that their reward circuits are not globally impaired (Murtra et al., 2000; Ripley et al., 2002).

However, significantly, the characteristic amphetamine-induced increase in striatal dopamine efflux is almost completely abolished in NK1R$^{-/-}$ mice, and corresponds to a reduction in locomotor activity (Yan et al., 2010).

5.1.5. Experimental aims

The principal aim of the experiments described in this chapter was to identify whether NK1R$^{-/-}$ mice develop sensitisation to amphetamine. Given the considerable impairment seen in NK1R$^{-/-}$ mice in amphetamine-induced striatal dopamine response, and their paradoxical behavioural response to amphetamine, it was hypothesised that the animals would not develop locomotor sensitisation to the same degree as their wild-type counterparts. Baseline locomotor activity and responses to acute amphetamine were also analysed, to determine whether the hyperactivity and paradoxical response to acute amphetamine previously observed would be replicated under altered experimental conditions and in different locomotor activity chambers.

5.2. Materials and methods

The methods given below are specific to the experiments carried out in this chapter. Full details of general methods used throughout this thesis, including details of animal housing conditions, colony maintenance, amphetamine and experimental design are given in Chapter 2.

Four experiments (A-D) investigating the locomotor activity of NK1R$^{-/-}$ and wild-type mice following saline or amphetamine are described in this chapter. The first three (A-C) are sensitisation experiments, in which animals received daily injections of amphetamine or saline for four days, followed by a 3 day withdrawal period before a final amphetamine challenge. The fourth experiment (D) measured the locomotor response to a single injection of amphetamine or saline. After baseline locomotor activity measurements were taken, the mice in Experiments A and B received saline or amphetamine at 4 mg/kg (as this had elicited a robust locomotor activity response in wild-type mice in a pilot study carried out in the same apparatus), while those in experiments C and D received saline or amphetamine at 2.5 mg/kg (the same dose as used in the study by Yan et al. (2010) in which amphetamine reduced the

hyperactivity of NK1R$^{-/-}$ mice). A full dose response curve was not carried out, however, as the primary aim of the experiments was to determine whether NK1R$^{-/-}$ mice would develop sensitisation to amphetamine at the same dose given by Yan et al. (2010). A summary of the four experiments is given in Table 5.1 and protocols are described in detail below.

Expt	*Description*	*i.p. injection*	*Activity chamber*	*Unit*	*n per group*
A	Sensitisation	Saline or AMPH 4 mg/kg	Rectangular chambers	cm	6-11
B	Sensitisation	Saline or AMPH 4 mg/kg	Circular corridors	¼ turn	7-9
C	Sensitisation	Saline or AMPH 2.5 mg/kg	Circular corridors	¼ turn	18
D	Acute	Saline or AMPH 2.5 mg/kg	Circular corridors	¼ turn	4

Table 5.1 Locomotor activity experiments.

Summary of the experiments referred to throughout this chapter. AMPH, amphetamine; i.p., intraperitoneal.

5.2.1. Equipment

5.2.1.1. *Rectangular locomotor activity boxes with EthoVision*

In the first experiment (A), horizontal locomotor activity was assessed in rectangular Perspex chambers measuring 20 x 9 x 9 cm, with black walls and transparent lids with air holes (Figure 5.1A-B). The chambers also had transparent floors, and were placed on an infrared light box in a room with dimmed light (10-30 lux in the chambers). Activity was analysed as distance moved (cm), using an automated system (Noldus EthoVision, Version 3). A maximum of eight chambers could be placed next to each other on the light box and analysed simultaneously. It was observed that animals tended to spend time in the corners of the chambers (Figure 5.1C), as shadows and dark corners were unavoidable, and it was thought that this may contribute to the considerable amount of within-group variability observed in this experiment. As a result, the equipment was changed to individually lit circular corridors (described below).

5.2.1.2. Circular corridors with beam breaks

A bank of eight circular corridors (Imetronic, Pessac, France) was used for the remaining three experiments (B-D). Each corridor measured 5 cm wide x 15 cm high, with an external diameter of 17 cm (Figure 5.2), with four photocells evenly spaced around the corridor and positioned 1 cm above the floor. Beam breaks were detected and recorded via an electronic interface to a computer running Imetronic software. A break in two adjacent beams consecutively (one quarter-turn) was considered a unit of locomotion. The bank was enclosed and ventilated, and all the corridors were individually lit at the same level (120 lux).

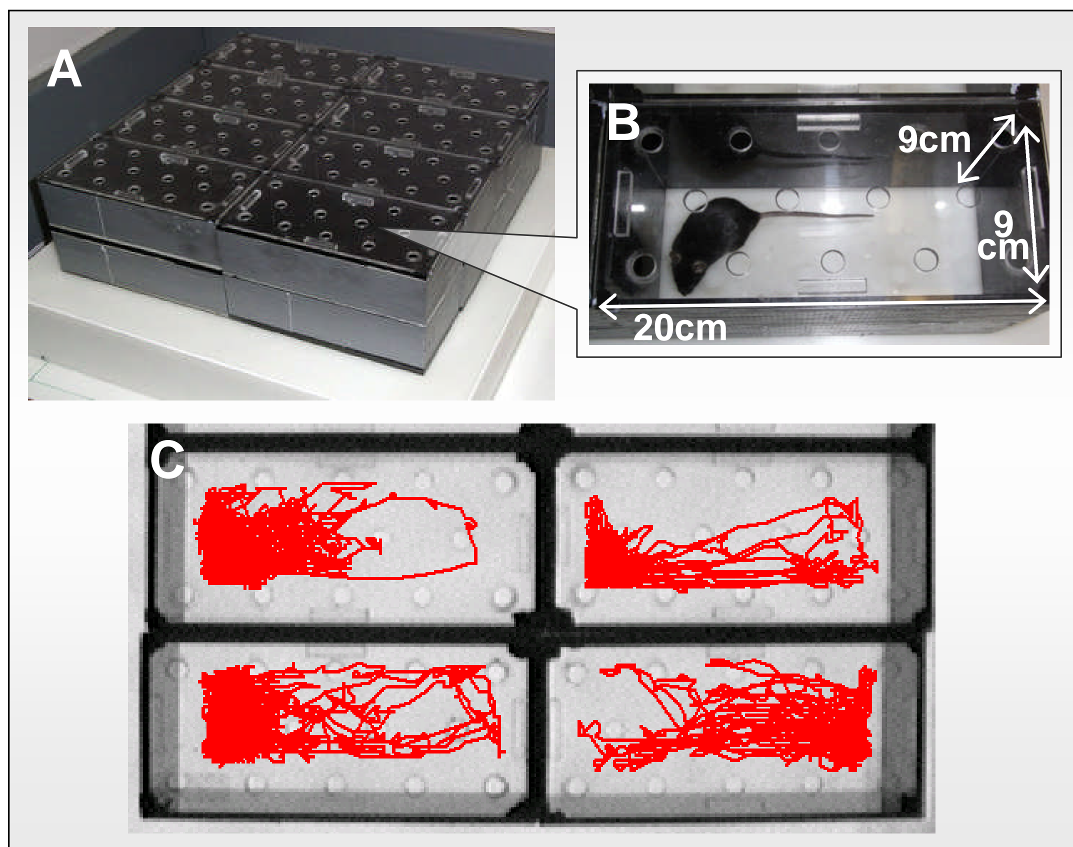

Figure 5.1 Rectangular locomotor activity chambers.

A-B, photographs of the rectangular locomotor activity chambers used in Experiment A. Some mice tended to stay in the corners of the boxes, which may have contributed to the high degree of within-group variability in this experiment. C, traces showing the movement of four mice over 60 minutes in the rectangular chambers.

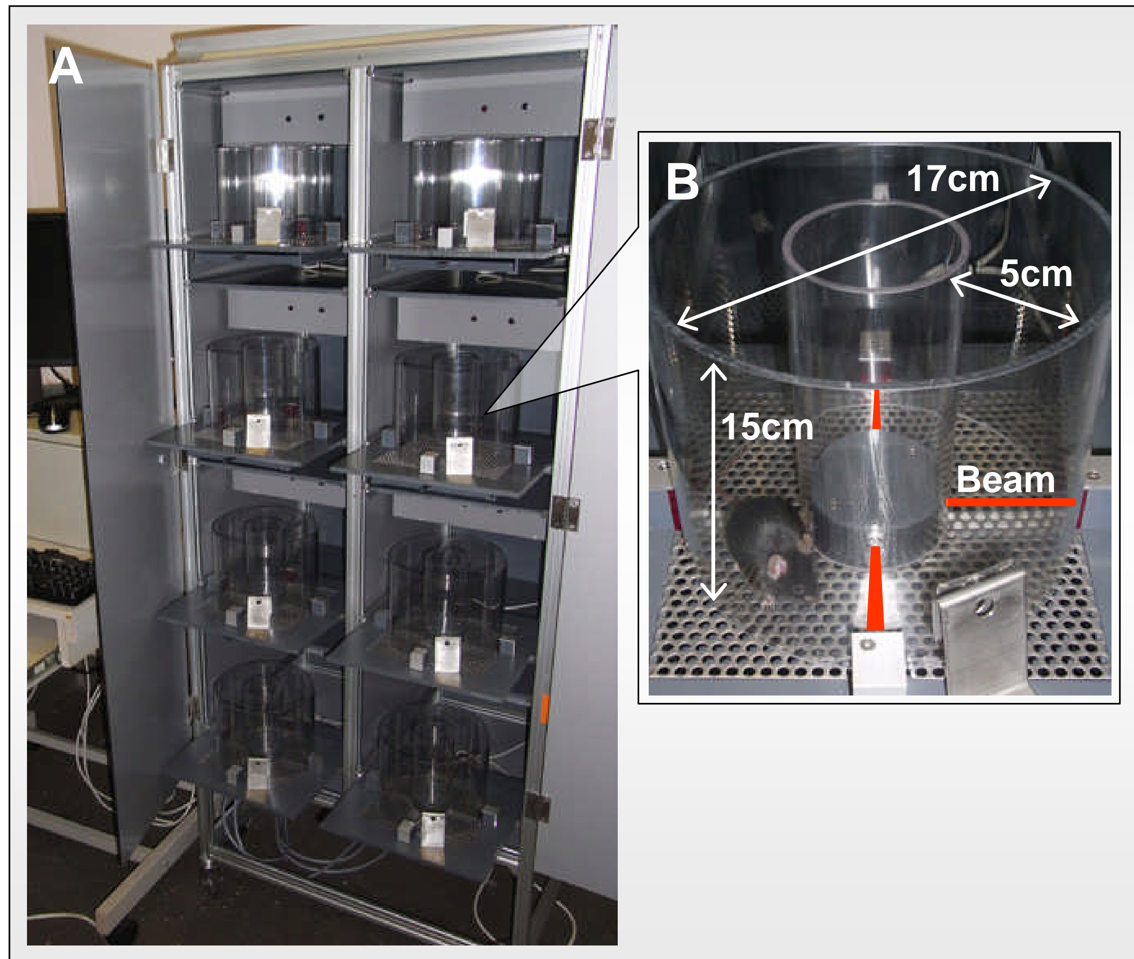

Figure 5.2 Circular corridors.

Photographs of the circular corridors used in experiments B-D, including an illustration of the beams used in the measurement of locomotor activity in this equipment.

5.2.2. Locomotor activity experiments

5.2.2.1. Habituation

All four experiments began with a 60 min habituation period at the start of each experimental day, during which baseline locomotor activity was measured. On Day 1 of Experiments A-C (sensitisation experiments), this corresponded with the activity of naïve mice, before any injections had been administered. In Experiment D (acute experiment), animals were habituated to saline injections in their home cage for three days before being placed in the activity chambers on the test day.

5.2.2.2. *Sensitisation experiments (A-C)*

A maximum of eight animals could be assessed at one time either in the rectangular chambers or in the bank of circular corridors, and the protocol for one set of eight animals lasted eight days in total. The protocol is summarised in Figure 5.3. On days 1-4, animals were placed individually into the activity chambers for a 60 min habituation period, with locomotor activity recorded for this time. A single i.p. injection of either saline or amphetamine (2.5 or 4 mg/kg, see Table 5.1 for details) was administered, and the activity of the animals was recorded for a further 2 h before the mice were placed back in their home cages. On days 5-7, the mice were left in their home cages and received only the amount of handling necessary to clean their cages. Finally, on Day 8, the animals underwent the same procedure as on Days 1-4, except that all animals, whether they had been previously treated with saline or with amphetamine, received an i.p. injection of amphetamine (challenge). Locomotor sensitisation to amphetamine was considered as increased locomotor activity in animals that had previously received injections of drug compared with those that had previously received injections of saline. The protocol is summarised in Figure 5.3.

5.2.2.3. *Acute locomotor activity experiment (D)*

Animals were habituated to saline injections in their home cages for three days, before being placed in the circular corridors on the test day and undergoing a similar procedure to the first day of the sensitisation experiments described above. Following a habituation period of 60 min, mice received an acute i.p. injection of saline or amphetamine (2.5 mg/kg). The activity of the animals was recorded for 2 h. This protocol is summarised in Figure 5.4.

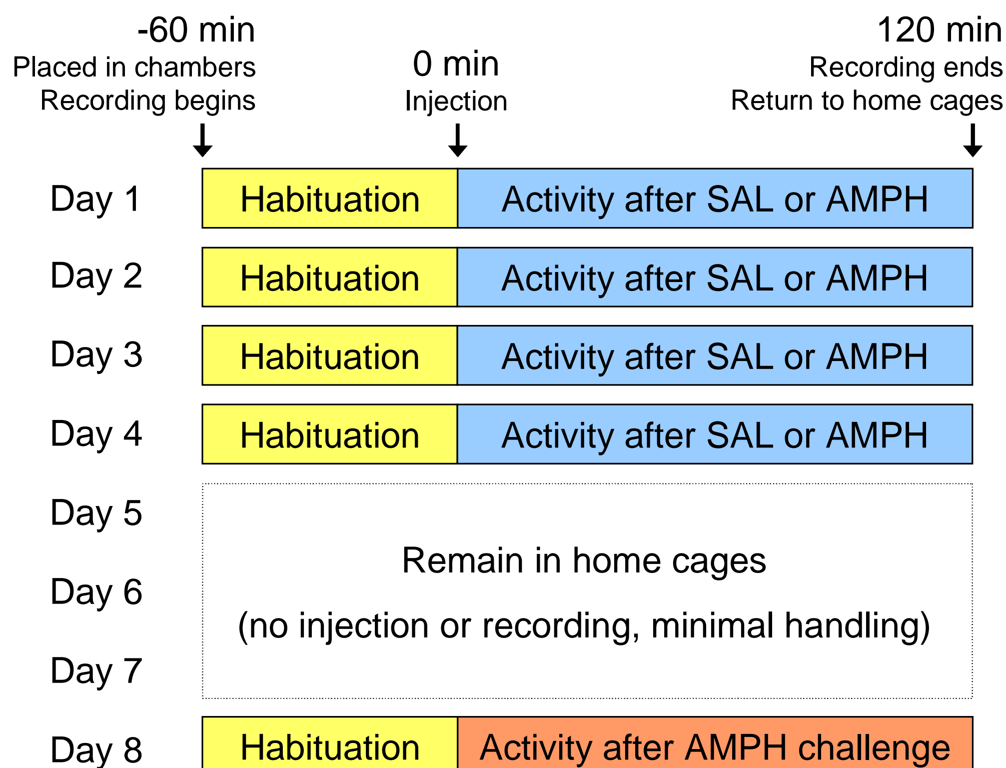

Figure 5.3 Amphetamine sensitisation protocol.

Summary of the sensitisation protocol used in Experiments A-C. On experimental days 1-4, the animals were placed into the chambers and their activity was recorded for 60 min (habituation period), before they were injected with either saline (SAL) or amphetamine (AMPH) (2.5 or 4 mg/kg). They were then recorded for a further 120 minutes before being placed back in their home cages. During days 5-7 the animals remained in their home cages and were handled only for routine husbandry. Day 8 (challenge) was carried out as on Days 1-4, except that all animals were injected with amphetamine (2.5 or 4 mg/kg).

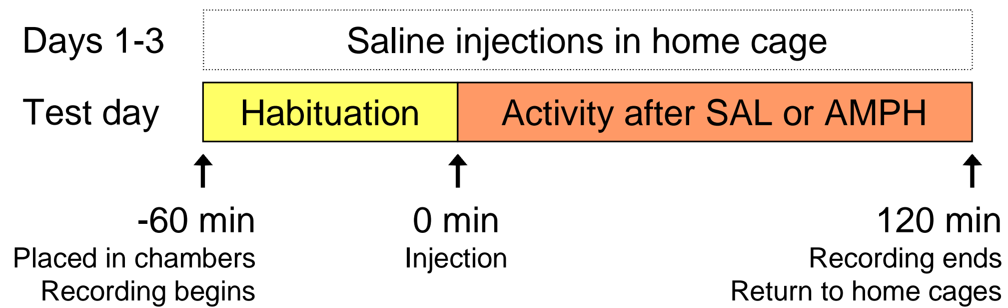

Figure 5.4 Acute amphetamine protocol.

Summary of the protocol used for the measurement of acute locomotor activity. Animals were habituated to saline injections for three days prior to the experimental day. On the test day, animals were placed in the locomotor activity chambers for a 60 min habituation period, injected with amphetamine (2.5 mg/kg) or saline, and recorded for a further 2 h.

5.2.3. Statistical analysis

An independent samples t-test was applied to the baseline locomotor activity data in each behavioural experiment, to compare activity between wild-type and $NK1R^{-/-}$ genotypes. Split-plot three-way repeated measures analysis of variance (ANOVA) was carried out on Day 1 and Day 8 of experiments A-C and on the test day of experiment D, with genotype and treatment as the between-subject variables and time as the within-subject variable. If significant main effects of genotype or treatment were observed, subsequent two-way ANOVA was carried out in each genotype to determine the effect of treatment in each genotype.

5.3. Results

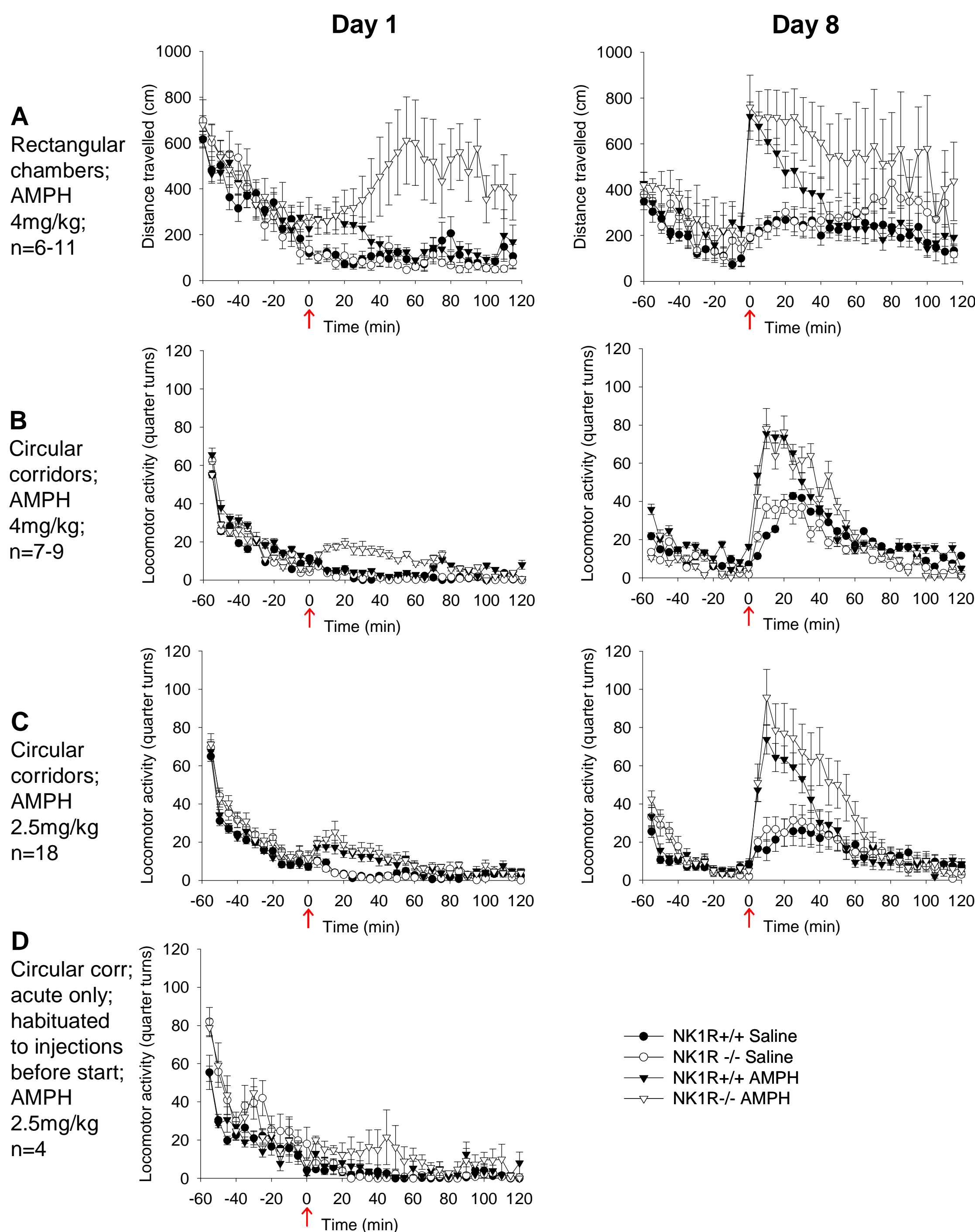

Figure 5.5 Time-dependent graphs of locomotor activity of NK1R$^{-/-}$ and wild-type mice. *Time course of locomotor activity on Day 1 and Day 8 of the three locomotor sensitisation experiments (A-C), and in the acute locomotor activity experiment (D).*

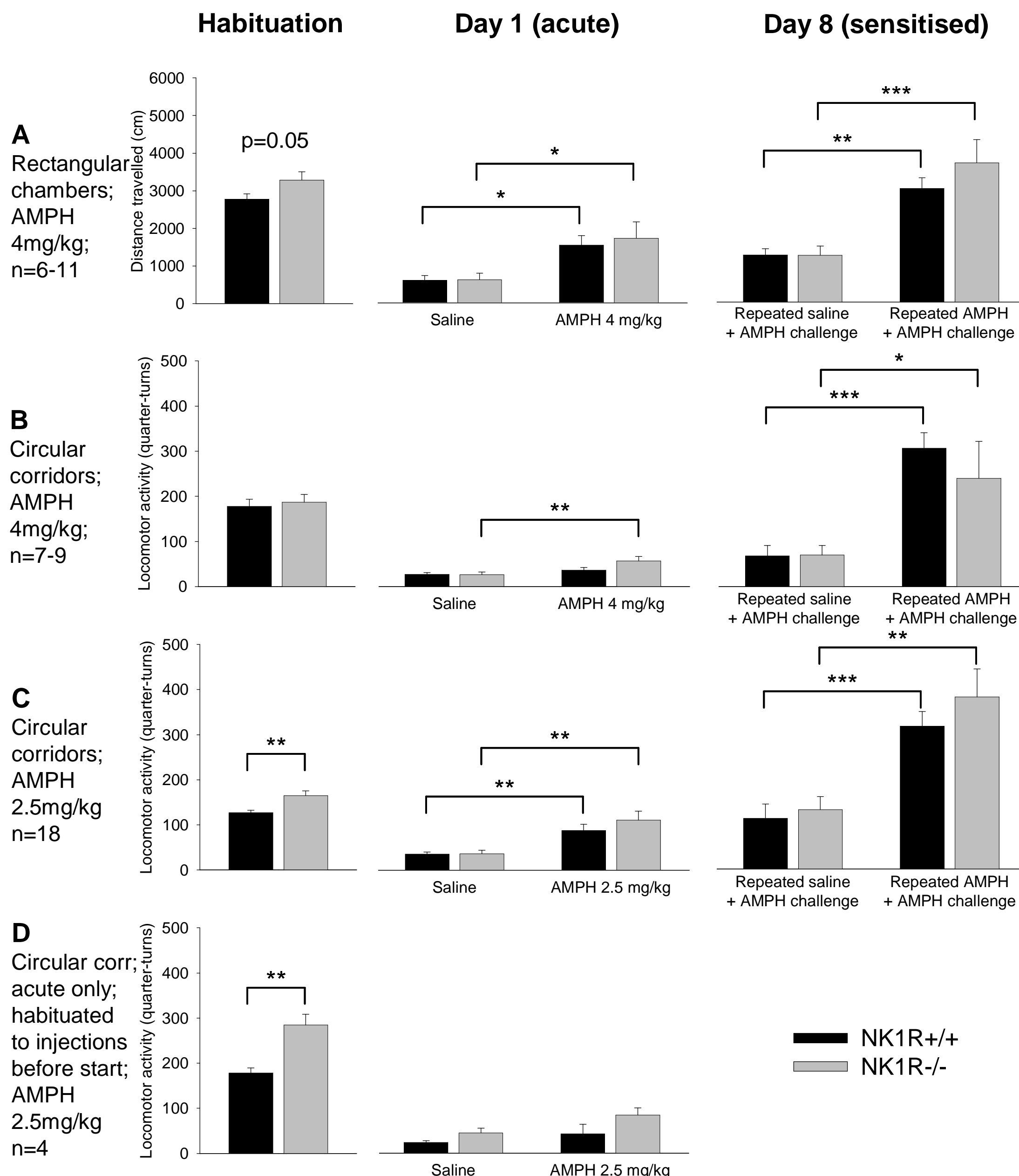

Figure 5.6 Total locomotor activity of wild-type and NK1R$^{-/-}$ mice.

*Baseline and stimulated locomotor activity in the four locomotor activity experiments. Graphs show total activity in the first 30 minutes of habituation, and in the first 30 min after i.p. injection of acute and repeated amphetamine or saline. *p<0.05; **p<0.01; ***p<0.001*

In Experiment A, the peak activity of NK1R$^{-/-}$ mice occurred at around 60 min following injection on Day 1, and an increased level of activity was maintained until the end of the recording on both Day 1 and Day 8. The peak of activity occurred within 30 min and returned to baseline levels by around 80 min for the remaining experiments; data from the first 30 min after injection are presented in Figure 5.6. A summary of mean ± SEM data is presented in Table 5.2.

Experiment	Genotype	Distance travelled in first 30 min				
		Day 1 Habituation	Day 1 after injection		Day 8 after injection	
			Saline	AMPH	Saline	AMPH
A: rectangular chambers; 4 mg/kg AMPH; n=6-11 (cm)	Wild-type	2778.1 ± 142.6	599.9 ± 123.3	1712.0 ± 330.5	1461.8 ± 193.2	3799.7 ± 376.7
	NK1R$^{-/-}$	3285.1 ± 217.6	614.5 ± 171.1	1719.2 ± 430.3	1448.6 ± 290.5	4319.3 ± 727.0
B: circular corridors; 4 mg/kg AMPH; n=7-9 (quarter-turns)	Wild-type	177.8 ± 15.5	34.2 ± 8.3	40.8 ± 6.6	120.6 ± 56.0	342.7 ± 47.2
	NK1R$^{-/-}$	187.2 ± 16.8	26.6 ± 5.6	79.9 ± 24.5	137.9 ± 50.7	283.3 ± 81.8
C: circular corridors; 2.5 mg/kg AMPH n=18 (quarter-turns)	Wild-type	126.9 ± 5.5	32.1 ± 5.3	79.6 ± 13.6	101.4 ± 29.3	285.5 ± 36.0
	NK1R$^{-/-}$	164.5 ± 10.6	33.1 ± 7.5	100.5 ± 19.1	118.7 ± 27.4	343.8 ± 61.7
D: circular corridors; 2.5 mg/kg AMPH n=4 (quarter-turns)	Wild-type	178.1 ± 11.1	23.8 ± 4.0	42.8 ± 21.1	(Acute only)	
	NK1R$^{-/-}$	284.6 ± 24.1	45.0 ± 10.7	84.8 ± 15.8		

Table 5.2. 30 min locomotor activity of wild-type and NK1R$^{-/-}$ mice.

Mean ± SEM of total locomotor activity of NK1R$^{-/-}$ and wild-type mice during the first 30 min of the habituation period on Day 1, as well as after a saline or amphetamine i.p. injection on Day 1 or Day 8 of the sensitisation experiments (A-C) and the test day of the acute experiment (D).

5.3.1. Baseline locomotor activity

NK1R$^{-/-}$ mice have previously been shown to be hyperactive in the novel, light zone of a light-dark exploration box. In order to establish whether this hyperactivity is present under different conditions, baseline locomotor activity was analysed over the first 30 min of the habituation period at the start of each of the four locomotor activity experiments. For every experiment, this was the first time the animals had encountered the locomotor activity chambers, meaning that baseline locomotor activity was always assessed in a novel environment. Independent-samples t-tests were performed on baseline locomotor activity data from each experiment. NK1R$^{-/-}$ mice displayed significant hyperactivity in experiments C and D compared with wild-types (p=0.001 and p=0.003 respectively; Figure 5.6C&D), while in Experiment A their hyperactivity fell just short of statistical significance (p=0.051; Figure 5.6A). In Experiment B, however, the activity of NK1R$^{-/-}$ mice was indistinguishable from that of wild-type animals (p=0.689; Figure 5.6B).

5.3.2. Acute amphetamine increases locomotor activity in NK1R$^{-/-}$ mice

Locomotor activity in response to acute amphetamine or saline was recorded on Day 1 of the three sensitisation experiments (A-C), when animals received their first injection of amphetamine or saline, as well as on the test day of Experiment D. The mice in Experiment D had received once-daily saline injections in their home cages for three days prior to the test day. A three-way split-plot repeated measures ANOVA on Day 1 was used to analyse locomotor activity response to acute amphetamine.

5.3.2.1. *Experiment A: Amphetamine (4 mg/kg) or saline in rectangular chambers*

A significant main effect of genotype (0.056) , treatment (0.001) and a genotype x treatment interaction (0.016) was observed with the three-way ANOVA. Subsequent two-way analysis in each genotype revealed a highly significant treatment effect in NK1R$^{-/-}$ (p=0.005) but not wild-type (0.155) mice (Figure 5.5A). Figure 5.6A (Day 1) illustrates the total locomotor activity over the first 30 min of the first day of Experiment A. Here it can be seen that during the first 30 min of the experiment the acute response to amphetamine is similar in wild-type and NK1R$^{-/-}$ mice.

5.3.2.2. *Experiment B: Amphetamine (4 mg/kg) or saline in circular corridors*

Three-way split-plot repeated measures ANOVA on Day 1 of Experiment B (Figure 5.5B) revealed a significant effect of treatment (p=0.011) and genotype (0.049). Two-way ANOVA in each genotype revealed a significant effect of treatment in NK1R$^{-/-}$ mice (p=0.026) but, surprisingly, not wild-types (p=0.592) (Figure 5.5B). In the first 30 min of the experiment (Figure 5.6B) amphetamine had a significant effect on NK1R$^{-/-}$ (p=0.004) but not wild-type (p=0.276) mice.

5.3.2.3. *Experiment C: Amphetamine (2.5 mg/kg) or saline in circular corridors*

In the third locomotor activity experiment, mice were given a lower dose of amphetamine. The three-way ANOVA showed a highly significant main effect of treatment (p<0.001) but not genotype (p=0.550) and no genotype x treatment interaction (p=0.159), demonstrating that, at 2.5 mg/kg, amphetamine increased locomotor activity of wild-type and NK1R$^{-/-}$ mice, with no difference between genotypes.

5.3.2.4. *Experiment D: Amphetamine (2.5 mg/kg) or saline in circular corridors, with prior habituation to saline injections*

No significant effects were observed following the three-way ANOVA, and a two-way ANOVA of total locomotor activity data from the first 30 min of the experiment was conducted with genotype and treatment as between-subjects factors, but did not reach statistical significance (p=0.063), precluding further probing of the data.

5.3.3. Wild-type and NK1R$^{-/-}$ mice consistently develop sensitisation to amphetamine

The animals in experiments A-C received a single daily i.p. injection of saline or amphetamine (2.5 or 4 mg/kg) for four days. After a 3 day withdrawal period, an amphetamine challenge (2.5 or 4 mg/kg i.p.) was administered to all animals. To determine the effect of repeated amphetamine injections compared with repeated saline injections, activity of the animals on Day 8 was analysed using a three-way split-plot repeated measures ANOVA in all three experiments. To determine whether animals sensitised to repeated amphetamine compared with a single dose, total activity over the first 30 min following injection on Day 1 and Day 8

was analysed using a three-way repeated-measures ANOVA, with day as the within-subjects factor and genotype and treatment as between-subjects factors. In all three sensitisation experiments, the locomotor activity of all animals receiving amphetamine was augmented on Day 8 compared to Day 1, and the response of amphetamine-treated animals was augmented compared to saline-treated animals on Day 8.

5.3.3.1. *Experiment A: Amphetamine (4 mg/kg) or saline in rectangular chambers*

Three-way split-plot repeated measures analysis of locomotor activity on Day 8 (Figure 5.5A) showed a significant effect of treatment (p=0.001) and a treatment x genotype interaction (p=0.016). The response in NK1R$^{-/-}$ mice was slightly increased compared with wild-types but the genotype comparison did not reach statistical significance (p=0.056). In the repeated measures analysis of total locomotor activity over the first 30 min on Day 1 compared with Day 8 (Figure 5.6A), a significant main effect of day (p<0.001), and a day x treatment interaction (p=0.001) were observed within subjects, while between-subjects analysis revealed a significant main effect of treatment (p<0.001) but not genotype (p=0.426).

5.3.3.2. *Experiment B: Amphetamine (4 mg/kg) or saline in circular corridors*

The second experiment used the same dose of amphetamine but different locomotor activity chambers. A main effect of treatment (0.025) but not genotype (0.926) or genotype x treatment interaction (0.914) was found using the three-way ANOVA on Day 8 (Figure 5.5B). A significant main effect of treatment (p<0.001) but not genotype (0.612) was revealed with between-subjects analysis between Day 1 and Day 8 (Figure 5.6B), while within-subjects analysis showed a significant main effect of day (p<0.001) as well as a day x treatment interaction (p<0.001) but no interaction between day and genotype (p=0.309), indicating that wild-type and NK1R$^{-/-}$ mice respond similarly to repeated amphetamine.

5.3.3.3. *Experiment C: Amphetamine (2.5 mg/kg) or saline in circular corridors*

Finally, in the third sensitisation experiment, a lower dose of amphetamine was used and the locomotor activity of animals was assessed in the same circular corridors used in Experiment B. Here, the three-way repeated measures ANOVA on Day 8 (Figure 5.5C) again showed a significant effect of treatment (p=0.004), and no effect of genotype (0.295) or genotype x

treatment interaction (p=0.281). The comparison between Day 1 and Day 8 (Figure 5.6C) again revealed a significant effect of day (p<0.001) and a day x treatment interaction (p<0.001) within subjects, as well as a main effect of treatment (p<0.001) but not genotype (p=0.272) or genotype x treatment interaction (0.492) between subjects, indicating that as for the 4 mg/kg dose, there is no difference in response to repeated amphetamine between wild-type and NK1R$^{-/-}$ mice.

5.4. Discussion

5.4.1. Variable baseline locomotor activity of NK1R$^{-/-}$ mice

Locomotor activity was measured in wild-type and NK1R$^{-/-}$ mice before each behavioural experiment. In Experiments C and D, significant hyperactivity is observed in NK1R$^{-/-}$ mice compared with their wild-type counterparts. In Experiment A, NK1R$^{-/-}$ mice are also more active than wild-type animals, although this fell just short of statistical significance (p=0.051), which can possibly be attributed to an underpowered NK1R$^{-/-}$ group, resulting from the exclusion of two mice as they escaped from the activity boxes during the recording. However, in Experiment B, the activity of NK1R$^{-/-}$ mice was indistinguishable from that of wild-types. This result is surprising given the hyperactivity observed in the other three experiments. It is also inconsistent with numerous previous studies from our lab, both in NK1R$^{-/-}$ mice and in wild-type mice given NK1R antagonists (Fisher et al., 2007; Herpfer et al., 2005; McCutcheon et al., 2008; Yan et al., 2010), although there has been some inconsistency in reports of hyperactivity from earlier studies also in our lab, which failed to find hyperactivity in NK1R$^{-/-}$ mice (Murtra et al., 2000; Ripley et al., 2002).

The simplest explanation for this variability is methodological differences such as the inclusion of a habituation period or lighting levels in the testing room. Other unavoidable external stressors may range from animal husbandry to the conditions of transport between the colony housing room and the test arenas. The experiments described in this chapter were carried out over a two year period, during which the alterations in environmental stressors such as these may have been considerable. There is a substantial body of evidence implicating substance P and the NK1 receptor in responses to stressful stimuli. However, in studies examining

corticosterone levels in NK1R$^{-/-}$ mice, one study found that exposure to a mild stressor (elevated plus maze) raised serum corticosterone in wild-type but not NK1R$^{-/-}$ mice (Santarelli et al., 2001) while another found no differences in corticosterone between the genotypes in the highly stressful restraint stress paradigm (McCutcheon et al., 2008). While these conflicting results could be explained by the fact that the experiments were carried out in mice on different genetic backgrounds, the implication for two different types of stressors having differential effects on the hypothalamic-pituitary-adrenocortical (HPA) axis is of interest with regard to the present results, as it appears that a mild stressor such as those to which the animals in the present experiments would have been exposed, may in fact have a greater influence in NK1R$^{-/-}$ animals than a more intense stressor.

5.4.2. Amphetamine stimulates locomotor activity of NK1R$^{-/-}$ mice

In the experiments described here, locomotor activity following amphetamine was consistently greater than that following saline. The augmentation of locomotor activity reached statistical significance in Experiments A-C, and in Experiment D, while significance was not reached likely due to the small sample size (n=4), a trend towards an increase in activity was observed. Furthermore, amphetamine-induced hyperactivity was seen in NK1R$^{-/-}$ but not wild-type mice in Experiment B (discussed further below). The increase in locomotor activity in NK1R$^{-/-}$ mice in response to amphetamine is surprising given the previously observed reduction in hyperactivity following acute amphetamine (Yan et al., 2010).

There are a number of methodological differences between the present experiments and those in which the hyperactivity of NK1R$^{-/-}$ mice is reduced with psychostimulants, which could account for the difference in results. The previous experiments were carried out in a light-dark exploration box, where the animal was habituated in the dark zone for 60 min, injected with saline or drug and returned to the dark zone. After a further 30 min the animal was moved into the novel light zone and its activity recorded. In the experiments described here, however, habituation, injection and activity assessment all occurred in the same lit chamber, and locomotor activity was measured over the first 30 min immediately after the animals had been injected, as in these experiments it was noted that the peak of activity occurs within 30 min.

There are two key differences between these two methods: the first is that in the previous study the activity of the animals was recorded in a novel environment, while in the present

experiments the animals were kept in the same (familiar) locomotor activity chamber throughout the whole experiment; the second is that the animals in the experiments described by Yan et al. (2010) were exposed to a 90 minute dark phase, while the experiments presented here were carried out entirely in the light – the animals were taken from their home cages in a lit room (lights on in the housing room from 8 a.m. until 8 p.m.) directly into the lit activity chambers. One explanation for the different results is that testing in a novel environment may uncover differences in stimulated locomotor activity, and that the addition of a habituation period normalises behaviour to that of wild-types. Indeed, the NK1R$^{-/-}$ mice in the present experiments were no longer hyperactive after the habituation period; this also suggests that the striatal dopamine response to amphetamine may be normal in NK1R$^{-/-}$ mice after a period of habituation. In addition, the animals in the study by Yan et al. were injected in the familiar (habituated) environment and placed back in the same environment for a further 30 min, before being introduced to the novel light zone at a time that corresponds with the peak of amphetamine-stimulated activity (around 30 min). This specific procedure may have heightened the novelty of the light zone, thus providing an opportunity to uncover a reduction in hyperactivity with psychostimulants. Alternatively, the addition of a dark “pulse” during the light phase of their normal 12 h light cycle could affect the arousal, and thus locomotor activity, of the animals (Mendoza et al., 2007; Tsai, 2009). Experiments are currently underway in our lab to establish whether having the circular corridor equipment lights off for the habituation period, before injecting the animals and monitoring their locomotor activity when the lights have been turned on, will reproduce the previously-observed reduction in hyperactivity with amphetamine.

If the amphetamine-induced responses of the NK1R$^{-/-}$ mouse are indeed so profoundly affected by the occurrence of a dark pulse, this may indicate that there is an interaction between NK1 receptor, drug response and circadian rhythms. Circadian rhythm alterations have been linked to the NK1 receptor (Challet et al., 1998). In addition to being expressed in the raphe nuclei, the NK1 receptor is found in the intergeniculate leaflet of the thalamus and the suprachiasmatic nucleus of the hypothalamus (Mick et al., 1994), both of which regulate circadian rhythms, and systemic administration of an NK1 receptor antagonist has been shown to inhibit light-induced phase advances in hamsters (Gannon & Millan, 2005). The involvement

of the NK1 receptor in circadian rhythm control is in line with its implication in ADHD, as circadian rhythms are disrupted in the disorder (Chiang et al., 2010).

5.4.3. NK1R$^{-/-}$ mice develop sensitisation to amphetamine at 2.5 and 4 mg/kg

Sensitisation is defined as the increased response to a drug following previous drug exposure. This is often taken as the response of an animal to its final (challenge) injection in a series, compared with its response to the first injection it received. In the experiments presented here, locomotor activity of animals of both genotypes was found to increase between Day 1 and Day 8 in all three sensitisation experiments with a significant effect of treatment. However, this method disregards any cross-sensitisation to stressors that may occur during the pre-treatment stage, such as multiple injections and exposures to the test arena. Indeed, an increase in locomotor activity can be seen between Day 1 and Day 8 in animals pre-treated with saline before their amphetamine challenge. For this reason, the degree of sensitisation is determined in this thesis by comparing the response of animals on Day 8, when all animals have received the same number of procedures (injections, handling and exposure to the test arena) and the specific response to the drug itself can be isolated.

On Day 8 of each sensitisation experiment, the locomotor activity response of both NK1R$^{-/-}$ and wild-type animals to an amphetamine challenge was greatly increased following repeated amphetamine pre-treatment compared with saline pre-treatment, demonstrating that NK1R$^{-/-}$ mice develop robust sensitisation to amphetamine to the same degree as wild-types, at two different doses of amphetamine.

It is well documented that an increase in striatal dopamine plays a key role in mediating locomotor activity responses following amphetamine in mice and other animals. Furthermore, evidence from both animal and human studies indicates that dopamine in the dorsal striatum is responsible for cue-conditioned behavioural responses and drug cue-elicited craving (Vanderschuren et al., 2005; Volkow et al., 2006; Volkow et al., 2008). Locomotor sensitisation has for many years been considered as a model of neurobehavioural plasticity that underlies aspects of drug addiction including reward and craving (Ito et al., 2002; Pierce & Kalivas, 1997; Robinson & Berridge, 1993; Vanderschuren & Kalivas, 2000). NK1R$^{-/-}$ mice developed the same degree of sensitisation to amphetamine as wild-types, despite the striking deficit in striatal dopamine response to amphetamine reported by Yan et al. (2010). This is in accordance with

the finding in the previous chapter that the animals exhibit normal striatal ERK activation following a single injection of amphetamine, as ERK activation has been demonstrated to mediate the long-lasting effects of a single exposure to a drug (Valjent et al., 2005). In addition, amphetamine-stimulated dopamine efflux in the ventral striatum has not been examined in these animals, and is also known to play a significant role in the development of sensitisation. Taking this into account, the behavioural results presented here may suggest that ventral striatal dopamine function is normal in NK1R$^{-/-}$ mice; this requires investigation. Furthermore, emerging evidence suggests that dopamine systems in the ventral striatum are involved in the initial stages of drug taking, while progressively dorsal areas are recruited with repeated drug consumption (Belin et al., 2009; Everitt & Robbins, 2005). The striatal efflux of dopamine following repeated administration of amphetamine, or other drugs of abuse, has as yet not been investigated in NK1R$^{-/-}$ mice. However, if the deficit in dorsal striatal dopamine efflux is maintained upon repeated drug exposure, it may be expected that the behaviour of NK1R$^{-/-}$ mice would differ from that of wild-types in drug self-administration and drug-seeking paradigms, which provide a more detailed model of the transition from initial drug taking to compulsive drug use and addiction.

5.4.3.1. *Non-dopaminergic contributions to sensitisation and hyperactivity*

Despite decades of research focusing on dopamine as the critical neurotransmitter in the development of sensitisation, there is a comparatively small yet convincing body of evidence that argues that other monoaminergic systems are in fact critical to the process. For example, studies using D1 receptor knock-out mice have shown that although the animals lack an acute locomotor response to psychostimulants, sensitisation can still develop at certain doses and treatment regimens (El-Ghundi et al., 2010; Karlsson et al., 2008). Similarly, the hyperactivity of DAT knock-out (DAT$^{-/-}$) mice is reduced by amphetamine and methylphenidate (Gainetdinov et al., 1999), despite the fact that they lack the primary site of action for psychostimulant drugs. Other studies have identified a critical role for $\alpha 1_b$ adrenoceptors and 5-HT$_{2A}$ receptors in the development of sensitisation (Bizot et al., 2007; Drouin et al., 2002). Together, such data indicate that sensitisation results from a separation of normally orchestrated serotonergic and noradrenergic systems (Lanteri et al., 2009; Lanteri et al., 2007; Lanteri et al., 2008; Salomon et al., 2007; Tassin, 2008).

5.4.4. Effect of previous saline injections on amphetamine-stimulated locomotor activity

Interestingly, in Experiment D, amphetamine failed to produce a significant effect in either wild-type or $NK1R^{-/-}$ mice, although there was a trend for it to increase activity in both genotypes. This could be due to the fact that there are only four animals per group, which could result in the statistical analysis being underpowered, but there is comparatively little variability within the groups. However another explanation could arise from the fact that this was the only experiment in which the animals were habituated to injections of saline in their home cages before being exposed to the activity chambers on the test day. This explanation is supported by research demonstrating that habituation to handling and injections in the home cage resulted in a reduced locomotor response to cocaine in the test chamber (Wise et al., 1996).

5.4.5. Activity of animals in the two different types of locomotor activity chamber

The failure of amphetamine to stimulate locomotor activity in wild-type mice in Experiment B is surprising, as not only was amphetamine-induced hyperactivity observed in the other experiments described in this chapter, but it is also generally accepted that amphetamine induces hyperactivity in mice and many other species. The sole difference between Experiments A and B was the equipment used.

There was a large degree of within-group variability in Experiment A, carried out in the small rectangular locomotor activity chambers (Figure 5.1). It was noted that shadows cast by the walls of the chambers were unavoidable, and that some animals would be drawn to the darker corners and often sleep there for a considerable proportion of the experiment. Furthermore, the ambient light level varied substantially from chamber to chamber. It was thought that these factors may influence the activity of the animals, and increase the variability of the results, thus potentially masking important differences between the groups. By way of addressing this, Experiment B was conducted using the same dose of amphetamine but in a bank of eight circular corridors ("donut"-shaped locomotor activity chambers, Figure 5.2). Each corridor was individually lit, providing uniform lighting within each chamber, as well as appreciably reducing the variability of light level between the chambers. The shape of these

chambers meant that there were no corners for the animals to hide in, and the eight corridors were contained in a ventilated cabinet, providing an enclosed setting which minimised external distractions. Indeed, the within-group variability was successfully reduced in the remaining three experiments conducted in the circular corridors.

Amphetamine was given at a dose of 4 mg/kg in Experiments A and B. This dose was chosen as it produced a clear hyperactivity in wild-type mice in a pilot study using the rectangular chambers used in Experiment A. It is a moderate dose, and although low to moderate doses of amphetamine have been shown to induce stereotyped behaviour in mice, such as repetitive licking or grooming (Beaulieu et al., 2005), the amount produced at such doses should be insufficient to interfere with locomotor activity (Yates et al., 2007). The same dose was used for Experiment B, yet produced different results. An explanation could be that despite their containment and consistent lighting, the circular corridors themselves may have in fact provided an aversive environment for the mice. The light in each corridor was bright (around 120 lux) and there was considerable background noise from the ventilation fans (although this was consistent, and may equally have served to block out external auditory stimuli). If the environment of the bright circular corridors was indeed aversive to the animals, it may have induced cross-sensitisation between stress and the higher dose of amphetamine in wild-type mice. This would lead to an enhanced drug response and a greater degree of stereotyped behaviours, possibly resulting in a reduction in locomotor activity. Conversely, the NK1R$^{-/-}$ mice, which are commonly reported to have an anxiolytic phenotype, may be more resilient to stressful stimuli and would not experience the same levels of stress or cross-sensitisation of stereotyped behaviours as the wild-type mice, allowing them to explore the locomotor activity chamber to a greater degree than the wild-types.

5.5. Conclusions

The results presented in this chapter demonstrate that the locomotor activity of NK1R$^{-/-}$ mice increases with amphetamine at 2.5 and 4 mg/kg, contrary to previous findings in NK1R$^{-/-}$ mice from our group, suggesting that the responsiveness of NK1R$^{-/-}$ mice to acute amphetamine is sensitive to experimental changes such as novel and familiar environments. In addition, NK1R$^{-/-}$ mice develop locomotor sensitisation to amphetamine to the same degree as wild-type mice

despite the previously reported severe impairment in their striatal dopamine signalling. This supports a recent study in the spontaneously hypertensive rat, a widely used model for ADHD, which was found to sensitise to methylphenidate (Barron et al., 2009). Taken together results may, with cautious extrapolation to the clinical setting, indicate that people with ADHD who are treated with amphetamine are at risk of developing an addiction to psychostimulants, but that they are no more at risk than people exposed to such drugs in the normal population.

Dopamine has been the focus of research into the locomotor activating effects of drugs for many years, though studies have agreed that other systems may contribute towards dopamine’s principle role in the control of sensitisation. However, in recent years a body of substantial evidence has begun to emerge that non-dopaminergic mechanisms play a more significant role in the control and development of sensitisation than previously thought. The results presented in this chapter support a non-dopaminergic contribution to sensitisation, as $NK1R^{-/-}$ mice show no impairment in their ability to sensitise to amphetamine, despite a significantly impaired acute striatal dopamine response.

6. Striatal cholinergic interneurones and transcription factors

6.1. Introduction

Previously in this thesis, NK1R$^{-/-}$ mice have been shown to express normal levels of dopamine transporter (DAT) in the striatum, exhibit a normal activation response of extracellular signal-regulated kinase (ERK) to acute amphetamine, and to develop normal sensitisation to repeated amphetamine, compared with wild-type mice. However, the basal forebrain cholinergic system was found to be altered in NK1R$^{-/-}$ mice, suggesting that cholinergic function is compromised in these animals. This chapter will present data from immunohistochemical studies which examine the expression of two different transcription factors in cholinergic interneurones of the striatum, a second central cholinergic system, either after acute amphetamine or after the sensitisation protocol described in the previous chapter.

6.1.1. Cholinergic interneurones

The NK1 receptor is extensively expressed on cholinergic interneurones in the striatum of rats, non-human primates and humans (Aubry et al., 1994; Gerfen, 1991; Parent et al., 1995), and in wild-type mice (C. Price, unpublished data), indicating that substance P exerts a direct effect on cholinergic transmission in the striatum. Indeed, both systemic and intra-striatal perfusion of the NK1 receptor antagonist CP-99,994 reduces striatal acetylcholine efflux (Anderson et al., 1995) and extremely low doses of substance P induce acetylcholine release from striatal slices, an effect that is inhibited dose-dependently by the NK1 receptor antagonists CP-96,345 and RP-67580 (Guzman et al., 1993).

Cholinergic interneurones, believed to be tonically active neurones (Inokawa et al., 2010), play a key role in the regulation of striatal dopamine release from the midbrain; conversely, dopamine regulates acetylcholine release, with the two neurotransmitters acting antagonistically to maintain a dopamine-acetylcholine balance in the striatum (DeBoer et al., 1996; Kudernatsch & Sutor, 1994; Pisani et al., 2000). Dopaminergic and cholinergic neurones respond in concert to salient events or stimuli that predict reward; cholinergic interneurones pause their tonic firing pattern, while dopamine neurones phasically increase their firing (Graybiel et al., 1994; Schultz et al., 1997). The lack of striatal dopamine response to amphetamine in NK1R$^{-/-}$ mice indicates that the genetic disruption of the NK1 receptor results in a disturbance of this orchestrated striatal control. This chapter examines whether the lack of dopamine response is reflected in an alteration of molecular changes that occur in cholinergic neurones following acute or repeated administration of amphetamine.

6.1.2. Myocyte-enhancer factor 2 (MEF2)

The DNA-binding regulatory protein myocyte-enhancer factor 2 (MEF2) was originally found in muscle where its role in muscle development was discovered (Black & Olson, 1998). However it is now established that MEF2 also plays a significant role in the central nervous system. MEF2 exists in four isoforms (MEF2A-D) that are expressed throughout the brain, and activate transcription of numerous genes including growth factor and stress-induced genes, thus contributing to neuronal differentiation and survival, as well as synapse formation, maintenance and regulation (Flavell et al., 2006; Gaudilliere et al., 2002; Lam & Chawla, 2007; Mao et al., 1999).

MEF2 is phosphorylated in its inactive form. It is activated by dephosphorylation by calcineurin, upon which it acts as a transcriptional activator of genes involved in synaptic remodelling such as activity-regulated cytoskeletal-associated protein (*Arc*) and synaptic RAS GTPase-activating protein (*Syngap1*) (Flavell et al., 2006). However, phosphorylated MEF2 (pMEF2) can also be modified by the small ubiquitin-related modifier (SUMO-1), or sumoylated, which switches its function to a transcriptional repressor. In this way, sumoylated pMEF2 can repress the expression of the transcription factor Nur77, which negatively regulates dendritic differentiation (Scheschonka et al., 2007; Shalizi et al., 2006).

Striatal dopamine depletion induces upregulation of active MEF2 resulting in MEF2-dependent loss of dendritic spines (Tian et al., 2010). Similarly, chronic elevation of dopamine levels in the striatum with psychostimulants increases dendritic spine density on medium spiny neurones (Kim et al., 2009), which is associated with an increase in the phosphorylation of MEF2 and consequently an attenuation of its activity in vivo (Pulipparacharuvil et al., 2008). The experiments presented in this chapter aim to determine whether a similar increase in pMEF2 expression is present in NK1R-expressing cholinergic interneurones in the striatum following administration of amphetamine, and whether the response of NK1R$^{-/-}$ mice differs from that of wild-types, given that they lack the normal dopamine response to amphetamine.

6.1.3. cAMP response element (CRE)-binding protein (CREB)

cAMP response element-binding protein (CREB) is a transcription factor whose role in neuronal plasticity, learning, memory and drug addiction is well characterised (Mayr & Montminy, 2001; Shaywitz & Greenberg, 1999). CREB activity in the dorsal striatum specifically plays a role in habit formation and striatum-dependent procedural learning and memory (Pittenger et al., 2006). CREB is activated by phosphorylation (pCREB) by a variety of stimuli including MAP kinases, protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), which all phosphorylate CREB at the same site, serine 133 (Shaywitz & Greenberg, 1999). Repeated exposure to certain drugs of abuse, including cocaine and amphetamine (Carlezon Jr et al., 1998; Shaw-Lutchman et al., 2003), activates CREB in the striatum. In both dorsal and ventral striatum, CREB acts as a negative regulator of the rewarding properties of drugs of abuse, with overexpression of CREB causing low doses of cocaine to be aversive (Carlezon Jr et al., 1998) and inhibition of CREB sensitising the behavioural response to drugs of abuse (Fasano et al., 2009). The experiments presented in this chapter examine the expression of pCREB in the striatum of NK1R$^{-/-}$ and wild-type mice following sensitisation to amphetamine.

6.1.4. Striatal sub-regions

Evidence is emerging that subdivisions other than those separating the dorsal and ventral striatum are necessary to account in full for the diverse functionality of the striatum. Preclinical experiments involving self-administration protocols have implicated dopaminergic projections to the ventral region of the striatum in goal-mediated (action-outcome) behaviours, in which instrumental actions such as drug or food seeking are made with the

intention of obtaining a goal (Everitt & Robbins, 2005). Conversely, habitual (stimulus-response) behaviours, that arise from over-training and are not related to a goal, are thought to be mediated by dopaminergic projections to the dorsolateral regions of the striatum. It has been suggested that with prolonged exposure to reinforcing stimuli, increasingly dorsolateral mechanisms are recruited that represent the shift from goal-mediated to habitual behaviours (Belin et al., 2009; Everitt & Robbins, 2005), and compulsive drug-taking, a hallmark of addiction, is thought to be an aberrant habitual behaviour (Robbins & Everitt, 1999). ADHD is highly comorbid with drug addiction, and impulsive behaviour in particular has been shown to predispose to compulsive drug taking (Belin et al., 2008). The behaviour of NK1R$^{-/-}$ mice in the light-dark exploration box indicates that they are more impulsive than wild-types in addition to being hyperactive (Yan et al., 2010).

Together with the lack of dopamine response observed in the dorsal striatum following amphetamine, this evidence suggests that there may be a sub-region specific effect of the genetic disruption of the NK1 receptor on striatal function. In the immunohistochemistry experiments presented in this chapter, the striatum was subdivided into ventromedial, ventrolateral, dorsomedial and dorsolateral sub-regions to determine whether any effects resulting from the genetic disruption of the NK1 receptor would predominate in a specific sub-region.

6.2. Methods

6.2.1. Amphetamine administration protocols

6.2.1.1. Acute administration

Animals were habituated to saline injections for three days prior to the test day. On the test day they were habituated to the test environment for two hours before receiving a single i.p. injection of either saline or amphetamine (2.5 mg/kg).

6.2.1.2. Sensitisation

Animals were given daily i.p. injections of amphetamine (2.5 mg/kg) or saline according to the sensitisation protocol described in Chapter 5. Daily injections were administered for four days

in the locomotor activity chambers, followed by a period of three days in the home cages with no injections, before all animals received an amphetamine challenge (2.5 mg/kg) on Day 8.

6.2.2. Immunohistochemistry

Animals were killed by perfusion approximately 4 to 5 h after the amphetamine challenge injection on Day 8. The brains were postfixed for 2 h before being placed in 30 % sucrose solution until sectioning. Forty µm sections were cut in sets of six, allowing 2 striatal sections per animal (around 0.4 to 0.9 mm caudal to bregma) to be used for analysis. Sections were stained for pMEF2, pCREB, ChAT and parvalbumin expression. Details of immunohistochemistry procedures can be found in Chapter 2 and the Appendix.

6.2.2.1. Quantification

Cell bodies expressing fluorescence-labelled antigens were counted under the appropriate filter for excitation of the fluorophore (FITC or Alexa 594). The region of interest was identified at x 10 magnification, and cells were counted at x 20, using a Nikon Eclipse E800 microscope (Nikon Ltd., Kingston-Upon-Thames, UK). A rectangular area of 440 x 600 µm, defined by the eyepiece graticule in the microscope, was selected for analysis in each quadrant of the striatum (Figure 6.1), and the number of ChAT-, pMEF2- or pCREB-immunoreactive cell bodies within this area was counted in two sections per animal, with each side counted separately (resulting in four counts per animal). For each animal, the mean number of counted cells per quadrant was calculated, and this mean was used in the analysis. All quantification was conducted blind to genotype and treatment. The images shown in Figures 6.5 and 6.6 are illustrative only. They were taken on a Leica DMR microscope with a x10 objective, connected to a CCD camera with a different-sized field of view to that of the Nikon eyepiece graticule, and cropped for illustration purposes.

6.2.2.2. Statistical analysis

A three-way multivariate ANOVA was used to analyse immunohistochemical data, with genotype, treatment and sub-region as factors and ChAT and the percent of ChAT cells that co-expressed pMEF2 or pCREB as dependent variables. Subsequent two-way ANOVAs were conducted in each sub-region, with genotype and treatment as between-subjects factors. A

three-way ANOVA was carried out on parvalbumin data with genotype, treatment and region as factors; significance was not reached and so no further analysis was conducted on this data.

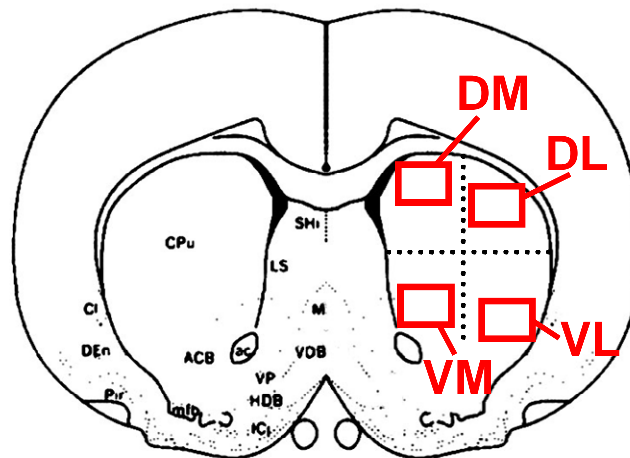

Figure 6.1 Striatal sub-regions as defined for immunohistochemistry.
Diagram showing the location of the counting area in each striatal quadrant used for quantification of immunohistochemical data. DM, dorsomedial striatum; DL, dorsolateral striatum; VM, ventromedial striatum; VL, ventrolateral striatum.

6.2.3. Western blots

Western blot analysis was carried out to determine global striatal levels of MEF2, pMEF2 and ChAT expression in tissue from a separate group of animals that had undergone the same sensitisation protocol detailed in Chapter 5. Animals were killed by i.p. injection of pentobarbital four to five hours after the challenge injection (amphetamine 2.5 mg/kg) on Day 8. Brains were snap-frozen in liquid nitrogen and stored at -80 °C until dissection. Frozen brains were cut into 1 mm coronal sections on dry ice and striatal tissue was dissected from the section that contained the largest portion of the striatum, using a 2 mm diameter tissue punch. Protein was extracted and Western blot analysis was performed on striatal extracts, as described in Chapter 2 and the Appendix.

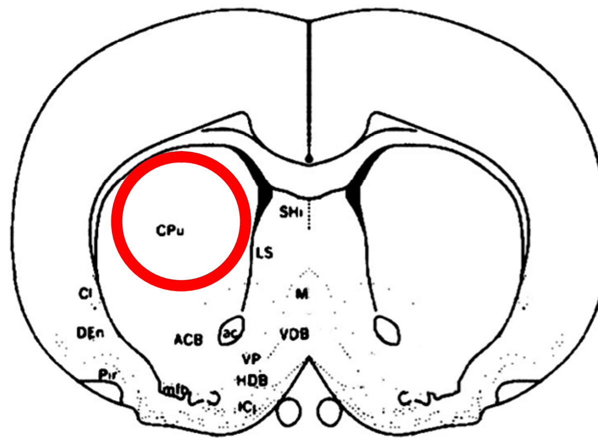

Figure 6.2 Striatal region as dissected for Western blot analysis.

Diagram illustrating the area of dorsal striatal tissue punched from a 1 mm frozen section for Western blot analysis.

6.3. Results

6.3.1. Three-way multivariate analysis of ChAT expression and pMEF2 co-expression

6.3.1.1. *After acute amphetamine*

A three-way multivariate ANOVA with genotype, treatment and sub-region as factors was carried out on neuronal counts of ChAT and the percentage of ChAT cells that co-expressed pMEF2. For ChAT data (Figure 6.3A), significant main effects of genotype ($p<0.001$) and sub-region ($p<0.001$) but not treatment ($p=0.669$) were observed. A genotype x treatment interaction (0.043) and a genotype x sub-region interaction (0.002) were also found. Subsequent analyses are described in the following sections. Significant main effects of treatment ($p=0.045$) and region ($p=0.007$) were found in the number of ChAT cells that co-expressed pMEF2 (Figure 6.4A), with no interactions between factors, however the general model fell short of significance ($p=0.06$) so no further analysis was carried out on this data. Mean (± SEM) data are summarised in Table 6.1.

6.3.1.2. After repeated amphetamine

In a separate experiment, effects of repeated amphetamine on ChAT expression and pMEF2 co-expression in the four striatal quadrants were analysed in the same way as the data from the acute amphetamine experiment. For ChAT data (Figure 6.3B), significant main effects of genotype ($p<0.001$) and sub-region ($p<0.001$) but not treatment ($p=0.159$) were found. No interactions between variables were observed in these data. A main effect of genotype only ($p=0.010$) was observed in the number of cholinergic cells co-expressing pMEF2 (Figure 6.4B), but the failure of the general model to reach significance ($p=0.209$) precluded further analysis. Summary data are presented in Table 6.2.

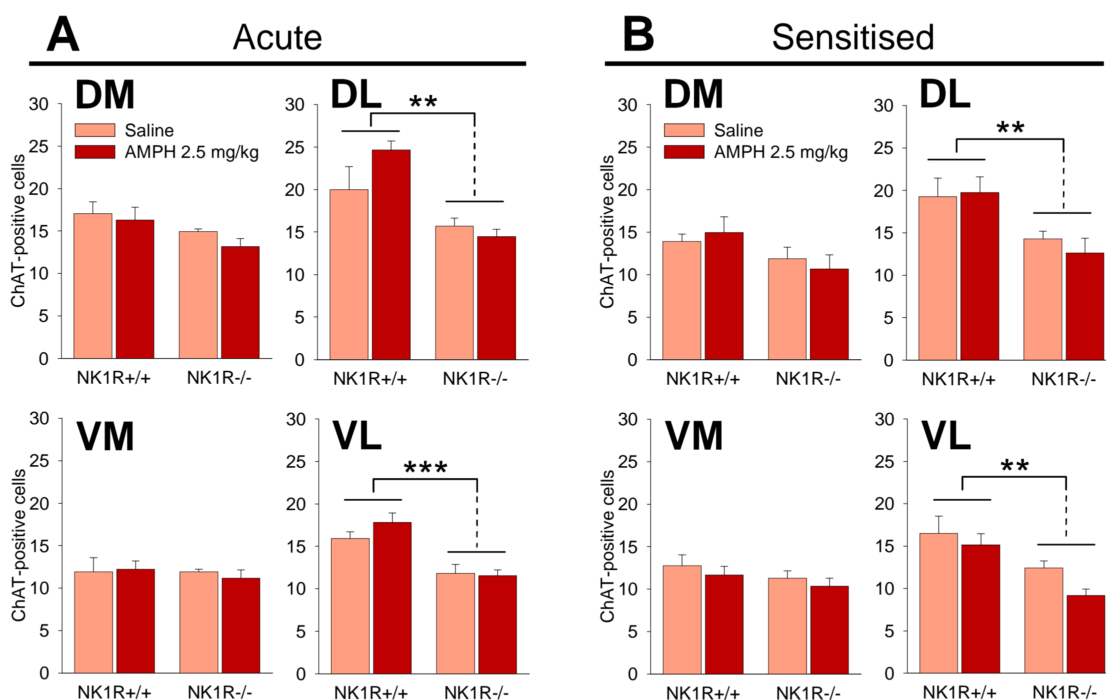

Figure 6.3 ChAT expression in striatal sub-regions.

*Mean (± SEM) number of ChAT-immunoreactive cells in the four quadrants of the striatum following a single i.p. injection (A, n=4) or repeated i.p. injections (B, n=5-6) of saline or amphetamine (2.5 mg/kg). DM, dorsomedial striatum; DL, dorsolateral striatum; VM, ventromedial striatum; VL, ventrolateral striatum. *p<0.05; **p<0.01; ***p<0.001.*

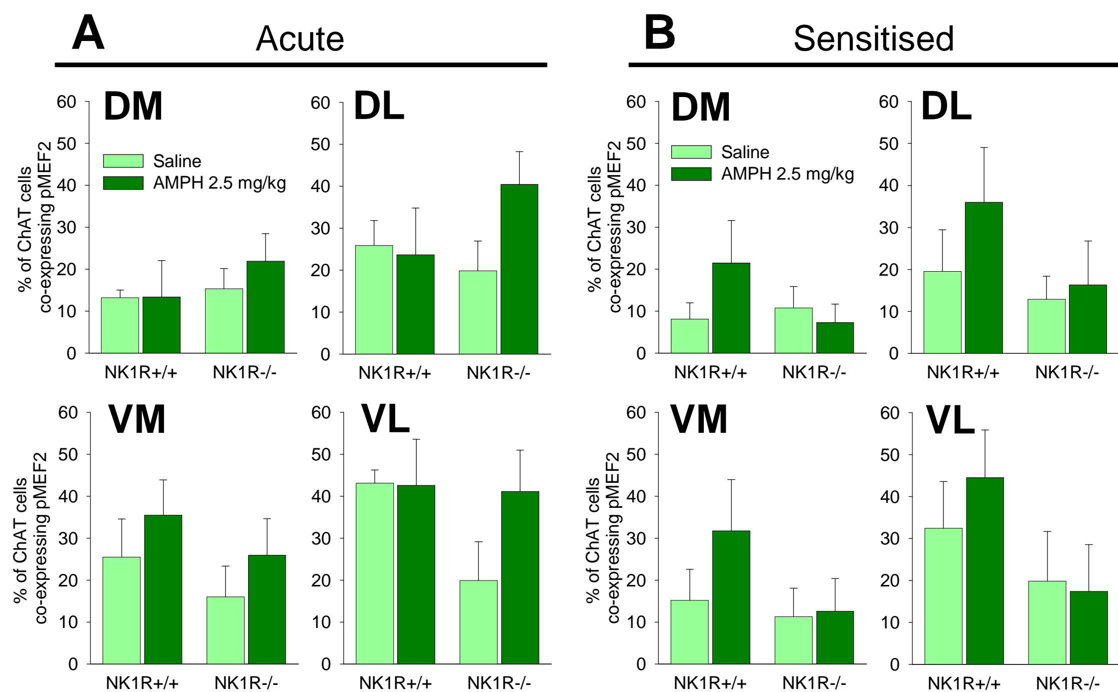

Figure 6.4 pMEF2 expression in striatal cholinergic interneurones.

Percent (Mean ± SEM) of ChAT-expressing cells that co-express pMEF2 following acute amphetamine or saline administration (A; n=4) or repeated amphetamine or saline administration (B; n=5-6) in the four quadrants of the striatum of wild-type and NK1R$^{-/-}$ mice. DM, dorsomedial striatum; DL, dorsolateral striatum; VM, ventromedial striatum; VL, ventrolateral striatum.

6.3.2. Pattern of striatal ChAT expression varies between NK1R$^{-/-}$ and wild-type mice

As the three-way ANOVA reported above was significant for ChAT data, subsequent two-way ANOVAs were carried out in each genotype to determine the effect of striatal sub-region on expression of ChAT. A two-way ANOVA in wild-types revealed a significant effect of region (p<0.001) but not treatment (p=0.167). Post-hoc analysis revealed that expression of ChAT was significantly greater in dorsolateral striatum than in dorsomedial (p=0.001), ventrolateral (p=0.002) and ventromedial (p<0.001) striatum. Similarly, ventromedial expression of ChAT was significantly lower than that seen in dorsomedial (p=0.006) and ventrolateral (p=0.005), while a similar level of ChAT was expressed in ventrolateral and dorsomedial striatum

(p=0.899). In NK1R$^{-/-}$ mice, as in wild-types, a significant effect of region (p<0.001) but not treatment (p=0.092) was observed in a two-way ANOVA. However, post-hoc analysis revealed the distribution of ChAT expression to differ more along a dorsal-ventral rather than a lateral-medial axis, with significant differences between dorsolateral and ventrolateral striatum (p<0.001) and between dorsomedial and ventromedial striatum (p=0.004), whereas expression was similar between dorsolateral and dorsomedial striatum (p=0.225) and between ventrolateral and ventromedial striatum (p=0.878).

Following the sensitisation protocol, a similar pattern was observed in wild-type mice to that following a single injection. However, in NK1R$^{-/-}$ mice, while ChAT expression in ventrolateral and ventromedial sub-regions after the sensitisation protocol was comparable to that seen in the same regions following a single injection, expression in dorsomedial and dorsolateral striatum was no greater than in the ventral sub-regions, and the two-way ANOVA in NK1R$^{-/-}$ mice revealed that the expression pattern was uniform across sub-regions (p=0.090) following sensitisation. A direct comparison between data following acute and repeated injections cannot be made as the experiments were carried out at separate times. The two-way ANOVA carried out in NK1R$^{-/-}$ mice was not significant (p=0.116) thus precluding further analysis of these data.

6.3.3. NK1R$^{-/-}$ mice have a reduction in number of ChAT-expressing cells in lateral striatum compared with wild-types

Two-way ANOVAs were carried out in each quadrant to identify region-specific genotype differences. A significant effect of genotype was found in the dorsolateral (p=0.001) and ventrolateral (p<0.001) quadrant in the acute experiment (Figure 6.3A) and in the sensitisation experiment (Figure 6.3B), where the significance of the genotype effect was p=0.002 and p=0.001 in dorsolateral and ventrolateral striatum respectively, with no treatment effect and no treatment x genotype interaction.

6.3.4. Striatal co-expression of ChAT and pMEF2

As the number of ChAT expressing cells varied throughout the striatum, the number of cells co-expressing pMEF2 was calculated as a percentage of the total number of counted ChAT cells (Figure 6.4). The three-way ANOVA described above revealed a main effect of treatment

(p=0.045) and region (p=0.007) in the acute experiment (Figure 6.3A), while in the sensitisation experiment a main effect of genotype (p=0.010) was observed (Figure 6.3B). Due to a high degree of within-group variability the general model failed to reach statistical significance which precluded further analysis of the data.

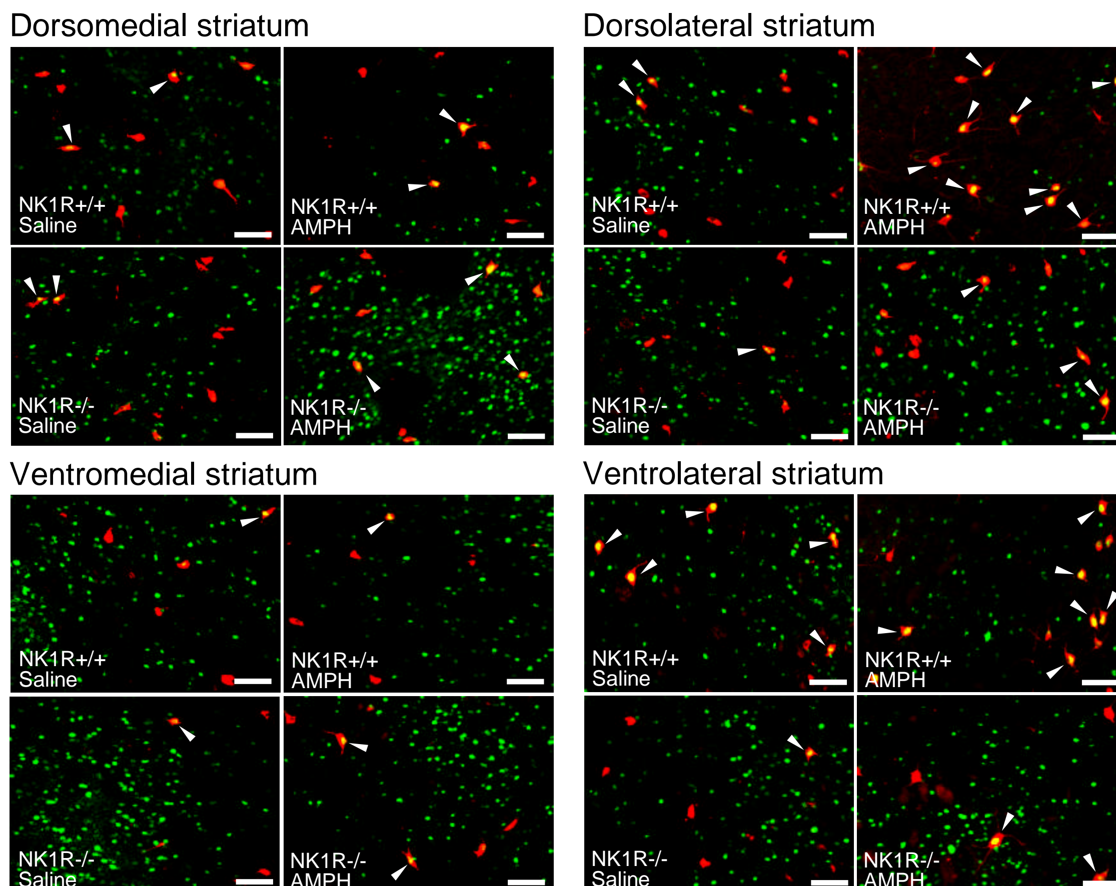

Figure 6.5 Striatal co-expression of ChAT and pMEF2 after acute amphetamine.

Images illustrating ChAT- and pMEF2-expressing cells in the four quadrants of the striatum in wild-type and $NK1R^{-/-}$ mice, following a single i.p. injection of saline or amphetamine (2.5 mg/kg). Red, ChAT-expressing neurones; green, pMEF2-expressing neurones; yellow, double-labelled cells expressing both ChAT and pMEF2. Scale bar, 200 µm.

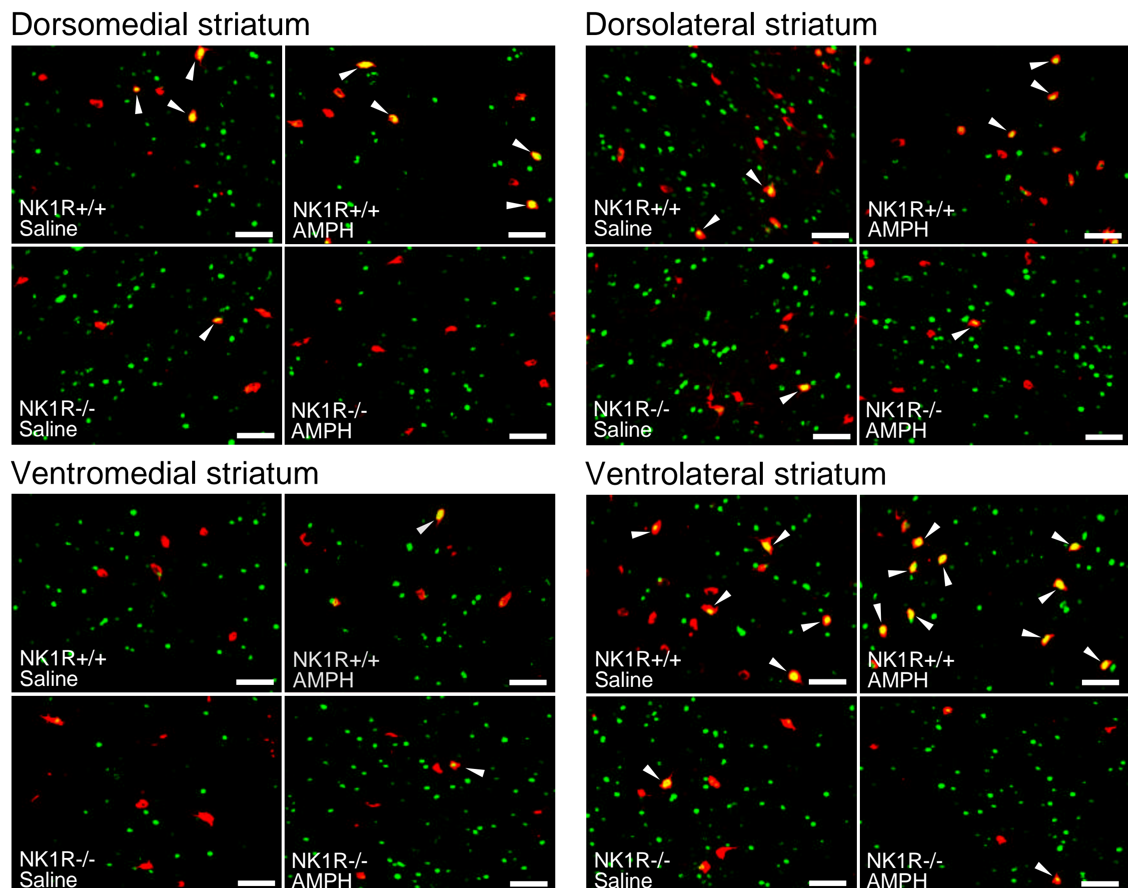

Figure 6.6 Striatal co-expression of ChAT and pMEF2 after repeated amphetamine. *Images illustrating ChAT- and pMEF2-expressing cells in the four quadrants of the striatum in wild-type and NK1R$^{-/-}$ mice, following sensitisation to amphetamine (2.5 mg/kg) compared with saline. Red, ChAT-expressing neurones; green, pMEF2-expressing neurones; yellow, double-labelled cells expressing both ChAT and pMEF2. Scale bar, 200 µm.*

Neuronal counts after a single injection (ChAT + pMEF2)	Genotype	Dorsolateral		Dorsomedial		Ventrolateral		Ventromedial	
		Saline	AMPH	Saline	AMPH	Saline	AMPH	Saline	AMPH
Number of ChAT-immunoreactive cells	Wild-type	20.0 ± 2.7	24.6 ±1.1	17.0 ± 1.4	16.3 ± 1.5	15.9 ± 0.8	17.8 ± 1.1	11.9 ± 1.7	12.3 ± 1.0
	NK1R$^{-/-}$	15.7 ± 1.0	14.4 ± 0.9	14.9 ± 0.3	13.2 ± 0.9	11.8 ± 1.1	11.6 ± 0.7	11.9 ± 0.3	11.2 ± 1.0
Number of pMEF2 + ChAT double-labelled cells	Wild-type	5.2 ± 1.7	5.4 ± 2.3	2.3 ± 0.4	2.2 ± 1.4	6.9 ± 0.4	7.1 ± 2.0	3.4 ± 1.6	4.4 ± 1.1
	NK1R$^{-/-}$	3.1 ± 1.2	5.7 ± 1.4	2.3 ± 0.8	3.1 ± 1.1	2.4 ± 1.2	4.8 ± 1.3	1.8 ± 0.8	3.0 ± 0.9
% cholinergic cells co-expressing pMEF2	Wild-type	25.9 ± 6.0	23.7 ± 11.1	13.2 ± 1.8	13.3 ± 8.7	43.1 ± 3.1	42.6 ± 11.0	25.5 ± 9.1	35.5 ± 8.4
	NK1R$^{-/-}$	19.8 ± 7.1	40.4 ± 7.8	15.4 ± 4.8	21.9 ± 6.6	19.9 ± 9.3	41.1 ± 9.8	16.1 ± 7.3	26.0 ± 8.7

Table 6.1 Striatal expression of ChAT and pMEF2 after acute amphetamine.

Summary table of mean (± SEM) number of ChAT immunoreactive and ChAT pMEF2 double-labelled cells in the four quadrants of the striatum, after a single injection of saline or amphetamine (2.5 mg/kg).

Neuronal counts after sensitisation (ChAT + pMEF2)	Genotype	Dorsolateral		Dorsomedial		Ventrolateral		Ventromedial	
		Saline	AMPH	Saline	AMPH	Saline	AMPH	Saline	AMPH
Number of ChAT-immunoreactive cells	Wild-type	19.3 ± 2.2	19.8 ± 1.8	13.9 ± 0.9	15.0 ± 1.9	16.5 ± 2.0	15.1 ± 1.3	12.8 ± 1.3	11.7 ± 1.0
	NK1R$^{-/-}$	14.3 ± 0.9	12.6 ± 1.7	11.9 ± 1.3	10.7 ± 1.7	12.4 ± 0.8	9.2 ± 0.7	11.3 ± 0.9	10.3 ± 0.9
Number of pMEF2 + ChAT double-labelled cells	Wild-type	4.3 ± 2.3	7.7 ± 2.8	3.2 ± 2.2	3.6 ± 1.8	6.1 ± 2.4	7.2 ± 2.0	2.1 ± 0.9	3.9 ± 1.6
	NK1R$^{-/-}$	2.0 ± 0.9	2.7 ± 1.9	1.0 ± 0.4	1.0 ± 0.7	2.7 ± 1.7	1.6 ± 1.1	1.2 ± 0.6	1.6 ± 1.0
% cholinergic cells co-expressing pMEF2	Wild-type	19.5 ± 10.0	36.0 ± 13.1	8.1 ± 3.9	21.4 ± 10.2	32.4 ± 11.1	44.5 ± 11.4	15.2 ± 7.4	31.7 ± 12.2
	NK1R$^{-/-}$	12.9 ± 5.6	16.3 ± 10.4	10.8 ± 5.1	7.3 ± 4.4	19.8 ± 11.9	17.4 ± 11.1	11.3 ± 6.8	12.6 ± 7.9

Table 6.2 Striatal expression of ChAT and pMEF2 after repeated amphetamine.

Mean (± SEM) number of ChAT-immunoreactive and ChAT + pMEF2 double-labelled cells in the four quadrants of the striatum after sensitisation to amphetamine (2.5 mg/kg).

6.3.5. Striatal co-expression of ChAT and pCREB

Neuronal counts from pCREB-expressing cholinergic cells are displayed in Figure 6.7, however due to the very high degree of within-group variability statistical analysis was not carried out on this data. A summary of mean ± SEM data is given in Table 6.3.

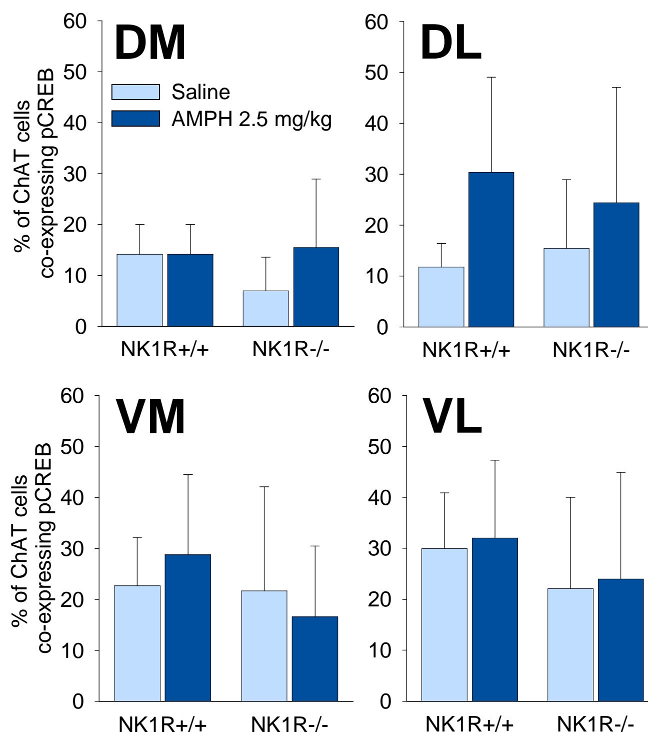

Figure 6.7 pERK expression in striatal cholinergic interneurones.

Percent (Mean ± SEM) of ChAT cells that co-expressed pCREB in the four quadrants of the striatum of NK1R$^{-/-}$ and wild-type mice following repeated i.p. injections of saline or amphetamine (2.5 mg/kg). DM, dorsomedial striatum; DL, dorsolateral striatum; VM, ventromedial striatum; VL, ventrolateral striatum. n=4 per group.

Neuronal counts after sensitisation (ChAT + pCREB)	*Genotype*	*Dorsolateral*		*Dorsomedial*		*Ventrolateral*		*Ventromedial*	
		Saline	*AMPH*	*Saline*	*AMPH*	*Saline*	*AMPH*	*Saline*	*AMPH*
Number of ChAT-immunoreactive cells	Wild-type	20.3 ± 2.4	18.3 ± 1.7	11.6 ± 2.7	10.9 ± 1.4	16.4 ± 1.3	16.6 ± 2.3	12.1 ± 1.4	11.6 ± 2.1
	NK1R$^{-/-}$	13.9 ± 2.9	13.3 ± 2.4	12.8 ± 2.0	10.1 ± 1.6	10.4 ± 1.8	9.9 ± 1.9	9.5 ± 1.5	9.0 ± 2.5
Number of pCREB + ChAT double-labelled cells	Wild-type	2.3 ± 1.0	5.6 ± 3.4	2.0 ± 1.2	1.9 ± 1.2	4.6 ± 1.4	5.9 ± 2.9	3.1 ± 1.4	4.0 ± 2.2
	NK1R$^{-/-}$	3.1 ± 2.8	4.6 ± 4.4	1.2 ± 1.1	1.9 ± 1.7	3.2 ± 2.8	3.5 ± 3.1	2.7 ± 2.5	2.4 ± 2.1
% cholinergic cells co-expressing pMEF2	Wild-type	11.8 ± 4.7	30.4 ± 18.7	14.2 ± 5.8	14.2 ± 5.8	29.9 ± 11.0	32.0 ± 15.3	22.7 ± 9.5	28.8 ± 15.7
	NK1R$^{-/-}$	15.4 ± 13.5	24.4 ± 22.6	7.0 ± 6.6	15.4 ± 13.5	22.1 ± 17.9	24.0 ± 20.9	21.7 ± 20.4	16.6 ± 13.9

Table 6.3 Striatal expression of ChAT and pCREB after repeated amphetamine.

Summary table of mean (± SEM) number of ChAT immunoreactive and ChAT + pMEF2 double-labelled cells in the four quadrants of the striatum, after sensitisation to amphetamine (2.5 mg/kg).

6.3.6. Expression pattern of parvalbumin-expressing neurones is similar in NK1R$^{-/-}$ and wild-type mice

In order to determine whether the reduction in cell number seen in NK1R$^{-/-}$ mice was specific to cholinergic cells, neuronal counts of parvalbumin-expressing cells were carried out in tissue from animals that had undergone the same sensitisation protocol. Data is summarised in Table 6.4. A univariate ANOVA on the number of parvalbumin-expressing cells was conducted, with genotype, treatment and sub-region as independent variables, and resulted in a significant effect of sub-region ($p<0.001$) but not genotype ($p=0.110$) or treatment ($p=0.564$) and no interactions between variables (Figure 6.8). A subsequent one-way ANOVA with post-hoc analysis was carried out to identify specific sub-region differences and revealed that the dorsomedial striatum expressed a greater number of parvalbumin-positive cells than the dorsolateral, ventrolateral or ventromedial striatum ($p<0.001$ for all three comparisons). No differences were found between numbers of parvalbumin-expressing neurones in the dorsomedial, dorsolateral and ventromedial striatum.

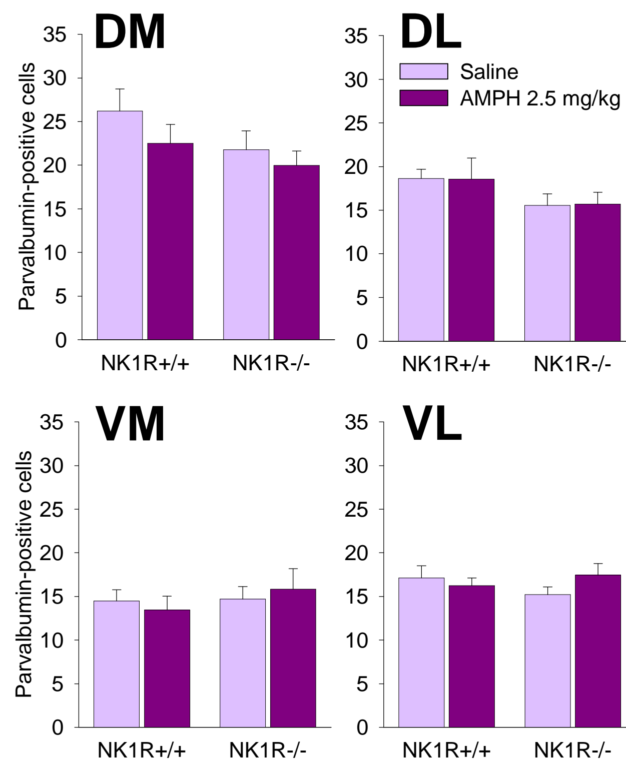

Figure 6.8 Parvalbumin expression in the striatum.

Mean (± SEM) number of parvalbumin-expressing cells in the four quadrants of the striatum in NK1R$^{-/-}$ and wild-type mice following repeated i.p. injections of saline or amphetamine (2.5 mg/kg) (n=5-8 per group).

Neuronal counts after sensitisation (Parvalbumin)	***Genotype***	***Dorsolateral***		***Dorsomedial***		***Ventrolateral***		***Ventromedial***	
		Saline	*AMPH*	*Saline*	*AMPH*	*Saline*	*AMPH*	*Saline*	*AMPH*
Number of PV-immunoreactive cells	Wild-type	18.6 ± 1.1	15.6 ± 3.5	26.2 ± 2.5	19.2 ± 3.8	17.1 ± 1.4	13.8 ± 2.6	14.5 ± 1.3	11.4 ± 2.4
	NK1R$^{-/-}$	15.4 ± 1.4	15.7 ± 1.4	21.4 ± 2.7	20.0 ± 1.6	15.0 ± 1.1	17.5 ± 1.3	15.3 ± 1.9	15.8 ± 2.4

Table 6.4 Striatal expression of parvalbumin after repeated amphetamine.

Mean (± SEM) number of parvalbumin-immunoreactive neurones in the four quadrants of the striatum after sensitisation to amphetamine (2.5 mg/kg).

6.3.7. Western blot analysis of ChAT, MEF2 and pMEF2 expression in striatal extracts following amphetamine sensitisation

Western blot analysis was carried out on dorsal striatal tissue extracts from animals that had undergone the amphetamine sensitisation procedure. A two-way ANOVA was used to analyse each western blot. No genotype or treatment differences were found in either ChAT ($p=0.249$ and $p=0.891$ for genotype and treatment respectively), pMEF2 ($p=0.983$ and $p=0.589$ respectively) or MEF2 ($p=0.724$ and $p=0.114$) expression, or in the ratio of expression of pMEF2:MEF2 ($p=0.623$ and $p=0.198$ for genotype and treatment respectively).

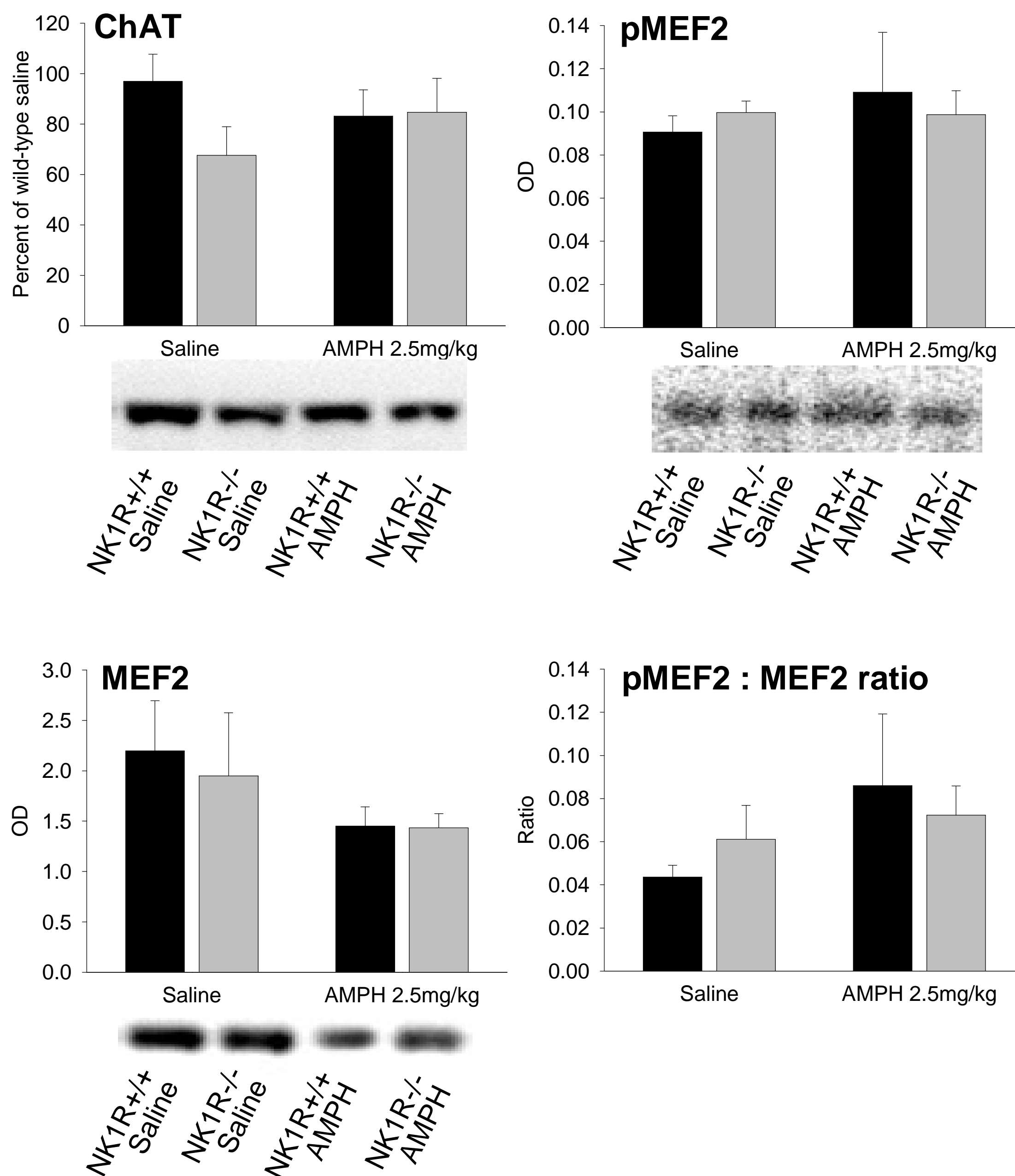

Figure 6.9 ChAT, pMEF2 and MEF2 expression in dorsal striatum.

Representative ChAT (n=7-8), pMEF2 (n=3-4) and MEF2 (n=3-4) western blots, with graphs optical densities following normalisation against total GAPDH protein. pMEF2:MEF2 ratio is also shown. Due to the larger sample number for ChAT analysis, separate gels were used and normalised against the mean value of wild-type saline, in order to minimise cross-gel variability.

6.4. Discussion

6.4.1. Fewer ChAT-expressing neurones in NK1R$^{-/-}$ mouse lateral striatum

The overall distribution pattern of ChAT-expressing cells differed between wild-type and NK1R$^{-/-}$ mice, with wild-type mice exhibiting an increase from dorsomedial to ventrolateral striatum, while that in NK1R$^{-/-}$ mice increased from ventral to dorsal rather than from medial to lateral (although while the difference between ventral and dorsal striatum was significant, the difference in cell number in each region was minor). Specifically, the lateral striatum in NK1R$^{-/-}$ mice contains fewer ChAT-expressing neurones than that in wild-types. The effect was highly significant both after acute and repeated amphetamine or saline. While treatment did not have a significant effect in either experiment, a genotype x treatment interaction was observed in the animals that had received a single injection, suggesting that in some way a single injection of amphetamine enhances the genotype difference, and indicating that there is a subtle difference between wild-type and NK1R$^{-/-}$ mice in the cholinergic response to amphetamine. While ChAT-expressing cell number did not differ significantly between saline or amphetamine treatments in either genotype, the response following amphetamine was enough to bring out differences between wild-type and NK1R$^{-/-}$ mice, which could potentially manifest in differences in behavioural response to amphetamine between the genotypes. While there is a risk of sampling error when a large structure such as the striatum is divided into small sections for analysis, the possibility that the present result could be a false positive due to sampling error is unlikely, as the neuronal counts were conducted blind to genotype and treatment, and the result was replicated in two separate experiments.

A decrease in ChAT-expressing cell number could simply mean that cholinergic neurones are synthesising a reduced amount of ChAT, rendering some neurones undetectable by the methods employed in the present experiments. As acetylcholine is synthesised by ChAT this would suggest that less acetylcholine is being produced. A number of other factors contribute to the synthesis and degradation of acetylcholine, including the activity (rather than simply the presence) of ChAT, regulated by different patterns of phosphorylation (Dobransky & Rylett, 2003), and the amount or activity of acetylcholinesterase at the synapse. None of these factors, nor the extracellular level of acetylcholine in the striatum, have been measured in NK1R$^{-/-}$ mice to date. Alternatively, assuming that ChAT is a stable marker of cholinergic

neurones, a reduction in the number of cells expressing ChAT suggests that there are fewer cholinergic cells. The effect could occur during embryonic development, as it is possible that the genetic disruption of the NK1 receptor interrupts the normal development or migration of striatal cholinergic neurones. Conversely, a form of cell death could be occurring. Indeed, an elevated level of NK1 receptor has recently been observed in cell lines from human malignant melanomas, while application of the NK1 receptor antagonist aprepitant specifically causes apoptotic cell death (Munoz et al., 2010), and the NK1 receptor has been shown to modulate methamphetamine-induced striatal apoptosis in mice (Zhu et al., 2009). As the NK1 receptor is expressed extensively on cholinergic interneurones in the striatum, this suggests that apoptosis may be occurring in some cells in which expression of the NK1 receptor has been disrupted. The insertion of the *LacZ* reporter gene into exon 1 of the NK1 receptor gene causes the production of beta-galactosidase (β-gal) in cell populations that would normally express the NK1 receptor, and the expression of β-gal could be used as a marker in $NK1R^{-/-}$ mice in order to verify whether there is cell death in these populations. In the present study, the genotype difference in neuronal number was specific to cholinergic cells, as no differences were found between $NK1R^{-/-}$ and wild-type mice in the number of parvalbumin-expressing neurones throughout the striatum. Parvalbumin neurones constitute a proportion of the GABAergic interneurones in the striatum, and do not express the NK1 receptor. However, the NK1 receptor is also expressed on striatal somatostatin interneurones, which are yet to be examined in $NK1R^{-/-}$ mice following amphetamine. Neuronal counts of somatostatin-expressing cells would determine whether the number of all cells expressing the NK1 receptor is reduced in $NK1R^{-/-}$ mice following amphetamine, or whether the effect observed is specific to the cholinergic population.

Enhanced β-gal activity has been linked to accumulation of autophagic vacuoles (Gerland et al., 2003), which in turn is associated with accumulation of protein aggregates in neurodegenerative diseases such as Alzheimer's and Huntington's (Funderburk et al., 2010; Turmaine et al., 2000). This may contribute to the loss of cholinergic cells observed here. Alternatively, rather than neurodegeneration of cholinergic cells, $NK1R^{-/-}$ and wild-type cholinergic interneurones may undergo different patterns of migration during development, which would explain the sub-region specific effect. Substance P is known to be involved in the active migration of tumour cells (Lang et al., 2004) and has been suggested to play a role in the

early neuronal differentiation (Karkkainen et al., 2009; Reynolds et al., 1992). In NK1R$^{-/-}$ mice, the lack of NK1 receptor during development may result in the aberrant development and distribution of striatal cholinergic interneurones observed here.

Striatal cholinergic interneurones, all of which express the NK1 receptor (Aubry et al., 1994; Gerfen, 1991), influence locomotor activity by modulating striatal dopamine release, which activates GABAergic medium spiny neurones (Pisani et al., 2007; Wang et al., 2006). As basal dopamine efflux is normal in NK1R$^{-/-}$ mice (Yan et al., 2010), the baseline hyperactivity exhibited by NK1R$^{-/-}$ mice must be caused via a mechanism other than increased basal dopamine resulting from the reduced cholinergic cell number (or lower level of acetylcholine). However, the results presented in this thesis indicate that upon the addition of a habituation period the previously observed hyperactivity diminishes, suggesting that neurochemical differences between NK1R$^{-/-}$ and wild-type mice may also diminish. Cholinergic neurones pause in firing in response to events of salience, which has been shown to coincide with burst-firing of midbrain dopaminergic neurones (Morris et al., 2004; Wang et al., 2006), so it is possible that the reduced number of cholinergic neurones affects responses only to novel environments or events. Reducing striatal acetylcholine has been shown to result in similar behavioural and neurochemical responses to those seen in NK1R$^{-/-}$ mice. For example, ablation of striatal cholinergic interneurones causes hyperactivity in a novel, but not habituated, environment (Kitabatake et al., 2003), and reduces striatal acetylcholine levels without affecting dopamine levels, but disrupts the convergent action of acetylcholine and dopamine on medium spiny neurones (Kaneko et al., 2000). A similar disruption of acetylcholine and dopamine regulation of medium spiny neurones in NK1R$^{-/-}$ mice would explain the hyperactivity seen in NK1R$^{-/-}$ mice without increased basal dopamine efflux.

However, amphetamine-stimulated striatal dopamine release is also abolished in NK1R$^{-/-}$ mice (Yan et al., 2010). Cholinergic interneurones are tonically active but pause in response to a salient event or reward, which has been shown to coincide with burst-firing of midbrain dopaminergic neurones (Morris et al., 2004; Wang et al., 2006). Zhou et al (2001) demonstrated in mouse striatal slices that when acetylcholine release from tonically active cholinergic interneurones is inhibited, stimulus-evoked dopamine release is dramatically reduced. The reduction in striatal ChAT seen in NK1R$^{-/-}$ mice (whether representing a reduction in cell number or reduction in acetylcholine production) may mean that upon a rewarding

stimulus such as amphetamine, the pause signal from cholinergic neurones is diminished, resulting in a lower level of burst-firing of dopaminergic cells and a lack of striatal dopaminergic response to amphetamine in NK1R$^{-/-}$ mice.

While the ventromedial striatum is associated with goal-mediated actions (action-outcome), and reinforcing stimuli, the lateral and dorsal sub-regions are associated with habitual responses (stimulus-response) and locomotor activity (Everitt & Robbins, 2005). The fact that the differences between ChAT expression in wild-type and NK1R$^{-/-}$ mice are confined to the lateral portions of the striatum supports behavioural studies presented both in this thesis and previously, that whilst NK1R$^{-/-}$ mice have a hyperactive phenotype, they do not differ from wild-types in paradigms that model aspects of reward to psychostimulants, such as self administration or conditioned place preference with cocaine (Ripley et al., 2002) and sensitisation to amphetamine (Chapter 5).

The results presented in this chapter provide further evidence that the NK1 receptor is essential for a fully functioning striatal cholinergic system, suggesting that the development of cholinergic cells without functional NK1 receptors affects either the number of cells that develop, or the production of ChAT, potentially leading to a lower level of acetylcholine in the striatum.

6.4.2. pMEF2 and pCREB expression

pMEF2 expression was measured in cholinergic interneurones by neuronal counts of cells co-expressing ChAT and pMEF2. The heaviest staining was located in the lateral sub-regions of the striatum. A significant main effect of treatment was found in the acute experiment, while in the sensitisation experiment no effect of treatment was observed, suggesting that amphetamine increases pMEF2 expression after a single injection, but that with repeated injections the effect diminishes. However, as the experiments were conducted at separate times they must be compared with caution. The trends observed suggest that any effect of treatment after acute amphetamine is restricted to NK1R$^{-/-}$ mice, however due to the large amount of variability in pMEF2 neuronal counts no statistical genotype effects were found in this experiment. Conversely, a significant genotype effect was found in pMEF2 expression in the sensitisation experiment, indicating that the number of cholinergic cells expressing pMEF2 is lower in NK1R$^{-/-}$ mice than in wild-types.

Interestingly, while a single injection of amphetamine appears to increase pMEF2 expression in cholinergic interneurones of NK1R$^{-/-}$ mice, the same effect is not seen in those mice in the sensitisation experiment who had received amphetamine for the first time after repeated saline injections. While statistical analysis cannot be carried out to compare the two experiments directly as they were conducted separately, the lack of amphetamine effect after repeated injections again suggests that following a period of habituation (i.e. repeated saline injections) responses of NK1R$^{-/-}$ mice become indistinguishable from those in wild-types.

The variability in pMEF2 expression in cholinergic neurones may be reduced by repeating the experiment with an increased number of animals, which may draw out genotype or region-specific differences. There is indication of a trend in which acute amphetamine increases pMEF2 expression in NK1R$^{-/-}$ mice whilst having no effect in wild-types, whereas the reverse trend is suggested following the sensitisation experiment, with amphetamine appearing to have a consistent effect of increasing pMEF2 expression in wild-type mice, while having no effect in NK1R$^{-/-}$ animals. The study reported by Pulipparacharuvil et al (2008) showed that pMEF2 expression increased in the nucleus accumbens following repeated administration of cocaine. The trends observed in the data presented in this chapter suggests that a similar response may be observed with amphetamine in wild-type mice but not in NK1R$^{-/-}$ mice, however due to the lack of statistical significance few conclusions can be drawn from this data. Similarly, the pCREB response following acute amphetamine was also too variable to determine any effects, either of drug or of genotype. Importantly, however, this is the first time either pCREB or pMEF2 have been identified in cholinergic interneurones, and further increases the importance of these neurones in striatal regulation.

Finally, Western blots were carried out to provide quantitative analysis of global dorsal striatal ChAT and pMEF2 expression. No genotype or treatment differences were observed in either ChAT, MEF2 or pMEF2 expression. Although a trend towards a reduction in ChAT expression in NK1R$^{-/-}$ mice was observed after saline, the inclusion of the whole dorsal striatum appeared to dilute the differences in ChAT expression observed between NK1R$^{-/-}$ and wild-type mice when individual quadrants were analysed, further indicating that the genotype and drug effects described above are specific to particular sub-regions.

6.4.3. Conclusions

The results presented in this chapter demonstrate that the striatal cholinergic system is compromised in NK1R$^{-/-}$ mice, particularly in the lateral striatum. The normal expression of parvalbumin-positive cells in these animals confirms that the effect is specific to cholinergic interneurones. Considering the NK1R$^{-/-}$ mouse as a model for ADHD, the present results suggest that the cholinergic system is responsible for the dopaminergic dysregulation observed in NK1R$^{-/-}$ mice, and provide evidence in support of a cholinergic hypothesis of ADHD.

CHAPTER SEVEN

7. General discussion

ADHD is a prevalent neuropsychiatric disorder encompassing behavioural and cognitive symptoms, as well as being highly comorbid with several other psychiatric disorders, including depression, obsessive compulsive disorder and addiction. Most commonly diagnosed in childhood, it is becoming recognised that the disorder frequently continues into adulthood. While the most widely used first-line treatments, amphetamine and methylphenidate, are effective in around 70 % of cases (Wilens, 2008), a large proportion of patients are left without successful treatment. Furthermore, diagnosis of the disorder is often problematic, particularly in adults, as a heterogeneous set of symptoms and a lack of biomarkers leaves scope for considerable differences in the interpretation of presenting symptoms (Parens & Johnston, 2009). Substantial research remains to be carried out in order to better understand ADHD and the risks associated with it.

There are a number of animal models of ADHD, however they frequently lack aspects of predictive, construct or face validity. The NK1R$^{-/-}$ mouse has recently been proposed as a new model for the disorder, given its hyperactivity which is reduced with a single dose of amphetamine or methylphenidate, lack of striatal dopamine response to amphetamine, and increased basal noradrenaline efflux in the frontal cortex, all of which mirror behavioural and neurochemical aspects of the disorder. In addition, four polymorphisms in the human *TACR1* gene have recently been found to be linked with ADHD, making the NK1R$^{-/-}$ mouse a promising model for the disorder (Yan et al., 2010).

The scope for research with a new model for ADHD is vast; the present work only touches upon the many possible directions for this subject, and centres on the behavioural and molecular responses of NK1R$^{-/-}$ mice to amphetamine. Over the course of this work, a fundamental difference between the striatal cholinergic neuroanatomy of NK1R$^{-/-}$ and wild-

type mice was discovered. While ADHD research has until recently focused on dysregulation of the dopaminergic system, this finding provides support for emerging evidence that central cholinergic systems play a role in the pathophysiology of the disorder.

7.1. Summary of results

7.1.1. NK1R$^{-/-}$ mice exhibit a normal level of DAT and pERK expression

NK1R$^{-/-}$ mice have previously been shown to lack the characteristic striatal dopamine response to a single dose of amphetamine (Yan et al., 2010). The principal site of action for amphetamine is the dopamine transporter (DAT), and it has been the subject of intensive research in ADHD, both clinically and preclinically, with varying results. Here, the striatal expression of DAT was investigated in NK1R$^{-/-}$ mice and found not to differ between NK1R$^{-/-}$ and wild-type mice. This is in accordance with the finding that basal dopamine efflux is similar in wild-type and NK1R$^{-/-}$ mice, and suggests that the reduced dopaminergic response to amphetamine seen in NK1R$^{-/-}$ mice (Yan et al., 2010) is likely due to impaired stimulated dopamine release rather than altered reuptake of dopamine through the transporter.

The expression of phosphorylated extracellular signal regulated kinase (pERK) in different brain regions in the NK1R$^{-/-}$ mouse was investigated in the immunohistochemical studies presented in Chapter 4. ERK plays an important role in synaptic plasticity and the long-term effects of drugs of abuse, including locomotor sensitisation, and is activated (by phosphorylation) within a characteristic pattern of brain regions in response to addictive drugs (Valjent et al., 2004). Hyperactive mice have been shown to have a paradoxical reduction in ERK activation following psychostimulant drugs (Beaulieu et al., 2006), however in the results presented here, pERK expression in NK1R$^{-/-}$ mice was not found to differ from that in wild-types. The level of pERK expression was significantly increased following amphetamine in the striatum and nucleus basalis of both genotypes, indicating that ERK activation is normal in NK1R$^{-/-}$ mice under the conditions tested. A different habituation period or a lower dose of amphetamine may have resulted in a lower level of ERK activation, but the present result corresponds with the findings presented in Chapter 5 that NK1R$^{-/-}$ mice develop normal sensitisation to repeated amphetamine administration.

7.1.2. Behavioural responses of NK1R$^{-/-}$ mice to amphetamine

Acute amphetamine has previously been shown to reduce the hyperactivity seen in NK1R$^{-/-}$ mice in a novel environment (Yan et al., 2010), however, the effects of repeated amphetamine have not been investigated previously in these animals. The effects of repeated amphetamine administration on locomotor activity of NK1R$^{-/-}$ mice were investigated here. NK1R$^{-/-}$ mice were hyperactive at the start of each experiment, but their activity diminished over the course of a 60 min habituation period, until it became similar to that of wild-type mice. Correspondingly, the locomotor response of NK1R$^{-/-}$ mice to the first dose of amphetamine, administered after the habituation period, was indistinguishable from that seen in the wild-type, suggesting that a novel environment is critical to the altered behavioural responses of NK1R$^{-/-}$ mice to amphetamine. In accordance with this, and with the results presented in Chapter 4 which demonstrated that pERK expression was normal in the striatum of NK1R$^{-/-}$ mice, both NK1R$^{-/-}$ and wild-type mice developed robust sensitisation to amphetamine, with no difference between genotypes.

7.1.3. Central cholinergic systems are altered in NK1R$^{-/-}$ mice

In the nucleus basalis, neuronal counts of cholinergic cells in wild-type mice were stable following amphetamine or saline administration, as expected. However in saline-treated NK1R$^{-/-}$ mice, expression of choline acetyltransferase (ChAT)-expressing cells in the nucleus basalis was greater than in wild-type mice. Furthermore, administration of a single moderate dose of amphetamine appeared to reduce the number of cholinergic cells compared with saline, while a high dose of amphetamine had no effect. In wild-type mice, cholinergic cell numbers were stable across treatments, with little intra-group variability. ChAT is considered a marker of cholinergic cells, and while it is unlikely that amphetamine would be neurotoxic specifically in the nucleus basalis after a single low, but not high, dose, this result suggests that central cholinergic systems are in some way compromised in NK1R$^{-/-}$ mice and subject to manipulation by psychostimulants. The result requires replication and further investigation with different doses of amphetamine, to confirm the suggested U-shaped amphetamine dose-response curve on cholinergic cell expression in the nucleus basalis of NK1R$^{-/-}$ mice.

The final chapter investigated the effects of the NK1 receptor knock-out in another major central cholinergic system, striatal interneurones, all of which express the NK1 receptor.

Neuronal counts were carried out to determine the number of cholinergic cells that co-expressed either phosphorylated myocyte enhancer factor 2 (pMEF2) or phosphorylated cAMP-response element binding protein (pCREB), two transcription factors that are involved in the development of responses to drugs of abuse. The striatum was divided into quadrants in order to determine whether differences between NK1R$^{-/-}$ and wild-type mice were confined to the limbic or motor portions of the striatum. A trend was observed in pMEF2 expression in cholinergic interneurones in all quadrants of the striatum, following acute and repeated amphetamine treatment: while acute amphetamine appeared to cause a trend towards an increase in pMEF2 expression in NK1R$^{-/-}$ mice, but not wild-types, repeated amphetamine appeared to cause a trend towards an increase in pMEF2 expression in wild-type but not NK1R$^{-/-}$ mice. However no significant difference between genotypes was found between pMEF2 or pCREB expression after acute or repeated saline or amphetamine.

Interestingly, NK1R$^{-/-}$ mice were found to have a lower number of ChAT-expressing cells in the lateral, but not medial, quadrants of the striatum compared with wild-type mice. This effect was specific to cholinergic cells, as the number of parvalbumin-expressing cells, which represent a different family of striatal neurones that do not express the NK1 receptor, did not differ between NK1R$^{-/-}$ and wild-type mice, providing convincing evidence of a deficit in this cholinergic system in NK1R$^{-/-}$ mice.

7.1.4. Elevated within-group variability of NK1R$^{-/-}$ mice

An elevated degree of within-group variability was observed in NK1R$^{-/-}$ mice, both in the behavioural and the immunohistochemical experiments described here. The animals are backcrossed onto an outbred strain and the colony is maintained as such, avoiding brother-sister pairings were possible, so a certain degree of variation is expected. However it is surprising to see that a large amount of variability is confined to the NK1R$^{-/-}$ genotype. Inter-individual variability has been inversely associated with neonatal stress: a moderately challenging (stimulating) neonatal period, for example involving brief periods of maternal separation or increased foraging demands, or similarly an increased level of neonatal corticosterone through maternal milk, has been found to reduce inter-individual variability in adult life (Macri et al., 2007). McCutcheon (2006) measured corticosterone release after stress in adult NK1R$^{-/-}$ and wild-type mice, and found that the release of corticosterone and its return

to baseline did not differ between genotypes after 30 minutes of restraint stress. However in the same study it was found that NK1R$^{-/-}$ mice express increased levels of hippocampal glucocorticoid receptor, suggesting that under different stressful conditions their corticosterone response may be terminated more quickly than that of wild-type mice. Assuming that glucocorticoid receptors are similarly elevated in the neonatal NK1R$^{-/-}$ mouse, this may provide an explanation for the increased variability observed in NK1R$^{-/-}$ mice in the experiments throughout this thesis.

7.2. Consideration of the NK1R$^{-/-}$ mouse as a model for ADHD

The behavioural experiments presented in this thesis support previous research demonstrating that NK1R$^{-/-}$ mice are hyperactive, however it was found that with a period of habituation the hyperactivity diminishes and responses to acute and repeated amphetamine are normal. This may indicate that the model is sensitive to environmental conditions, an effect that is also reported in ADHD (Russell et al., 2005), and it could permit the study of activity levels and associated neurobiological changes in novel and familiar environments in relation to the disorder. However, the observation that amphetamine at different doses consistently stimulates locomotor activity in NK1R$^{-/-}$ mice to a similar level to that observed in wild-type mice, does not support the hypothesis that genetic disruption of the NK1 receptor provides a model of ADHD. One explanation for the discrepancy between the present results and those reported in Yan et al. (2010) may be that the mice, while from the same line and bred in the same laboratory, have developed different phenotypes due to the breeding strategy used, in which the wild-type and NK1R$^{-/-}$ mice were bred in separate colonies. While this was the best method available in our laboratory, it provided opportunities for genetic drift and the potential creation of two separate substrains. Alternatively, the removal of a dark “pulse” or the introduction of a habituation period during which the amphetamine was administered, in the present results compared with those from Yan et al. (2010), may have brought about the differences observed from the previous studies from our lab using NK1R$^{-/-}$ mice. Repeating the sentisitation experiments using NK1 receptor antagonists to verify the present results was not possible due to time constraints, but such studies are planned for future work in NK1R$^{-/-}$ mice.

However, it is also likely that, while repeatedly attempted throughout molecular psychiatry, the disruption of a single gene is not sufficient to model every aspect of a highly heterogeneous disorder such as ADHD. Despite this, it is possible to model certain aspects of the disorder, such as the hyperactivity of $NK1R^{-/-}$ mice observed in the present results and previously, and the behavioural and neurochemical responses to amphetamine under certain conditions as reported previously (Yan et al. 2010).

Work is ongoing to further characterise the potential of $NK1R^{-/-}$ mice to model ADHD. Lesions of the striatum, and specifically the lateral striatum, have been shown to cause profound impairments in reward-related learning and tests of visual attention using the 5-choice serial reaction time task (5-CSRTT) (Kitabatake et al., 2003; Rogers et al., 2001). Work in our lab is ongoing to determine the attentional deficits of $NK1R^{-/-}$ mice in the 5-CSRTT. Preliminary results indicate that in the early stages of the task, in which the inter-trial interval is constant, $NK1R^{-/-}$ mice do not exhibit differences in the number of correct responses, premature responses or omissions. However, in the advanced stages of the task, during which the inter-trial interval is variable and requires sustained attention, $NK1R^{-/-}$ mice make an increased number of omission errors and premature responses than wild-types. This suggests that under increased attentional demand $NK1R^{-/-}$ mice are both inattentive and impulsive, providing additional evidence that the $NK1R^{-/-}$ mouse has face validity as a model for ADHD. The deficits observed in the more advanced stages of the 5-CSRTT reflect the deficits found in the lateral striatum of $NK1R^{-/-}$ mice. While the difference in cholinergic cell number was statistically significant, the actual difference in the number of neurones counted between $NK1R^{-/-}$ and wild-type mice was relatively small. As such, it corresponds that any behavioural or neurochemical differences between wild-type and $NK1R^{-/-}$ mice are only revealed under circumstances where increased attentional effort is demanded, such as a novel environment or the advanced stages of the 5-CSRTT.

Another finding from the 5CSRTT is that $NK1R^{-/-}$ mice consistently perform an elevated number of perseverative responses, i.e. repeatedly responding to a stimulus that has already been rewarded, and to which repeated responding is not further rewarded. This mirrors aspects of compulsive disorders which are often comorbid with ADHD. Compulsive behaviour is associated with dopaminergic circuitry specifically in the dorsolateral striatum (Koob & Volkow, 2010). In this context, the cholinergic deficit seen in the dorsolateral striatum of

NK1R$^{-/-}$ mice, which may lead to a dysregulation of dopaminergic control in this region, could contribute to the compulsivity or perseverative responses seen in NK1R$^{-/-}$ mice.

The inter-individual variability seen in NK1R$^{-/-}$ mice mirrors increased behavioural variability thought to be seen in people with ADHD (Sagvolden et al., 2005b), further substantiating its validity as a model for ADHD. The spontaneously hypertensive rat (SHR), another model for ADHD, also exhibits increased variability in behavioural tests (Russell et al., 2005).

While a great number of models of ADHD exist, many result from neurochemical or neuroanatomical manipulation in the neonate or adult. However, ADHD is proposed to be a developmental disorder with a high degree of hereditability (Sagvolden et al., 2005a; Sharp et al., 2009), and the use of a genetic model has the potential to address such developmental characteristics in a way that may not be possible from later-life manipulation.

7.2.1. Cholinergic hypothesis of ADHD

The dopaminergic hypothesis of ADHD is still the mainstay of research in to the disorder from molecular studies to the clinic (Genro et al., 2010). Psychostimulants affect the dopaminergic system acutely and chronically, and in many cases successfully counteract the overt hyperactivity and impulsivity symptoms of ADHD. DAT knock-out mice exhibit a number of the behavioural characteristics of ADHD, and a paradoxical response to psychostimulants is found in a number of models of ADHD. However, clinical evidence for a dopaminergic basis of ADHD is not consistent. Some imaging studies with people with ADHD have demonstrated a decrease in striatal DATs (Hesse et al., 2009; Volkow et al., 2007a) while others have reported an increase (Krause et al., 2000) and other still have described no change (van Dyck et al., 2002). Family-based association studies found no evidence in support of an association between the dopamine transporter DAT1 gene and ADHD (Langley et al., 2005; Loo et al., 2008), or with the D5 dopamine receptor (Loo et al., 2008), although the seven-repeat allele of the D4 receptor was found to be associated with poor performance in a number of cognitive measurements in some studies (Johnson et al., 2008; Loo et al., 2008) but not in others (Bakker et al., 2005; Brookes et al., 2005). While the improvement of ADHD symptoms by psychostimulants is a key argument for the dopamine hypothesis of the disorder, the principal symptoms improved by these drugs are the overt behavioural symptoms of hyperactivity and impulsivity, while they have less effect on the cognitive symptoms (Spencer et al., 1996). In addition, the mechanisms

by which their effects are produced remain unclear, and improvements in a number of functions are not specific to people with ADHD but are also improved in the general population (Zahn et al., 1980).

Over the last few years a convincing body of evidence has begun to emerge that supports a cholinergic hypothesis of ADHD. The basal forebrain cholinergic system is known to play an important role in cognitive functions and in particular attention (Everitt & Robbins, 1997), and the striatal cholinergic interneurone network exerts a powerful control over dopamine release and control of GABAergic medium spiny neurones (Perez et al., 2009; Pisani et al., 2001; Wang et al., 2006) thus influencing cognitive and limbic functions as well as locomotor activity. Studies in muscarinic receptor knock-out mice have demonstrated that M3, M4 and M5 receptors are specifically and differentially involved in the regulation of striatal dopamine release, locomotor activity and behavioural response to amphetamine (Woolley et al., 2009; Zhang et al., 2002). An association has been demonstrated between attentional network function and genetic variations within exon 5 of the α_4 subunit of the nicotinic acetylcholine receptor gene CHRNA4 (Winterer et al., 2007), and ADHD is highly comorbid with nicotine addiction, which may indicate self-medication of a dysregulation between dopaminergic and nicotinic-cholinergic circuits (McClernon & Kollins, 2008).

Clinical studies of the efficacy of cholinergic agents are still largely restricted to small patient sizes and have had promising results. ABT-418, a selective $\alpha_4\beta_2$ nicotinic receptor agonist, was shown to improve symptoms, in particular those reflective of attention, in a group of adults with ADHD (Wilens et al., 1999). Ultra-low doses of the nicotinic antagonist mecamylamine have been demonstrated to improve cognitive symptoms in people with ADHD (Potter et al., 2009). Donepezil, an acetylcholinesterase (AChE) inhibitor, improved ADHD symptoms in small samples of patients (Doyle et al., 2006; Wilens et al., 2000), athough a further small open-label study with donepezil found that patients stabilised on psychostimulants did not show improvement in residual symptoms with adjunctive donepezil use (Wilens et al., 2005). This highlights the need both for further preclinical research and larger-scale clinical studies in the development of a greater understanding of the influence of central cholinergic systems in the pathogenesis of ADHD, and ultimately the development of novel therapeutic agents for the disorder.

7.3. Future directions

While the results presented here are an important development in the study of the NK1 receptor and its putative role in ADHD, they only touch upon the number of investigations that could be carried out in a new model for ADHD, and there is much scope for further research.

It is possible that the amount of ChAT is reduced because of increased activity of the enzyme, so the activity of ChAT would need to be measured to determine whether the production of acetylcholine is increased or decreased. Similarly, acetylcholinesterase activity could be assayed to determine whether acetylcholine is being degraded more slowly to compensate for the lower level of ChAT. Immunohistochemical studies could be carried out in younger animals to investigate the possibility of a reduction in cholinergic cell number into adulthood, either with ChAT or with other markers of cholinergic cells such as the vesicular acetylcholine transporter (VAChT), considered to be a more stable marker of cholinergic neurones, or morphological studies as cholinergic interneurones are a characteristic shape. Finally, microdialysis could be used to determine whether acetylcholine efflux in the lateral striatum is correspondingly reduced in NK1R$^{-/-}$ mice.

7.3.1. Studies in NK1R$^{-/-}$ mice

7.3.1.1. Furthering the present findings

Primarily, the finding that there are fewer ChAT-expressing cells in the lateral quadrants of the striatum in NK1R$^{-/-}$ mice should be followed up by further histological or neurochemical studies. Microdialysis studies in the striatum would confirm whether basal and stimulated acetylcholine efflux is correspondingly altered in the lateral striatum of NK1R$^{-/-}$ mice, and cholinergic lesioning in the lateral striatum would determine whether a selective reduction in interneurone number would result in behavioural effects similar to those observed in NK1R$^{-/-}$ mice. It remains to be determined whether there are indeed fewer cholinergic interneurones, or whether there is simply less ChAT being produced per cell and the number of cells are similar between genotypes. This would provide evidence as to whether the effect is developmental, degenerative or functional in nature. Recent findings in cell lines from human malignant melanomas have implicated the NK1 receptor in tumour proliferation, and have shown that blockade of the receptor induces cell apoptosis (Munoz et al., 2010). This suggests

that the effect observed in the present work may be due to induced apoptosis of striatal cholinergic cells following the removal of functional NK1 receptors. An investigation of whether the same decrease in ChAT expression is observed in wild-type mice maintained chronically on NK1 receptor antagonists would go towards determining whether this is a developmental effect or a whether this putative loss of neurones is a later consequence of a lack of functional NK1 receptors. In addition, the reason for the cell loss being confined to the lateral part of the striatum remains to be determined.

The finding that a low dose of amphetamine reduces ChAT-expressing cell numbers in the nucleus basalis also warrants further investigation. The suggestion of a U-shaped dose-response curve should be investigated with different doses of amphetamine, and acetylcholine microdialysis in the frontal cortex would determine whether any drug-induced change in the nucleus basalis would be reflected in the cholinergic terminal field.

Finally, an interesting analysis would be to determine whether individual differences in behaviour (hyperactivity) correlate with individual levels of protein expression, particularly in the experiments in which trends were observed but the difference between genotypes did not reach statistical significance. A post-hoc analysis of the present results was unfortunately not possible due to insufficient animal numbers for a reliable correlation.

7.3.1.2. *Impulsivity, compulsivity and habit formation*

Impulsivity is hypothesised to be under the control of a dopaminergic circuit involving the nucleus accumbens. While no differences were observed between wild-type and NK1R$^{-/-}$ mice in the ventromedial striatum in the present work, the impulsivity observed in the later stages of the 5-CSRTT in ongoing research suggests that the neurobiology of the nucleus accumbens may be affected in NK1R$^{-/-}$ mice. There has been little research to this effect in the NK1R$^{-/-}$ mouse, despite the receptor being abundantly expressed in this region, so this warrants further investigation.

There is convincing evidence that dorsolateral striatal circuitry contributes to habit formation, considered the mediating stage between actions and compulsions (Everitt & Robbins, 2005). One way of testing this is using a cross maze in which one arm is blocked (resulting in a T-maze), with visual cues surrounding the maze. During the training phase, an animal is trained

to turn consistently in one direction to gain a food reward. In the test phase, the animal is started from the opposite arm of the maze (effectively rotating the T-maze 180 degrees), and will either enter the arm that corresponds to the previous location of the reward (place strategy, or action-outcome), or turn in the same direction as it turned during the training phase (stimulus-response) (Yin & Knowlton, 2006). This latter response strategy recruits dorsolateral striatal circuits and is adopted by animals that have been over-trained in the task. Given the deficit in cholinergic cell numbers in the dorsolateral sub-region of the striatum and possible dysregulation of dopaminergic control, it could be hypothesised that NK1R$^{-/-}$ mice have an increased propensity to develop habitual (stimulus-response) behaviour more readily than their wild-type counterparts. Such research could inform investigations in ADHD as well as addictive disorders.

7.3.1.3. Other tachykinin receptors

Little work has been carried out in NK1R$^{-/-}$ mice to determine the role of the other neurokinin receptors that may have developed alterations in expression patterns or ligand specificity to compensate for the lack of functional NK1 receptors. Both NK2 and NK3 are stimulated by substance P, albeit to a lesser degree than NK1 (Saffroy et al., 2001; Salome et al., 2006).

7.3.1.4. Different background strains

As with all studies in knock-out mice the experiments presented in this thesis should be repeated in other strains of mice to determine the extent of interactions between genetic background and the NK1 receptor. If the aim of the studies is to elucidate the specific functions of the NK1 receptor, replication in different strains is of great importance, illustrated by McCutcheon et al (2008), whose studies revealed that genetic disruption of the NK1 receptor on a backcrossed C57BL/6 background and on a mixed C57BL/6 x 129/Sv background resulted in different behavioural, neurochemical and drug-induced responses. Conversely, for the purposes of creating a model of ADHD, the importance of the effects of background strain on the NK1R knock-out is reduced. In this case, of paramount importance is that the model complies as far as possible with the three core validity criteria, whichever strain is used.

7.3.2. Clinical research

When considering the NK1R$^{-/-}$ mouse as a model for ADHD, it is important to consider the possibility of extending the present findings to the clinical setting. The findings of a deficit in striatal cholinergic neurones in NK1R$^{-/-}$ support evidence that a dysfunction in central cholinergic systems contributes to the aetiology of ADHD. Brain imaging has the potential to find a diagnostic biomarker for ADHD. Currently, research has focused on the dopaminergic system and in particular the DAT. Success, however, has been limited as studies have shown conflicting results, with some showing increases, some decreases, and others no change in DAT levels in people with ADHD (Spencer et al., 2007; Volkow et al., 2007a). The present results have the potential to contribute a new approach of assessing the structural integrity of the cholinergic system in patients with ADHD. Currently, ChAT has not been successfully imaged in vivo due to a lack of radioligands, but other cholinergic markers, VAChT and AChE, have successfully been used to map cholinergic cells in human brain (Wevers, 2010). After further studies in NK1R$^{-/-}$ mice to determine whether these markers reflect the deficit seen in ChAT, this could potentially provide a powerful new approach to assess cholinergic function in people with ADHD.

7.4. Final conclusions

An intimate association exists between central cholinergic and dopaminergic systems. While current treatments for ADHD often successfully reduce hyperactivity, a large proportion of patients remain without appropriate treatment, and the cognitive deficits commonly seen in ADHD are frequently overlooked. The research presented in this thesis demonstrates a deficit in central cholinergic systems in NK1R$^{-/-}$ mice, and provides evidence in support of the hypothesis that a dysregulation in the dopamine and acetylcholine balance contributes to the complex combination of locomotor and cognitive symptoms in ADHD and its comorbid disorders.

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Detailed laboratory protocols

A.1. General laboratory solutions

Phosphate buffer (0.1 M; pH 7.4)

190 mM NaH_2PO_4 (BDH Laboratory Supplies, Poole, UK)

810 mM Na_2HPO_4 (BDH)

Paraformaldehyde

4 % paraformaldehyde (BDH)

0.1 M phosphate buffer

Heparinised saline

5 IU/ml heparin (CP Pharmaceuticals, Wrexham, UK)

0.9 % NaCl (Baxter, Lessines, Belgium)

Sucrose with sodium azide

30 % or 5 % sucrose (BDH)

0.02 % NaN_3 (Sigma-Aldrich, Poole, UK)

NB: 30 % sucrose is used for cryopreserving whole brains before sectioning; 5 % sucrose is used for storing sections prior to immunohistochemistry.

A.2. Immunohistochemistry

Preparation of gelatinised slides

Dissolve 2.5 g gelatine (BDH) in 500 ml dH2O by heating gently to no more than 50 °C. Add 0.5 g chrome alum (chromic potassium sulphate; Sigma-Aldrich). Filter. Dip twin-frost slides in the solution for 30 seconds each. Dry overnight.

Tissue preparation

Cut 40 µm coronal sections using a freezing microtome and place into 5 % sucrose with NaN_3. Immunohistochemistry should be performed as soon as possible after sectioning to ensure preservation of the tissue.

Immunohistochemistry solutions

Blocking solutions

For use with fluorescent detection

0.1 M	phosphate buffer
3 %	normal serum from the species in which the secondary antibody was raised
0.3 %	Triton X-100 (BDH)

For use with chromogenic detection using DAB

0.1 M	phosphate buffer
3 %	normal serum from the species in which the secondary antibody was raised
2 %	H_2O_2 to quench endogenous peroxidase activity
0.3 %	Triton X-100

Tris/Triton-buffered saline (TTBS)

0.05 M	Tris base
0.3 %	Triton X-100
0.9 %	NaCl

Single antigen labelling protocol

All steps are performed on a rocking platform at room temperature unless otherwise specified

Primary antibody

- Select sections and rinse in phosphate buffer to remove sucrose and azide
- Block non-specific binding sites in tissue by incubating in appropriate blocking solution for 1 h
- Place sections in primary antibody made up in TTBS. Incubate either at room temperature for 1 h or at 4 °C for 3 days

Fluorescent detection

Directly conjugated Alexa-594

- Wash sections in phosphate buffer 3 times for 10 min each time
- Incubate in AlexaFluor594 (Molecular Probes, Oregon), 1:500 in TTBS, for 2 h in the dark
- Wash sections in phosphate buffer 3 x 10 min in the dark
- Rinse in 0.01 M phosphate buffer before mounting onto gelatinised slides and leave to air dry in the dark
- Coverslip with Fluoromount (Sigma-Aldrich)

Avidin-biotin method with FITC

- Wash sections in phosphate buffer 3 x 10 min
- Incubate sections in appropriate biotinylated secondary antibody (raised against the species in which the primary antibody was raised) diluted 1:500 in TTBS, for 2 h
- Wash sections in phosphate buffer 3 x 10 min
- Incubate sections in Fluorescein Avidin D (Vector Laboratories, Burlingame, CA, USA), 1:200 in TTBS, for 2 h in the dark
- Wash sections in phosphate buffer 3 x 10 min in the dark
- Rinse in 0.01 M phosphate buffer before mounting onto gelatinised slides and leave to air dry in the dark
- Coverslip with Fluoromount

Chromogenic detection with DAB

- Wash sections in phosphate buffer 3 x 10 min
- Incubate sections in appropriate biotinylated secondary antibody (raised against the species in which the primary antibody was raised) diluted 1:200 in TTBS, for 2 h
- Wash sections in phosphate buffer 3 x 10 min
- Make up avidin-biotin complex (ABC) using the ABC Vectastain kit (Vector Laboratories).
    - Mix 1 µl/ml Reagent A (avidin) and 1 µl/ml Reagent B (biotinylated horseradish peroxidase, HRP) with 0.1 M phosphate buffer and incubate for 30 min to allow reaction to take place
- Incubate sections in ABC for 1 h
- Wash sections in phosphate buffer 3 x 10 min
- Make up DAB solution using the DAB Vectastain kit (Vector Laboratories)
    - Add 2 drops buffer, 4 drops DAB, 2 drops peroxidase and 2 drops nickel to 5 ml dH_2O
- Incubate sections in DAB until a suitable level of staining is achieved, normally within 3-10 min
- Quench reaction in dH_2O and transfer to 0.1 M phosphate buffer for 5 min
- Rinse in 0.01 M phosphate buffer before mounting onto gelatinised slides and leave to air dry overnight.
- Dehydrate sections through dH_2O, 70 % ethanol (twice), 95 % ethanol (twice) and 100 % ethanol (twice), dipping the slides for 2 min at each stage
- Clear in Histoclear (National Diagnostics, Hull, UK) twice for 2 min
- Coverslip immediately using DPX mounting medium (BDH)

Double antigen labelling protocol

All steps are performed on a rocking platform at room temperature unless otherwise specified

First antigen

- Select sections and rinse in phosphate buffer to remove sucrose and azide
- Block non-specific binding sites in tissue by incubating in appropriate blocking solution for 1 h
- Place sections in first primary antibody made up in TTBS. Incubate either at room temperature for 1 h or at 4 °C for 3 days
- Wash sections 3 times for 10 min each time
- Incubate sections in appropriate biotinylated secondary antibody (raised against the species in which the primary antibody was raised) diluted 1:400 in TTBS, for 90 min
- Wash sections in phosphate buffer 3 x 10 min
- Incubate in tyramide signal amplification (TSA) reagent (PerkinElmer, MA, USA), 1:75 in TTBS, for 7 min
- Wash sections in phosphate buffer 3 x 10 min
- Incubate sections in Fluorescein Avidin D (Vector Laboratories, Burlingame, CA, USA), 1:200 in TTBS, for 2 h, in the dark
- Wash sections in phosphate buffer 3 x 10 min in the dark

Second antigen

All stages must be carried out in the dark to preserve the fluorescent labelling of the first antigen

- Place sections in second primary antibody made up in TTBS. Incubate either at room temperature for 1 h or at 4 °C for 3 days
- Wash sections in phosphate buffer 3 x 10 min
- Incubate in AlexaFluor594 (Molecular Probes, Oregon), 1:500 in TTBS, for 2 h
- Wash sections in phosphate buffer 3 x 10 min
- Rinse in 0.01 M phosphate buffer before mounting onto gelatinised slides and leave to air dry
- Coverslip with Fluoromount

A.3. Western blots

Western blot solutions

RIPA buffer

100 mM NaCl
100 mM NaF
20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4
5 mM ethylenediaminetetraacetic acid (EDTA)
1 mM Na_3VO_4
1 % v/v NP-40

Loading buffer

50 % v/v glycerol
10 % w/v sodium dodecyl sulphate (SDS; Sigma-Aldrich)
0.5 % w/v bromophenol blue (Sigma)

MOPS running buffer (pH 7.3)

0.05 M 3-(N-morpholino) propane sulphonic acid (MOPS) (Invitrogen, Oregon)
0.05 M Tris base
3.5 mM SDS
1 mM EDTA

Transfer buffer

48 mM Tris base
39 mM glycine
0.037 % SDS
10 % v/v methanol

Tris-buffered saline

0.05 M Tris base
0.9 % NaCl

PBS-Tween

0.1 M	phosphate-buffered saline (Sigma-Aldrich)
0.1 % v/v	Tween 20

Blocking solution

0.1 M	phosphate-buffered saline
4 % w/v	skimmed milk powder

Tissue preparation

- Remove brains and snap-freeze in liquid nitrogen until required.
- Section by placing brain dorsal side down in a mouse brain matrix with 1 mm coronal section slice intervals (Zivic Instruments, Pittsburgh, PA, USA; Figure A.1A)
- Use a razor blade to slice through the tissue, keeping everything on dry ice
- Once sections have been cut, dissect striatal tissue using a biopsy punch (Zivic Instruments; Figure 1B), keeping everything as cold as possible to ensure preservation of the tissue
- Once tissue has been dissected it can be stored at -80 °C until use

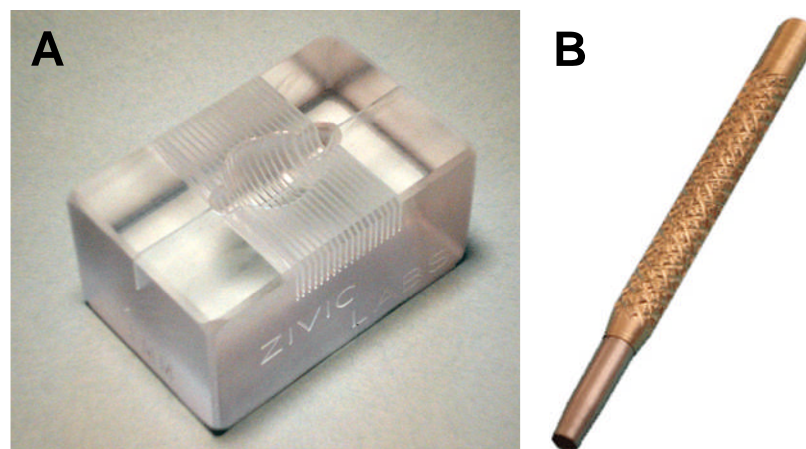

Figure A.1 Instruments for frozen dissections.

(A) Adult mouse brain matrix with 1 mm coronal section slice intervals. (B) 2 mm diameter biopsy punch used to dissect striatal tissue from 1 mm coronal sections. Photographs from www.zivicinstruments.com.

Homogenisation

- Place each tissue sample and about 12 beads of Lysing Matrix D (MP Biomedicals Europe, Illkirch, France) in a 2 ml impact resistant screw-cap tube (MP Biomedicals) and place in ice
- Add around 60 µl RIPA + 1 µl protease and phosphatase inhibitor cocktail (Sigma-Aldrich) to each tissue sample
- Homogenise using the FastPrep Biopulverizer (MP Biomedicals Europe, Illkirch, France), 2 x 10 sec bursts on speed 4
- Incubate homogenised samples on ice for up to 2 h
- Centrifuge at 4 °C for 15 min at 12,000 RPM and keep the supernatant
- Measure protein content in samples using the BCA Protein Assay kit (Pierce, Rockford, IL)
    - Add 10 µl of six protein standards of known concentration (constituting a standard curve), and 5 µl of each sample, in duplicate to a 96-well plate
    - Mix bicinchoninic acid and 4 % copper sulphate ($CuSO_4.5H_2O$) solution in a 50:1 ratio and add 200 µl of the mixture to each well containing standards or samples
    - Incubate plate for 30 min at 37 °C, and run the plate through a colorimeter to determine the concentration of protein in the samples
    - Correct the resulting concentrations for the reduced volume of sample compared to standard

Running the gel

- Mix the volume of homogenised sample that contains 20 µg protein with 2 µl loading buffer and boil for 5 min to denature proteins
- Load the sample and loading buffer mixture onto 10 % SDS polyacrylamide gel
- Run gel at 100 V for 90 min

Transfer of proteins

- Prepare polyvinylidine difluoride (PVDF) membrane (Bio-Rad, Hertfordshire, UK) by soaking in 100 % methanol for 2 min followed by transfer buffer for 5 min
- Gently remove gel from running apparatus and place, with membrane, between transfer buffer-soaked filter paper and sponges and place in transfer apparatus with an ice block to prevent overheating which causes the gel to stick to the membrane

- Run for 45 min at 100 V

Immunoblotting

All stages are carried out on a rocking platform or a tube roller at room temperature unless otherwise stated

Protein of interest

- Incubate membrane in blocking solution for 1 h
- Add primary antibody to blocking solution and incubate overnight at 4 °C
- Wash membrane in PBS-Tween 6 times for 5 min each time
- Incubate membrane in appropriate HRP-conjugated secondary antibody (raised against the species in which the primary antibody was raised), 1:2000 in blocking solution, for 45 min
- Wash membrane 6 x 5 times in BPS-Tween
- Rinse in PBS without Tween before developing
- Identify bands using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Buckinghamshire, UK) and ChemiDoc-XRS molecular imaging system (Bio-Rad)

Normalisation against housekeeping protein

- Rinse membrane with PBS-Tween
- Incubate with primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) raised in mouse, 1:2000 in blocking solution, for 90 min at room temperature or overnight at 4 °C
- Wash membrane 6 x 5 times in BPS-Tween
- Incubate with secondary antibody (HRP-conjugated anti-mouse IgG), 1:2000 in blocking solution, for 45 min
- Wash membrane 6 x 5 times in BPS-Tween
- Identify bands as for the first primary antibody, using the ECL detection system and ChemiDoc-XRS

A.4. Genotyping using PCR

PCR solutions

Tail buffer

50 mM Tris
100 mM EDTA
100 mM NaCl
1 % SDS
Ultrapure water (18 MΩ/cm)

TE buffer

10 mM Tris base
1 mM EDTA

dNTP mix (10 mM)

10 mM dATP (Promega)
10 mM dCTP (Promega)
10 mM dGTP (Promega)
10 mM dTTP (Promega)

PCR reaction master mix (total 45 µl)

32.5 µl	ultrapure water (18 MΩ)
5 µl	*Taq* DNA polymerase buffer (Promega)
3 µl	25 mM $MgCl_2$ (Promega)
1 µl	dNTP mix (10 mM)
1 µl	NeoF: 5’-GCAGCGATCGCCTTCTATC-3’ (0.5 µg/µl; Sigma Genosys, Cambridge, UK)
1 µl	NK1-F: 5’-CTGTGGACTCTAATCTCTTCC-3’ (0.5 µg/µl; Sigma Genosys)
1 µl	NK1-R: 5’-ACAGCTGTCATGGAGTAGATAC-3’ (0.5 µg/µl; Sigma Genosys)

0.5 µl	*Taq* DNA polymerase (Promega) – add last

Loading buffer

0.25 %	bromophenol blue (Sigma)
0.25 %	xylene cyanol FF (Sigma)
30 %	glycerol (BDH)

DNA extraction from tail tips

- Spray tip of tail with ethyl chloride and place in 1.5 ml microfuge tube with 750 µl tail buffer and 22.5 µl proteinase K (20 mg/ml; Sigma-Aldrich)
- Incubate at 55 °C overnight
- Vortex and centrifuge for 3 min at 13,000 RPM
- Transfer supernatant to fresh tubes
- Add 200 µl protein precipitation solution (Puregene, Minneapolis, USA) to each tube and vortex for 20 s
- Centrifuge for 3 min at 13,000 RPM and discard supernatant
- Add 300 µl 70 % ethanol
- Centrifuge for 3 min at 13,000 RPM
- Discard supernatant and leave to air dry for 1-2 h
- Add 250 µl TE buffer and agitate tube to dislodge the pellet from the bottom of the tube
- Dissolve precipitate overnight at 4 °C to obtain DNA sample

NK1 receptor PCR protocol

- Add 5 µl DNA sample to 45 µl PCR master mix
- Place in PCR temperature cycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) and run the following programme, repeating steps 2-4 34 times:
  1) 95 °C 5 min
  2) 60 °C 30 s
  3) 72 °C 30 s
  4) 94 °C 30 s
  5) 60 °C 30 s
  6) 72 °C 5 min

Running the gel

- Add 10 µl of loading buffer to each sample and mix well
- Load 15 µl of each sample and loading buffer mixture onto a 2 % agarose gel, in TBE buffer (National Diagnostics) containing 10 µg/ml ethidium bromide (Sigma)
- Run the gel at 120 mV for approximately 45 min
- Visualise and photograph under ultraviolet transillumination