# The effect of repeated early injury on reward-related processing in the adult rat

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**Declaration:**

**I, Lucie Low, 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.**

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# Abstract

Pain during early life can affect the developing central nervous system, leading to altered neural function in the adult organism. In this thesis, I investigate the long-term effects of repeated early pain on reward-related processing in the adult rat. I hypothesised that the reward system was likely to be sensitive to early activation of pain pathways, as the brain systems involved in both pain and reward overlap extensively, and virtually all centrally acting analgesic drugs are also drugs of reward.

To begin, I investigate the extent to which the developing reward system is activated by a classic analgesic and drug of abuse, morphine. Comparing neonatal and adult activation of the dopaminergic system, results show that a single morphine challenge activates neonatal reward pathways, but that there are qualitative differences in the neonatal response to repeated morphine. Next, I show how reward-related behaviours of adult animals repeatedly injured as neonates differ from those of uninjured littermates, and finally propose the lateral hypothalamic orexin system as a biomarker reflecting this behaviour. The results provide evidence that neonatal injury interferes with the normal development of reward systems during a critical period of development, resulting in characteristic changes in reward behaviour and cell signalling in the adult animal.

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# 1  Introduction

The experiments presented in this thesis are designed to investigate the long-lasting effects of repeated pain during early development on adult reward-related processing in the rat. Human data has clearly shown that pain during childhood, particularly in very young and premature neonates, can cause changes in sensory and nociceptive processing in the older child that can persist into adulthood. Pain processing is intricately linked with reward processing in a number of ways, and so the central hypothesis tested in this thesis is that exposure to injury in infancy will alter CNS reward processing in adult rats, and that this will be reflected in reward behaviours and the neurobiological reward signalling in the adult brain.

## 1.1  The problem of early pain

Pain at any age is unpleasant, yet most adult humans have methods of treating this pain, either by requesting or taking medication, or by engaging internal coping mechanisms that can help diminish the emotional, affective components of pain. However, young human infants do not have the abilities to either self-administer nor to engage executive coping mechanisms. Pre-verbal infants cannot report an internal pain state, meaning the responsibility for pain relief must come from the carer or clinician.

Over the past 20 years, there has been an increase in the incidence of premature birth (Langhoff-Roos et al., 2006), and the technology to aid survival has meant that infants as young as 24 weeks post-menstrual age (PMA) can survive and develop in the neonatal intensive care unit (NICU). However, the NICU is a very different environment to the womb, and premature infants are exposed to a mass of stressful stimuli such as light, noise, tactile stimulation, surgery, medication, and maternal separation that could feasibly affect development (see Grunau et al., 2006). Evidence exists to show that very young neonates can respond to pain, and also that pain during this vulnerable time of development can cause long-term alterations in pain processing, extending well into childhood and adulthood (see Fitzgerald, 2005; Fitzgerald and Walker, 2009; La Prairie and Murphy, 2010 for reviews).

### 1.1.1 Pain management in neonates

Treatment of pain in neonates is a clinical challenge for a number of reasons. Firstly, pre-verbal infants cannot report their pain experience, so surrogate measures of pain must be taken in order to decide upon the appropriate treatment. In older infants, visual analogue scales (VAS) and self-report measures can be used for pain measurement, although these rely on a certain level of cognitive development for optimal usefulness (see Howard, 2003).

Pain measures in pre-verbal neonates that are used in clinical practice are based upon behavioural and physiological reactions to a noxious stimulus, and include monitoring gross body movements, muscle and limb reflex withdrawal magnitude, heart rate and blood pressure, cerebral haemodynamic responses, and facial grimacing (see Fitzgerald and Walker, 2009). These measures are incorporated into assessment tools, and current tools include the Premature Infant Pain Profile (PIPP), the Neonatal Infant Pain Scale (NIPS), and the Behavioural Indicators of Infant Pain (BIIP), amongst others (Holsti and Grunau, 2007; Shah and Ohlsson, 2007). Whilst pain scales have been validated for older children (von Baeyer and Spagrud, 2007) and neonates (Grunau et al., 2006), there is no 'gold standard' as yet for pain assessment in neonates. As there can be a robust activation of the cerebral cortex after noxious stimulation, but no facial pain response at all (Slater et al., 2008), this suggests that measures are best taken in conjunction with each other to enable adequate treatment of vulnerable infants (see Howard, 2003).

Further difficulties in treatment arise through differences in CNS organisation and synaptic organisation between neonates and adults: dosage, metabolism of medication, efficacy and specificity of action are all affected by brain development (see Walker, 2008).

Current treatments to manage pain in neonates include systemic opioids such as morphine, and use of epidural analgesia administration, delivered directly into the cerebrospinal fluid surrounding the spinal cord, is often employed for post-operative pain (Murat et al., 1987). In addition, morphine is used in the NICU to provide sedation in neonates requiring mechanical ventilation, to improve tolerance of ventilation and comfort of the infant, although further research on its use in this situation has been

recommended (Bellú et al., 2008). Opioids are the most frequent form of analgesia used in the NICU, with analgesia rarely given for non-surgical invasive procedures (Johnston et al., 1997).

Regional nerve blocks with local anaesthetic can be used for minor procedures, as can the application of topical local anaesthetics such as lidocaine, in combination with opioids or paracetamol if necessary (Howard, 2003), but the efficacy and benefits of non-steroidal anti-inflammatory drugs (NSAIDs) is unclear in neonates and infants less than 3 months old so their use is not as widespread (see Fitzgerald and Walker, 2009). The stress and/or pain of procedures such as heel lance (to take blood), which can be performed multiple times a day, can be alleviated by comfort measures such as non-nutritive suckling or sucrose given shortly before the procedure (Blass and Watt, 1999; Stevens et al., 2004; Stevens et al., 2010), as can cuddling, swaddling, and tactile stimulation (Howard, 2003; D'Apolito, 2006). In older children, distraction techniques and teaching of coping skills can be helpful for pain management. For example, a recent systematic review has indicated that cognitive behavioural therapy is effective in treatment of headache in children and adolescents (see Eccleston et al., 2003).

## 1.1.2 Early pain in humans causes long-term alterations in pain circuitry and sensitivity

Evidence suggests that repeated pain during the neonatal period and childhood can cause long-lasting effects on sensory and pain processing (Grunau et al., 1994; Oberlander et al., 2000; Grunau et al., 2001; Grunau et al., 2007). Effective pain management in neonates therefore becomes important to minimise the consequences of early pain experience. In a clinical setting, multiple painful procedures may be performed daily for routine monitoring i.e. heel lance, to monitor blood oxygenation levels of very young infants. Simons et al (2003) found that, during the first 14 days of NICU admission, neonates were subject to an average of 14 potentially painful procedures per day. A more recent study (Carbajal et al., 2008) confirmed these findings, showing that neonates were exposed to an average of 16 painful and/or stressful procedures, and that up to 80% of neonates were not given specific analgesia for these procedures.

Taddio and colleagues (1997) studied male infants that had been circumcised when they were vaccinated at 4-6 months. Infants who had been treated with a topical anaesthetic (EMLA cream) when circumcised showed a lower pain score than those who received no anaesthetic, and the circumcised groups showed higher pain scores than uncircumcised controls. This double-blind, randomised, controlled trial (RCT), together with an earlier paper showing that parental ratings of pain sensitivity were lower at 18 months of age in infants born at extremely low birth weights (Grunau et al., 1994), highlighted the potential consequences of early pain, and the importance of neonatal analgesia.

Premature infants with NICU experience show lasting behavioural sensitivity to mechanical stimuli that persists for at least the first year of life (Abdulkader et al., 2007), and longitudinal studies find evidence for alterations in sensory processing in ex-NICU children at school age (9-12 years)(Hermann et al., 2006; Hohmeister et al., 2008). Buskila and colleagues (2003) showed ex-premature 12-18 year olds had significantly more 'tender points' and lower tenderness thresholds than matched full-term children, and Walker et al (2009a) recently presented data from a cohort of extremely preterm infants born at less than 26 weeks PMA in 1995 (the UK EPICure cohort); quantitative sensory testing (QST) in these children showed that ex-premature children had decreased sensitivity to thermal stimuli, but no differences in mechanical sensitivity when tested at the thenar eminence (at the base of the thumb). Decreases in mechanical and thermal sensitivity were found adjacent to neonatal thoracotomy scars, although no differences between ex-premature and term children were seen in current pain experience or pain coping styles. This decrease in baseline sensitivity both globally and at the site of scarring is replicated by evidence in children aged 9-12 who had undergone neonatal cardiac surgery (Schmelzle-Lubiecki et al., 2007). Local sensitivity changes are proposed to be due to altered peripheral pain processing, and global changes in thermal and mechanical sensitivities due to centrally-mediated alterations in nociceptive pathways (Walker et al., 2009a).

## 1.1.2.1 Effects are mediated by alterations in central nociceptive processing

Evidence for centrally-mediated alterations in pain processing after neonatal injury is provided by studies investigating pain responses to noxious stimuli, or a new injury, in post-premature or post-NICU children. Hermann and colleagues (2006) showed that post-NICU premature infants had decreased thermal sensitivity and enhanced perceptual sensitisation to a prolonged heat stimulus when tested at 9-12 years of age, compared to non-hospitalised control children. Furthermore, these children were more likely to catastrophise (exaggerate the negative aspects of the pain experience) and did not show increased pain thresholds when in the presence of their mothers, unlike full-term control children with no NICU experience (Hohmeister et al., 2008).

Major surgery to infants within the first three months of life caused an increase in pain sensitivity when infants underwent subsequent surgery in either the same or a different dermatome (an area of skin supplied by a single spinal nerve) at a later date (Peters et al., 2005). In this study, pain sensitivity was measured by noradrenaline plasma concentrations and anaesthetic demand during surgery, and observation and self-report measures plus morphine intake post-surgery. Anaesthetic and analgesic demand was highest in children with later surgery in the same dermatome, but was also higher when surgery was performed in a different dermatome compared to infants with no surgical history, suggesting changes in both spinal and supraspinal processing following neonatal surgery.

However, the severity and type of injury during infancy can produce differing long-term effects. Deep somatic and visceral noxious stimulation, such as that described by Peters et al (2005), may lead to sensitisation of pain responses, but acute inflammatory types of neonatal noxious stimulation, such as heel lance, may lead to reduced nociceptive responsiveness (see La Prairie and Murphy, 2010). To illustrate, heel lancing led to dampened behavioural responses in infants at 32 weeks' post-conceptional age, with a larger degree of hypo-responsiveness in neonates with a greater number of previous invasive procedures (Grunau et al., 2001). A dampened response, as seen by decreased facial responsiveness to immunisation and blunted nociceptive sensitivity to everyday bumps, has also been seen in older infants at 4, 8, and 18 months of age in ex-preterm

neonates compared to full-term controls (Oberlander et al., 2000; Grunau et al., 1994). This dichotomy is also illustrated by a study of school-age children (9-16 years) who had suffered moderate or severe burn injuries in infancy (6-18 months of age) (Wollgarten-Hadamek et al., 2009). Moderately burned infants showed increased mechanical pain sensitivity, whereas severely burned neonates showed elevated thermal pain thresholds.

Taken together, the effects of early surgery, prematurity and resulting length of NICU stay can all affect the type and degree of later effects. Invasive early procedures such as surgery may decrease sensitivity to pain at baseline, but a re-injury to a previously injured infant, especially to an area served by the same spinal innervation, leads to increased hypersensitivity compared to controls (Peters et al., 2005).

## 1.1.2.2 Prematurity can affect the stress response

Premature birth is also proposed to lead to alterations in generalised stress-arousal systems. Premature infants are, as already mentioned, subject to many stressors such as light, noise, maternal separation and noxious procedures. Grunau and colleagues (2006) propose that these early stressors could increase the 'allostatic load' of the neonate. 'Allostasis' refers to the cumulative effects of exposure to repeated stressors, and is the process by which an organism will strive to maintain homeostasis (bodily equilibrium), for example via the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response (see McEwen, 1998). Long-term, repeated stress exposure causes an increased 'allostatic load', which will lead to an elevation of stress responses mediated by the HPA axis (see McEwen, 2004). Evidence for this comes from studies showing that cortisol levels (a stress hormone released on HPA axis activation) were higher in ex-premature infants at 8 months when exposed to novel toys (Grunau et al., 2005), and higher at baseline in infants born extremely premature (23-28 weeks PMA) when tested at 3, 6, 8 and 18 months (Grunau et al., 2007). The authors propose that the stress response systems are 'reset' by the stressful NICU experience, and that this could contribute to the increased pain reactivity in older infants.

## 1.1.2.3 NICU experience can alter long-term cognitive development

Prematurity, and the associated necessary healthcare, are also linked to problems with cognitive development. A large proportion of ex-NICU preterm infants show cognitive

impairments later in development (Wood et al., 2005), and up to half will require some form of educational support in childhood (Rivkin, 2000), with a high incidence of behavioural disorders reported in post-premature adolescents (Indredavik et al., 2004). Further, a meta-analysis of 227 studies showed that ex-premature infants were more at risk from attention deficit disorders, and the earliest pre-term infants showed the most cognitive deficits when tested at school age (Bhutta et al., 2002). Attempts to find an anatomical substrate for these deficits has used imaging techniques to study brain morphology in previously premature infants. Magnetic resonance imaging (MRI) studies have demonstrated abnormalities in the size and/or growth rate in the cerebral cortex, cerebellum and basal ganglia (Ajayi-Obe et al., 2000; Dyet et al., 2006; Boardman et al., 2006), and diffusion tensor imaging (DTI), which can be used to assess brain connectivity, has shown abnormalities in white matter tracts in the ex-premature brain, which could help explain the increased incidence of cognitive difficulties in ex-premature infants (Counsell et al., 2007; Berman et al., 2005; Dubois et al., 2006).

## 1.1.3 Animal models provide information on the development of nociceptive processing

The data from humans on the effects of neonatal pain clearly show that injury during the early life period can cause changes in sensory and nociceptive processing that can last into adulthood. However, elucidation of the mechanisms of these changes relies critically on modelling pain in animals. Animal models of neonatal pain identify cellular mechanisms that would be impossible to study in humans, and provide researchers with novel drug targets for the development of new pharmaceutical agents for pain management. In addition, animal models are used as preclinical screening systems, where potential new drugs are tested for efficacy and toxicity.

The gestational period for a rat is 21.5 days, and the development of the CNS over the first 7-10 days of life in the rat is approximately equivalent to that of an infant from 24 through to 40 weeks of gestation (see Fitzgerald and Walker, 2009; McCutcheon and Marinelli, 2009 for reviews). This comparability means that study of the development of spinal nociceptive circuits and brain connectivity in animals can inform researchers on the developmental stage of premature infants.

## 1.1.3.1 Nociceptive transmission pathways

Pain is transmitted from the periphery to the brain via the spinal cord. Primary afferent nociceptors at the periphery are activated upon intense, potentially damaging stimuli, and action potentials within these nerve fibres are transmitted to the spinal cord. Primary afferent fibres are divided into those that are myelinated (Aβ and Aδ fibres) and those that are not (C fibres). Myelinated A fibres are involved in transmitting mechanical information, and unmyelinated C fibres in transmitting thermal information, with Aδ and C fibres implicated in pain processing. Cell bodies of all fibre types constitute the dorsal root ganglion (DRG)(Wall and Melzack, 2005). The terminals of the nociceptors are located in specific spinal layers (laminae), and when activated, the primary afferents release peptides and glutamate, which activate second order neurons. Dorsal horn neurons of the spinal cord relay the nociceptive signal to the contralateral side of the spinal cord, and from there the spinothalamic tract, consisting of ascending projection fibres, conveys the signal to the brainstem and thalamus (see Figure 1-1) (Wall and Melzack, 2005). From here, the nociceptive signal is passed to a variety of nuclei and cortical areas, which mediate different aspects of the pain experience. The intensity of a noxious stimulus is coded for by the firing rate of pain transmission neurons (Julius and Basbaum, 2001).
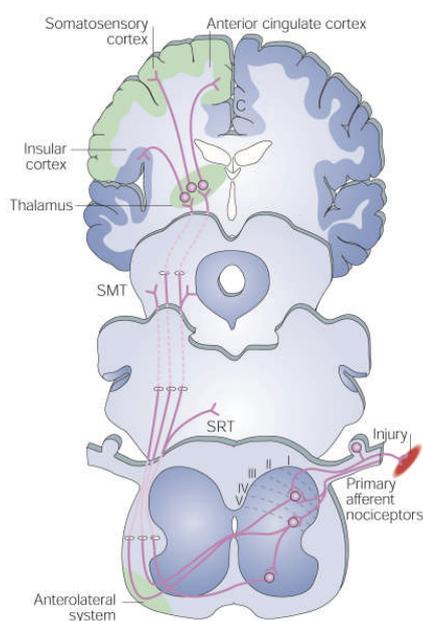


**Figure 1-1 – A schematic diagram to illustrate the afferent projections from the dorsal horn of the spinal cord to supraspinal areas, via the spinothalamic tract (consisting of the spinoreticular (SRT) and spinomesencephalic (SMT) tracts). Nociceptive stimuli are transduced by primary afferent nociceptors at the periphery and transmitted to the spinal cord, from where the signal ascends to the thalamus and is then passed onto higher cortical areas for processing. Adapted from Fields (2004).**

However, nociception and the sensation of pain are modulated by a number of mechanisms at different sites, and the brain exerts a large descending modulatory influence on spinal cord nociceptive processing, depending on both external stimuli and the motivational state of the organism (Scholz and Woolf, 2002; Fields, 2004). The

periaqueductal grey (PAG) of the midbrain receives direct input from the hypothalamus (which has a role in maintaining the internal homeostatic state of an organism) and limbic forebrain, which consists of a collection of cortical and subcortical structures that are implicated in memory and emotional processing, and includes the hippocampus and amygdala. From the PAG, descending modulatory signals are sent to the rostroventral medulla (RVM) of the brainstem, which transmits either excitatory or inhibitory signals to the nociceptive neurons of the dorsal horn of the spinal cord, with a net effect to either increase or decrease ascending nociceptive signals (see Figure 1-2).
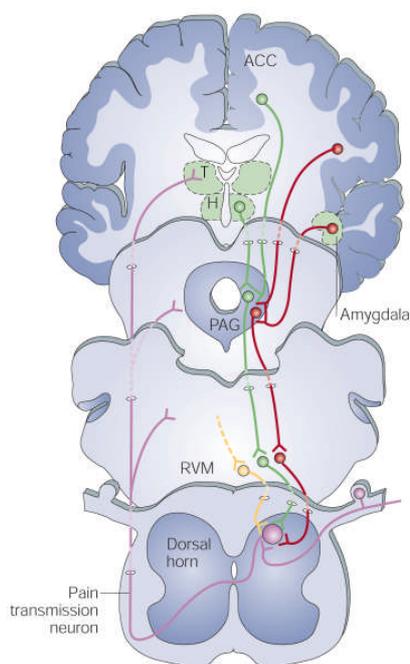


**Figure 1-2 – A schematic diagram to illustrate the descending pathways that can modulate nociceptive processing. The anterior cingulate cortex (ACC), hypothalamus (H), thalamus (T) and amygdala project to the periaqueductal grey (PAG), which can indirectly control pain transmission in the dorsal horn of the spinal cord through the rostroventral medulla (RVM). Excitatory modulation is shown in red, inhibitory in green. The yellow pathway represents a separate control channel via serotonergic neurons in the RVM. Ascending transmission is shown in purple. Adapted from Fields (2004).**

## 1.1.3.2 Spinal nociceptive processing develops over the first few weeks of an animal's life

The spinal cord is not mature at birth in the rat, and undergoes considerable change over the first 2-3 weeks of life (see Fitzgerald, 2005). In the adult, both Aδ and C fibres regulate nociceptive processing, and the Aβ fibres are involved in mechanosensation. However, in the neonatal rat, differences in the developmental rate of penetrance of the afferents from the dorsal root ganglia mean that nociceptive reflexes are predominantly mediated by Aβ and Aδ fibres, rather than by the C fibres, as in the adult. This is because myelinated afferents enter the spinal cord earlier than unmyelinated C fibres during embryogenesis (Ozaki and Snider, 1997; Jackman and Fitzgerald, 2000), and C fibre innervation is not present in lamina II until embryonic day (E) 18-19 (Fitzgerald, 1987). In addition, Aβ fibres grow terminal arbors that are larger than they will be in

adulthood, extending across spinal cord laminae that will not be innervated by these fibres in later life (Beggs et al., 2002; Granmo et al., 2008). Destroying developing C fibres with capsaicin, or blocking cell activity with NMDA receptor antagonists show that the dendritic withdrawal process is dependent on cell signalling between A and C fibres within the spinal cord (Torsney et al., 2000; Beggs et al., 2002).

The immaturity of dendritic projections of Aβ fibres in the developing spinal cord is likely to contribute to increased receptive field size (the area of the body that produces a response in a specific neuron when stimulated) of dorsal horn neurons (Fitzgerald and Jennings, 1999), and an overlap of A and C fibre signalling. This in turn leads to reflex withdrawal from normally innocuous stimuli and disorganised reflex responses to noxious stimuli (Jennings and Fitzgerald, 1996; Levinsson et al., 1999; Holmberg and Schouenborg, 1996; Waldenstrom et al., 2003). Indeed, in rats, a focal heat source directed to the tail will produce reflex withdrawals both towards and away from the stimulus, which becomes more organised over time (Waldenstrom et al., 2003). The fact that disordered withdrawal reflexes are also seen in young infants (Andrews et al., 2002) suggests that similar developmental spinal mechanisms are present in the human.

The enhanced, less organised spinal withdrawal responses in the neonate compared to the adult is also likely to be due to an immature balance between excitatory and inhibitory signalling (see Fitzgerald, 2005). Excitatory transmission is mediated by glutamatergic synapses in the spinal cord, and excitatory postsynaptic receptors such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid) and NMDA (N-methyl-D-aspartate) receptors are found at higher levels in the neonate than the adult (Jakowec et al., 1995; Gonzalez et al., 1993). In addition, the composition of receptor subunits changes over development, which will alter the relative permeability of these receptors to calcium ions (Burnashev et al., 1992; Wang et al., 1994). Inhibitory modulation by glycine and GABA (γ-aminobutyric acid) is not mature at birth, and high levels of chloride ions within the cell (due to a developmental lag in expression of the KCC2 transporter protein that regulates these ions within the cell) mean that GABA receptor activation in the neonate can be excitatory, instead of inhibitory (Ben-Ari, 2002). Spinal GABAergic and glycinergic inhibition is functional in neonatal rats, but undergoes significant maturation over the first days of life (Baccei et al., 2003; Ririe et al., 2008).

## 1.1.3.3 Descending inhibitory control from the brainstem is not mature in the neonatal rat

The brainstem plays a major role in modulation of ascending excitatory signalling from the spinal cord (Basbaum and Fields, 1984; Ren and Dubner, 2002), and modulation by the periaqueductal grey (PAG) and rostroventral medulla (RVM) is channelled down descending fibres via the dorsolateral funiculus (DLF) to the spinal cord (Basbaum and Fields, 1984; Sandkuhler, 1996) (see Figure 1-2). This descending inhibition does not reach maturity in the rat until 3 weeks of age (postnatal day (P)21) (Fitzgerald and Koltzenburg, 1986; Boucher et al., 1998).

Furthermore, the RVM undergoes a developmental shift beginning at around P21, from an exclusively facilitatory role in spinal pain transmission, to a biphasic role, both facilitating and inhibiting ascending spinal input (Hathway et al., 2009). PAG stimulation will not produce analgesia until 3 weeks of age, further suggesting that the brainstem nuclei involved in descending inhibition are not functional until the third postnatal week (Van Praag and Frenk, 1991b).

Together, this evidence demonstrates that pain processing is immature in the neonatal rat, and that before P21, excitatory spinal processing predominates, leading to enhanced responses to noxious and non-noxious stimuli. This excitability and lack of descending control may be an important factor in allowing central plasticity following early pain and injury.

## 1.1.3.4 Long-term effects of neonatal noxious stimuli in rodents

As in humans, interference with the neonatal rodent CNS causes long-term alterations in nociceptive responses. Similar to neonatal humans, animals cannot report their pain, but behavioural responses can be measured and monitored, and drug effects and physiological mechanisms investigated.

The consequences of neonatal injury in rodents depend, as in humans, on the type of injury (e.g. nerve damage or inflammation), and the sensory modality being investigated (e.g. heat or mechanical sensitivity). Inflammation induced by repeated paw prick (much like a human heel lance) causes thermal hyperalgesia (an exaggerated response to

a painful stimulus) in rat pups that lasts from several weeks up to adulthood (Anand et al., 1999; Walker et al., 2003a). Neonatal inflammation lasting up to 7 days, induced by injection of Complete Freund's adjuvant (CFA), results in enhanced spinal neuronal responses to paw pinch in the adult, as well as increased primary afferent nerve fibre innervation of the dorsal horn (Ruda et al., 2000), suggesting that the hypersensitivity exhibited after both short- and long-term inflammation may be mediated by spinal circuits.

Further evidence for spinal circuitry mediating later hypersensitivity comes from studies of skin wounds to neonatal rat pups. Wounds caused drops in mechanical withdrawal thresholds and increases in dorsal horn receptive field size long after the wound had healed (Reynolds and Fitzgerald, 1995; Torsney and Fitzgerald, 2003). Tissue injuries such as skin wounding cause release of nerve growth factors, and this increase in growth factors causes hyper-innervation of the skin, leading to the increased sensitivity to noxious stimuli seen in later life (De Lima et al., 1999). This is consistent with human studies showing hypersensitivity after injury in children, especially those with previous surgical history (Taddio et al., 1997; Andrews and Fitzgerald, 2002; Peters et al., 2005).

As in humans, global hypoalgesia in adult rats can also result from pain experience as pups, and is proposed to be mediated by changes in descending modulation (La Prairie and Murphy, 2009) or alterations in the stress response to injury (see Fitzgerald, 2005). Bhutta et al (2001) found that repeated formalin injections to the rat pup hindpaw within the first week of life caused decreased thermal sensitivity in the adult, the effect of which was decreased by pre-treatment with morphine. Daily foot-shock to rat pups from birth until P21 produced adult animals with a marked reduction in thermal sensitivity (Shimada et al., 1990), and the authors also found that morphine analgesia in adulthood was increased in the foot-shocked rats. The preventative role of analgesia in the long-term effects after this type of injury is also seen in mice (Sternberg et al., 2005) and humans (Taddio et al., 1997; Taddio and Katz, 2004).

Ren et al (2004) modelled both the baseline adult hypoalgesia after neonatal injury, and hypersensitivity after re-injury, by injecting carrageenan (which causes short-term inflammation lasting ~24 hours) to the rat hindpaw within the first two weeks of life.

When mechanical and thermal thresholds were tested at baseline in adulthood (P120-125), significant hypoalgesia was seen in both the ipsilateral (same) and contralateral (opposite) paws, an effect replicated by La Prairie and Murphy (2007). When the adults were given a further carrageenan challenge, however, hypersensitivity to noxious stimuli was seen only in the ipsilateral paw. The authors suggest that the local hypersensitivity is mediated by spinal segmental changes after neonatal inflammation, i.e. alterations in the developmental connectivity of the spinal cord and/or sprouting of nerve fibres at the skin, resulting in an increased nociceptive response. The hypoalgesia they attribute to alterations in descending inhibitory tone or alterations in stress regulation, support for which comes from studies showing that early stress alters later nociceptive processing (Pieretti et al., 1991; Coutinho et al., 2002). Similar baseline hypoalgesia after neonatal CFA and hypersensitivity to re-inflammation was shown by Lidow and colleagues (2001).

## 1.1.3.5 Critical periods in development and long-term effects

Consistent with evidence on the maturation of spinal nociceptive processing, plus human studies showing that pre-term infants experience lasting effects of noxious stimulation but that full-term infants do not (Taddio et al., 1997; Walker et al., 2009a), it would appear that it is stimulation during a 'critical period' of development which predicts long-term changes (La Prairie and Murphy, 2010). Critical periods are defined as time windows within which an organism develops in a particular way. Interference with development during this window will cause long-lasting changes, yet outside of the critical period, the same stimulation will not lastingly affect development. Critical periods have been described in a variety of modalities, including vision, hearing, the stress response (see Hensch (2004) for a comprehensive review), and more recently pain (La Prairie and Murphy, 2007; Walker et al., 2009b).

The concept of a critical period is demonstrated by the effect of nerve injury on neonates at different stages of development. In adults, peripheral nerve damage causes significant allodynia (a withdrawal response to a previously innocuous stimulus) and neuropathic-like pain behaviours that last for weeks to months (Decosterd and Woolf, 2000). However, the same nerve damage to rat pups performed within the first three weeks of life produces only transient increases in sensitivity. If, however, surgery is performed at P33, lasting allodynia is produced (Howard et al., 2005). This

phenomenon is consistent with human data showing that brachial plexus damage, which injures the nerves innervating muscles and skin of the arm and upper torso, does not cause chronic pain in neonates as it does in adults (Anand and Birch, 2002).

From the evidence discussed above, long-term alterations in nociceptive processing only occur within certain critical periods. In the rat, this is within the first three weeks of life. In the human neonate, long-term effects occur mainly in post-premature infants, born between 24 and 38 weeks PMA. As the first two weeks of the rat's life correspond approximately to the final trimester in human gestation, this strongly suggests that the critical periods are the same for both species, and means that the developmental mechanisms described in the rat that mediate nociceptive processing (i.e. development of spinal cord connectivity, maturation of spinal neuronal responses, and appearance of descending inhibition) are likely to be similar to those in the human. This, in turn, could help inform clinical practice in the treatment of preterm infants in the NICU.

## *1.2  Pain and reward processing are linked*

The experience of pain is unpleasant – indeed, the International Association for the Study of Pain (IASP) define pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". This definition inherently accepts that pain is a subjective emotional experience, but also that the relief from pain will be the opposite of unpleasant, i.e. pleasurable, or rewarding. The main groups of analgesics in use in clinical practice, such as opioids, are also intensely rewarding and subject to addiction and abuse (Franklin, 1989), and there is a huge overlap in brain areas involved in pain and reward processing (see Borsook et al., 2007; Leknes and Tracey, 2008 for reviews). Furthermore, many drugs of abuse have analgesic actions, such as cocaine, amphetamine, nicotine, ketamine, and alcohol (Franklin, 1989).

### 1.2.1 Areas of the brain involved in pain and reward processing overlap

Rewarding and painful (i.e. aversive) stimuli are positive and negative reinforcers of behaviour in animals, and the dopaminergic and opioidergic systems of the brain are critical for both types of reinforcer, as they provide both the 'pleasure' of rewards and are involved in antinociception and analgesia (see Borsook et al., 2007). Human

imaging studies have shown that the same areas activated by reward are also activated by pain (Becerra et al., 2001; Seymour et al., 2005; Scott et al., 2007) and much data now suggests that rewarding and aversive stimuli affect similar pathways in the CNS, leading to a robust demonstration that pain and reward processing involves many overlapping areas. These areas include the hypothalamus, amygdala, RVM, PAG, hippocampus, prefrontal cortex, anterior cingulate cortex (ACC), insular cortex, ventral tegmental area (VTA), and nucleus accumbens (NAcc) (see Leknes and Tracey (2008) for a comprehensive review).

To illustrate the overlapping regions in brain pain and reward, brief evidence is presented for dual processing roles in three structures: the nucleus accumbens, amygdala and anterior cingulate cortex.

### 1.2.1.1 NAcc

The NAcc is particularly well-situated to mediate pain and reward processing. It receives direct input from dopaminergic neurons in the mesolimbic dopamine system and injection of opioids directly into the NAcc, which activates reward pathways, induces analgesia (Dill and Costa, 1977). Opioid antagonism by accumbal injection of naloxone attenuates the analgesic effect of morphine (Gear and Levine, 1995), and two distinct regions of the NAcc shell have been identified: one that mediates pleasure, and one that causes a negative shift in affect (Reynolds and Berridge, 2002). Dopamine analgesia is mediated through the NAcc (Altier and Stewart, 1998; 1999).

### 1.2.1.2 Amygdala

The amygdala is part of the descending pain modulatory system (see Figure 1-2) is activated in humans during both acute and chronic pain (Becerra et al., 2001; Harris et al., 2007a), and is associated with the emotional reactivity to pain (see Finn et al., 2003; Neugebauer et al., 2004). The amygdala also shows activation following both rewarding and aversive stimuli (Breiter et al., 1997), possibly due to its role in orienting to motivationally salient stimuli (see Rolls, 2000).

### 1.2.1.3 Anterior cingulate cortex

The ACC is critical for learning aversive teaching signals i.e. the cue that predicts when an animal will receive a noxious stimulus (Johansen et al., 2001; Johansen and Fields,

2004), and human imaging studies have shown that the ACC is implicated in aberrant pain signalling in chronic pain patients (Apkarian et al., 2001). Furthermore, lesions of the ACC decrease the physical perception of pain, implicating a role in analgesia (Wilkinson et al., 1999). Shidara and Richmond (2002) showed that ACC neurons in monkeys code for reward expectancy, illustrating a role for the ACC in reward processing.

## 1.2.2 Analgesia and relief from pain are rewarding

As pain is potentially damaging to an organism, a pain state is one that an organism will work to avoid. Conversely, a reward is something that an organism will work to achieve, and includes pain relief, i.e. analgesia. As pain is a deviation from the standard internal state, the motivation for pain relief can be thought of in terms of the animal striving to regain homeostasis as it would for other systems, i.e. hunger and thirst. Together, this leads to conceptualisation of pain and reward as opposite ends of the same spectrum, with homeostatic balance being striven for. In general, the reward value of a stimulus increases the more effective that stimulus is at restoring homeostasis (see Craig, 2003), which would mean that analgesics which are most effective in reducing pain are those with the highest reward value. That analgesia is rewarding is supported by several lines of evidence, as detailed below.

### 1.2.2.1 Termination of a pain stimulus is rewarding

Firstly, termination of a painful stimulus is rewarding (Seymour et al., 2005; Leknes and Tracey, 2008). For example, Leknes and colleagues (Leknes et al., 2008) performed a human psychophysical experiment into the pleasantness of pain relief. A noxious thermal stimulus of varying intensity (ranging from pain threshold to intense pain, as rated by self-report using a visual analogue scale) was briefly applied to the arm. Subjects were asked to self-report the maximal relief felt when the stimulus was terminated. The authors found a strong positive correlation (r=0.81) between the pain intensity and sensation of relief (see Figure 1-3), showing that the termination of the noxious stimulus was more rewarding when the pain intensity was higher. These findings are consistent with data showing that relief from other unpleasant states such as hunger activates reward processing areas in the brain (Kringelbach et al., 2003), as does expectation of pain relief (Seymour et al., 2005). In addition, psychophysical data has shown that pain perception increases as stimulus intensity increases (Price, 1999),

therefore directly linking stimulus intensity, pain sensation, and reward upon pain cessation.
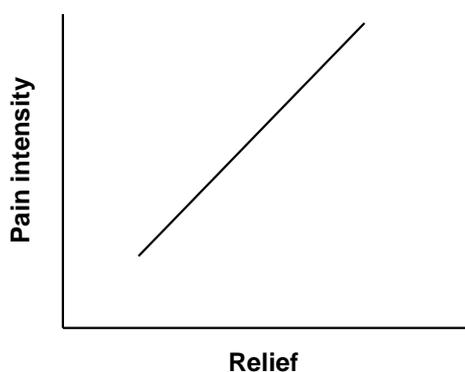


**Figure 1-3 - A schematic illustration of the positive correlation found by Leknes et al, whereby subjective relief upon pain cessation increases as pain intensity increases. Adapted from Leknes et al (2008).**

## 1.2.2.2 The placebo effect relies on reward processing

The placebo effect occurs when patients perceive benefit from treatment, despite it having no genuine therapeutic effect. The effect therefore relies on expectation of pain relief, and is mediated by opioidergic mechanisms, as similar brain areas are activated by opioid agonists and placebo analgesia (Levine et al., 1978b; Petrovic et al., 2002; Benedetti et al., 2005). Human imaging (fMRI) studies over the past decade have implicated crucial brain areas involved in the placebo response, most of which are classically implicated in reward processing. For example, the release of endogenous opioids and dopamine from mesolimbic reward areas such as the nucleus accumbens and ventral striatum has been shown during placebo analgesia (Zubieta et al., 2005; Scott et al., 2008). In addition, the magnitude of the placebo effect is predicted by the release of dopamine from the nucleus accumbens (Scott et al., 2007) and grey matter density in brain reward processing areas (Schweinhardt et al., 2009). Dopamine-related personality traits (such as sensation-seeking and reward responsiveness) are also linked to the extent of placebo analgesia (Schweinhardt et al., 2009).

## 1.2.2.3 Rewarding stimuli induce analgesia

Analgesia can also result from a variety of classically rewarding stimuli, such as pleasurable music, odours, images, and sexual behaviour (Leknes and Tracey, 2008). Palatable food can also induce analgesia. A classic study by Dum and Herz (1984)

showed that thermal nociceptive withdrawal thresholds of rats are raised when they are expecting a chocolate reward, as the animals took longer to move off a hot-plate when they had previously received a chocolate reward under the same conditions. This effect was reversed by naloxone (an opioid antagonist), and is therefore opioid-mediated. As discussed previously, sucrose can be used as an analgesic in neonatal care (see Stevens et al., 2010). The fact that sucrose is not a 'classic' analgesic i.e. does not directly influence pain transmission (Fitzgerald, 2009), suggests that sucrose may be acting via supraspinal reward pathways in the neonate, evidence for which comes from the fact that sucrose analgesia in animals is blocked by naloxone (Blass et al., 1987).

### 1.2.2.4 Chronic pain states reduce reward responses

Whilst opioids are highly rewarding, chronic pain patients do not show the same levels of addiction to opioids as non-patients (Noble et al., 2010). In both inflammatory and nerve injury pain states, morphine loses its rewarding effects (Suzuki et al., 1996). The mechanisms for this are thought to involve dysregulation of mu opioid receptor function in the VTA via an ERK-mediated signalling pathway, caused by continuous release of endogenous opioids caused by nerve injury (Ozaki et al., 2002; Ozaki et al., 2004; Niikura et al., 2008). The loss of the rewarding effects of opioids in an inflammatory pain state is also due to sustained activation of the kappa opioidergic system, which is anti-analgesic (Narita et al., 2005).

### 1.2.3 Endogenous opioids modulate pain and reward

As many of the above studies show, opioids are essential for modulation of both pain and reward (Fields, 2007). Exogenous opioids, such as morphine, are potent analgesics and drugs of abuse, and endogenous opioids, produced and released internally, are critically involved in the descending modulatory control of nociceptive processing (see Fields, 2004). The analgesic effect of opioids are produced by direct action on spinal cord nociceptive circuits, and supraspinal disinhibition of brain reward areas, which leads to increased dopamine release in the forebrain, and results in the rewarding effects of opioids.

There is robust evidence for the importance of endogenous opioids in nociception. Opioid receptors are found throughout the brain, but particularly in areas involved in both nociceptive and reward processing such as the RVM, PAG, and ventral striatum

(see Le Merrer et al., 2009). Injection of opioid receptor agonists produces analgesia, particularly in the PAG (Yaksh et al., 1976), and electrical stimulation of areas rich in opioid receptors such as the PAG produce increases in nociceptive withdrawal thresholds in animals (Mayer and Liebeskind, 1974) and analgesia in humans (Hosobuchi et al., 1977). Antagonism of opioidergic signalling in the PAG and RVM blocks analgesia (Kiefel et al., 1993). Furthermore, the brain releases endogenous opioids in response to painful stimuli, suggesting an internal anti-nociceptive circuit is being activated (Zubieta et al., 2001).

However, descending modulation of pain can excite or inhibit pain responses due to specific subsets of cells within the PAG and RVM exerting opposite effects on spinal cord nociceptive signalling. RVM stimulation can both inhibit or facilitate nocifensive behaviours and dorsal horn neuronal responses (Urban et al., 1999; Zhuo et al., 2002). The two classes of neurons responsible for this opposite signalling are termed ON and OFF cells, with a third class of serotonergic neurons classified as NEUTRAL cells, which have a variable response to noxious stimuli (Potrebic et al., 1994) (see Figure 1-2).

ON cells are characterised by a burst of activity that begins just before a nociceptive withdrawal reflex, and OFF cells show a pause in firing that begins just prior to the withdrawal reflex (Heinricher et al., 1989). Both classes of cells project to specific laminae of the spinal cord that relay nociceptive signals (Fields et al., 1995), and firing rates are affected by opioid receptor agonists, which cause continuous OFF cell firing and silence ON cells (Heinricher et al., 1994). The net activity of one set of neurons over the other is thought to rely on the behavioural state of the animal, as activity is controlled by the higher order brain areas reciprocally connected to the PAG and RVM, such as the amygdala, hippocampus and frontal cortices, which assimilate information from multiple areas to control behaviour (see Fields, 2004).

## 1.2.4 Motivation-decision model

The systems controlling the facilitation or inhibition of spinal cord nociceptive processing are best understood when looking at pain as a behavioural motivator, in that escape from painful stimuli and seeking of relief from pain are highly motivating drives. The motivation-decision model, as put forward by Fields (2004; 2006), states that

anything that is potentially more important for survival than pain should exert anti-nociceptive effects. For example, if an animal is subject to a mild pain state in order to gain a reward such as food or sex, the pain state will be ignored as the reward exerts an anti-nociceptive effect. Conversely, if the tissue damage, or threat of damage, is severe enough to threaten the animal's survival, any reward will be ignored as the animal engages in self-protective strategies such as escape, avoidance, and guarding behaviour of any injury site. Fields (2006) states that the control over the reward or survival behaviours is mediated by cortical brain structures involved in reward and decision-making, and signals sent via the biphasic descending opioid modulatory system.

Evidence for this model comes from a variety of sources. Animal studies have shown that rewards elicit an increased tolerance of pain, and that this is mediated by endogenous opioid release, as shown by Dum and Herz (1984). Stimuli that elicit innate (i.e. predator-driven) and conditioned (i.e. learnt) fear block pain responses via opioid release, indicating that the blockade of pain responses is in order to protect the organism against a greater immediate threat to survival (Fanselow, 1986). Indeed, animals conditioned to receive a foot-shock show longer withdrawal latencies upon presentation of the conditioned stimulus, which relies on endogenous mu opioid signalling in the amygdala, PAG and RVM (Bellgowan and Helmstetter, 1998; Foo and Helmstetter, 1999). This phenomenon, termed stress-induced analgesia, has been the subject of much research, and is modulated by the descending inhibitory pain pathway in both animals and humans (Willer et al., 1981; Butler and Finn, 2009).

In humans, further evidence for the motivation-decision model comes from findings that the anticipation of pain activates descending inhibitory pathways. Keltner et al (2006) showed that pain expectation affected the activation of classic nociceptive areas such as the thalamus and somatosensory cortices, and that this modulation converged on the nucleus cuneiformis, a brainstem region that receives input from the PAG and projects to the RVM. Fairhurst et al (2007) also found that pain anticipation caused increased activation of the PAG, and that actual receipt of the pain activated the PAG, RVM and ventral tegmental area. The authors proposed that the anticipatory activity in the PAG was to activate descending inhibitory pathways to help modulate the pain. Supporting this, the anticipation of a reward or pain relief, as already shown, causes release of endogenous opioids in the human brain (Zubieta et al., 2005).

Becerra et al (2001) provided a potential neurobiological substrate for the 'decision' aspect of the motivation-decision model. The authors showed that noxious thermal stimuli activated both classical reward and pain areas, but that an early phase of activation was seen in 'reward' structures such as the nucleus accumbens and ventral tegmental area, and a later phase of response was seen in 'pain' areas such as somatosensory and anterior cingulate cortices, thalamus and insula. Fields (2006) proposes that this early phase activation is when the response 'decision' is made, taking into account salient external factors and motivational drives. This proposal fits well with evidence that dopaminergic signalling in brain reward areas is involved in incentive salience (i.e. how important a stimulus is to an organism), valence (i.e. whether a stimulus is positive or negative in nature) and behavioural motivation (see Wise, 2004; Schultz, 2007). Once the 'decision' is made, the late phase activation of 'pain' areas seen by Becerra and colleagues controls the behavioural response, as higher cortical areas such as the anterior cingulate, amygdala and prefrontal cortex send output to the midbrain structures that biphasically modulate spinal cord nociception.

## 1.2.5  Does early pain lead to altered reward processing in later life?

The evidence presented above shows that pain during a critical period of development causes long-lasting changes in pain responses, but the effects of early pain on alternative modalities is an area of much research.

Studies have shown the long-term effects of repeated neonatal pain and stress on the adult stress response, in terms of the responsiveness of the HPA axis. Infants with prior NICU experience show alterations in stress responsivity later in childhood (Grunau et al., 2005; Grunau et al., 2007), and animal studies have investigated the impact of stressors such as maternal deprivation and repeated handling on the organism's neurobiology and later adult behaviours (Pieretti et al., 1991; Coutinho et al., 2002; Champagne and Meaney, 2006; Lippmann et al., 2007; Enthoven et al., 2008). Meaney et al (2001) studied the impact of early maternal deprivation on dopaminergic processing, explicitly linking early stress and reward, and found changes in dopamine transporter proteins and adult response to cocaine and amphetamine. As pain is inherently stressful, this strongly suggests that neonatal pain could alter reward processing in the adult.

Maternal style in rats and humans also impacts upon later responses to stress, novelty and pain, (Meaney, 2001; Champagne et al., 2003; Champagne and Meaney, 2007; Clinton et al., 2007; de Medeiros et al., 2009), the effects mediated by epigenetic influences on gene expression and translation (Weaver et al., 2004; Fagiolini et al., 2009). Furthermore, evidence from rodent, primate and human studies suggests that the infant-parent attachment is rewarding for both mother and infant, and that this bond is mediated by opioidergic and dopaminergic systems (Moles et al., 2004; Barr et al., 2008; Noriuchi et al., 2008), both of which are critically involved in pain and reward processing. Due to the large overlap between pain and reward processing, we hypothesise that pain during early life will lead to alterations in reward processing in the adult animal, and this hypothesis forms the basis of this thesis.

There have been suggestions in the literature that early pain can affect later reward-related processing, although to date no published literature exclusively investigating this question. Studies into the long-term effects of early pain on adult pain processing have shown changes in preference for alcohol as adults (Bhutta et al., 2001) or alterations in opioid tone (La Prairie and Murphy, 2009). However, these studies are investigating pain, gender and the effects of early analgesia, and do not discuss their results in terms of reward processing. They do, however, provide intriguing suggestions that early pain may affect the opioid-mediated descending modulation of pain, which, as discussed above, shares common brain regions with areas involved in reward processing.

## 1.3  The aims of this thesis

To summarise the above evidence, it is known that early pain causes long-term effects on pain processing, and that pain and reward processing are neurobiologically linked. We therefore hypothesise that repeated painful experience will affect reward processing in the adult.

This thesis will investigate this question in three main ways:
1) To test the feasibility of the hypothesis that early life pain can interfere with developing reward pathways, we needed to establish the functionality of reward pathways in the neonate. Chapter 2 will therefore investigate the functionality of

reward pathways in the neonate using a classic analgesic and drug of abuse, morphine, to activate the system.

2) To test the hypothesis that early pain experience can alter adult reward behaviour, Chapter 3 will investigate the effect of repeated neonatal pain on adult reward behaviours, using a model of neonatal injury designed to mimic clinical procedures. Reward behaviour will be investigated using a conflict paradigm that places an animal's motivation for reward in competition with its drive for safety and survival.

3) Finally, to test the hypothesis that early pain experience can alter adult reward cell signalling in the CNS, Chapter 4 will investigate whether early pain experience alters the activation of the orexin system of the lateral hypothalamus during reward behaviours. The orexin system impacts upon opioid and dopaminergic transmission and is strongly implicated in reward processing.

# 2 Chapter 2 – Investigating the effect of morphine on neonatal reward pathways

## 2.1 Introduction

The aim of this chapter is to investigate the function of the neonatal mesolimbic dopaminergic system and how it differs from that of the adult, using morphine to stimulate and activate the system. Activity of the dopaminergic cells of the ventral tegmental area (VTA) of the rat midbrain have been used to investigate the functional role of dopaminergic pathways after acute and chronic morphine administration in young rats. Morphine is not only a potent analgesic but also a drug of abuse, and is well-known to activate the reward pathways in the adult brain (see Le Merrer et al., 2009).

The response of the adult dopaminergic system to rewarding stimuli is well-documented, but the development in the neonatal system is less well-known. As the overall hypothesis of this thesis is that pain and reward processing overlap, and that pain during early life affects later reward-related processing, then it is important to see to what extent the early dopaminergic reward system can be activated in the newborn. Morphine is a useful and classical method of testing this.

In addition, it is important to understand the central effects of morphine in the newborn, as this is a critical period for the development of the pain system and morphine is frequently prescribed in neonatal intensive care units (NICU) to sedate premature infants and provide analgesia for some procedures (see Anand, 2007).

### 2.1.1 Dopamine (DA)

Dopamine is a catecholamine and, as well as being an important neurotransmitter in its own right, it is also the precursor to adrenaline and noradrenaline. Dopamine-releasing cells are located mainly in the substantia nigra pars compacta (SNpc), the hypothalamus, and ventral tegmental area (VTA) and dopamine is broken down by the enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).

Figure 2-1 shows the biosynthetic pathway for dopamine:

L-Tyrosine → L-DOPA → Dopamine → Noradrenaline → Adrenaline

(Tyrosine hydroxylase) (DOPA decarboxylase) (Dopamine β-hydroxylase) (Phenylethanolamine *N*-methyltransferase)

**Figure 2-1 – the biosynthetic pathway for dopamine, showing precursors and enzymes**

Dopamine acts on $D_1$ to $D_5$ G protein-coupled receptors (GPCRs). D1 and D5 are classified as 'D1-like receptors' and D2, 3 and 4 as 'D2-like receptors'. The two groups are similar in terms of structure and pharmacological profile, but differ in terms of localisation within the brain and relative distribution. Furthermore, 'D1-like' receptors couple to the $G_s$ GPCR, stimulating adenylate cyclase to activate protein kinase A (PKA), whereas 'D2-like' receptors couple to the $G_i$ GPCR signalling pathway, which inhibits PKA (see Rho and Storey, 2001). The D1 and D2 receptors are most strongly implicated in reward and addiction, as shown by animal (Maldonado et al., 1997; Pruitt et al., 1995) and human imaging studies (see Volkow et al., 2009).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in DA production from its precursor (see Figure 2-1). It is widely-used as a dopaminergic cell marker the expression of TH has been used in this investigation to map dopaminergic cells of the ventral tegmental area at different ages (Narita et al., 2004a; Berhow et al., 1996).

### 2.1.1.1 Dopamine in the brain

The dopaminergic system has been the subject of a huge number and variety of experiments and various controversies over the years, and is now known to be involved in a variety of brain systems. In the basal ganglia, dopamine cells are crucial in the initiation of movement (see Lewis and Barker, 2009). Imbalances of dopamine in the prefrontal cortex can lead to psychosis, and a lack of dopamine has been linked to depression (see Totterdell, 2006). It also has a major role in reward and motivational processing, via the mesolimbic dopaminergic system (see Wise, 2004).

Tracing and lesion studies of dopaminergic projections have elucidated the anatomy of connections well, showing that dopaminergic fibres project to many areas of the brain in both rats (Deutch et al., 1988) and man (Haber and Knutson, 2009).
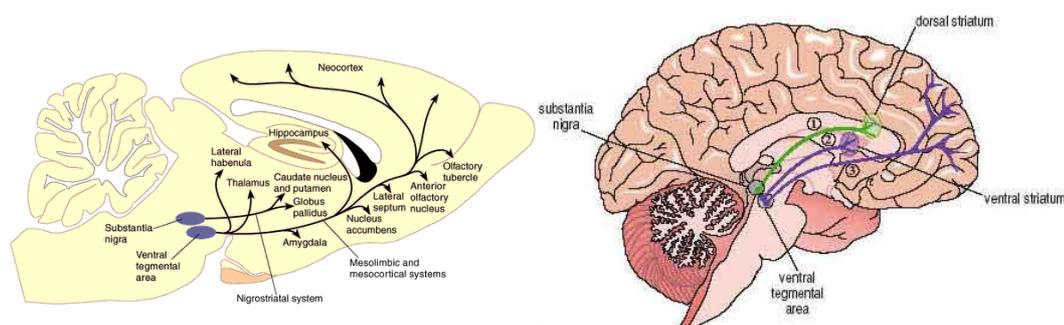
**Figure 2-2 - a schematic to show the nigrostriatal and mesocorticolimbic projections in the rat (left) and human brain (right). In the human brain schematic, the green pathway is the nigrostriatal projection and the purple the mesolimbic projections from the VTA to the ventral striatum and prefrontal cortex (left: adapted from http://homepage.psy.utexas.edu/homepage/class/Psy332/Salinas/Neurotransmitters/Slide08.GIF; right: adapted from http://openlearn.open.ac.uk/mod/resource/view.php?id=192954).**

Many addictive drugs, including many analgesics, affect the dopaminergic system. They increase dopamine levels in the brain by a variety of methods. Cocaine and amphetamine block the dopamine reuptake transporter (Giros et al., 1996), which allows DA to remain in the synaptic cleft for longer. Nicotine acts on presynaptic cholinergic receptors to increase DA release, and mu opioids inhibit GABAergic interneurons that, at basal levels, inhibit the DA cells of the VTA.

Dopaminergic drugs also have analgesic potential. In contrast to the opioids, which cause sedation, dopaminergic drugs increase alertness whilst having a similar efficacy to opioids in the relief of suffering. Combinations of opioids and dopaminergic drugs have been suggested as an effective therapeutic avenue, as dopaminergic drugs such as amphetamine are stimulatory, and can counteract opiate-induced respiratory depression. There is some reluctance to include dopaminergic drugs in clinical practice, perhaps as it has been argued that the analgesia they produce is mediated through the rewarding effects of the drugs, and their abuse potential when combined with opioids for maximal analgesia, is too high (see Franklin, 1989; 1999).

Clinically, dopamine antagonists are often used as anti-psychotics and indirect agonists (i.e. cocaine) as psychostimulants. As DA cannot cross the blood-brain barrier, its precursor, L-DOPA, is given as a form of medication for diseases where dopaminergic function is lost i.e. Parkinson's disease.

Reinforcement, reward, incentive motivation, conditioned reinforcement, and anhedonia are all thought to depend upon the function of dopamine in the brain and these proposals are all supported and refuted by a massive body of literature spanning 60 years. The evidence for a critical role for dopamine in reward processing, however, is very strong, as discussed below (see Wise, 2004).

## 2.1.2 Mesolimbic dopaminergic system

Dopaminergic neurons can be broadly subdivided into two groups that form two pathways. One originates in the zona compacta of the substantia nigra (SNc) and gives rise to the nigrostriatal pathway, which is crucial for the control of movement, orchestrating a delicate balancing act between the nuclei of the basal ganglia to produce and refine movement.

The second pathway originates in the ventral tegmental area (VTA) and is comprised of two interlinked and overlapping pathways; the mesolimbic and mesocortical pathways, thought to be important for motivational functioning. The mesolimbic pathway runs from the VTA to the nucleus accumbens (NAcc) and olfactory tubercle, further innervating the septum, amygdala and hippocampus. Medial VTA cells project to medial prefrontal, cingulate and perirhinal cortices and this projection is known as the mesocortical pathway. Because there is large overlap between the VTA cells that project to these various targets, the pathways are collectively referred to as the 'mesocorticolimbic' dopamine system.

The mesocorticolimbic system has been the subject of many investigations over the years. Olds and Milner (1954) first found that intracranial electrical stimulation of the hypothalamus and associated mesolimbic structures can be a rewarding stimulus for an animal, and later studies have shown that animals trained to stimulate themselves electrically in areas through which dopaminergic neurons are known to pass will repeatedly stimulate themselves, above other motivational factors such as food and sex (Franklin, 1978; Liebmann and Butcher, 1974).

Blocking dopaminergic signalling via receptor antagonism, using neuroleptic drugs, can reduce the effect of rewarding stimuli and self-stimulation (Fouriezos et al., 1978; Wise et al., 1978) and ablation through the administration of 6-OHDA, an excitotoxic

dopamine agonist, can abolish certain reward-seeking behaviours (Berridge et al., 1989).

## 2.1.2.1 Genetic manipulation of the DA system

The use of genetically modified mice where different dopamine receptor subtypes have been deleted highlight their varying roles in reward processing (see Holmes et al., 2004).

The rewarding effects of cocaine are lost in mutant mice where the dopamine D1 receptor has been knocked out (Xu et al., 1994; Drago et al., 1996). Mutant mice lacking the D2 receptor have severe motor problems (Baik et al., 1995) and show a decreased response to cocaine (Caine et al., 2002). These D2 knockout mice do not self-administer morphine nor learn morphine-cued conditioned place preference (Maldonado et al., 1997), and they also show abnormal responses to ethanol and cocaine (see Holmes et al., 2004).

Deleting the gene that codes for tyrosine hydroxylase produces dopamine-deficient (DD) mice, of which 90% will die during gestation unless DA signalling is rescued by maternal L-DOPA administration. Once born, they are indistinguishable from wild-type litter-mates until around P10, but will die by P15 without DA rescue (by L-DOPA injection), as they will not feed enough to survive (Zhou et al., 1995). If reared to adulthood by repeated L-DOPA administration, DD mice can learn place preferences, but appear apathetic and show minimal interest in their environment, and do not seem to engage in goal-oriented behaviour i.e. nesting or exploratory behaviours, suggesting a lack of motivational drive (see Palmiter, 2008). Dopamine also has a role in fear conditioning via the amygdala. Adult DD mice do not learn fear conditioning in a fear-potentiated startle paradigm, suggesting a role for dopamine in reward and aversion learning (Fadok et al., 2009).

## 2.1.3 Ventral Tegmental Area

The ventral tegmental area (VTA) is located in the midbrain (mesencephalon) and neurons in the VTA are reciprocally connected to almost all of the areas to which they project, including the nucleus accumbens, substantia nigra, limbic structures such as the

amygdala, and cortex. It contains the dopaminergic cells that give rise to the mesocorticolimbic system (see Figure 2-2 and Figure 2-4 for localisation of the VTA).

## 2.1.3.1 Firing properties of VTA DA cells

Dopamine cells fire at two different frequencies – tonic and phasic (Grace, 1991; Grace et al., 2007) Tonic firing is spontaneous, low frequency activity of ~4Hz that releases low levels of dopamine steadily into the synaptic cleft (Brischoux et al., 2009). Phasic firing is the burst of activity that occurs after a stimulus and releases large amounts of dopamine transiently into the synaptic cleft, thus activating post-synaptic receptors. Phasic firing typically occurs in bursts of around 5 spikes, at frequencies above 12Hz (Ungless et al., 2004), starting with an interspike interval (ISI) of less than 80msecs and finishing with an ISI of 120msecs (Brischoux et al., 2009). Phasic and tonic firing are proposed to be inversely related, such that an increase in tonic extracellular DA levels attenuates subsequent phasic firing (see Wood, 2006).

The work of Schultz on awake monkeys (Schultz et al., 1998; 2001; 2007) has suggested that dopaminergic neurons of the VTA encode reward, showing that VTA cells will fire on receipt of an unexpected reward, and are inhibited when the expected reward does not appear. Cells in the rat VTA will also respond to aversive stimuli i.e. footshock (Mirenowicz and Schultz, 1996; Guarraci and Kapp, 1999). Originally, these cells (3-49% of VTA cells recorded) were declared non-dopaminergic (Ungless et al., 2004) but later research has confirmed the existence of dopaminergic cells that fire for both rewarding and aversive stimuli (Brischoux et al., 2009).

Recent suggestions help resolve the debate by putting forward the view that there are two functionally and anatomically distinct dopamine systems in the VTA (Ikemoto, 2007). An important paper by Brischoux et al (2009) shows that dorsal VTA dopaminergic cells are inhibited by noxious footshock, consistent with their role in reward processing, as a non-rewarding stimulus inhibits cell firing. However, ventral VTA cells are phasically excited by footshock, suggesting that dopaminergic signalling is involved in both rewarding and aversive events. The suggestion that there are two functionally and anatomically discrete cell populations in the VTA that signal either rewarding or aversive stimuli fits well with data showing that the amygdala, an area

receiving input from the ventral VTA, is implicated in noxious processing and is involved in the learning of aversive stimuli (Barr et al., 2009).

Taken together, electrophysiological research has led to ideas that the DA neurons of the VTA fire according to the expectation and receipt of a reward (reward error-prediction) (Schultz, 2007; Berridge and Robinson, 2003; Robinson and Berridge, 1993; McClure et al., 2003) and also the incentive salience of a stimulus, i.e. how important it is to the organism, as seen by studies showing the rewarding and aversive signalling properties of DA neurons (Ungless, 2004)

## 2.1.3.2 Behavioural studies

Many studies have investigated the effect of dopamine modulation in the nucleus accumbens, which receives input from the VTA (Di Chiara and Bassareo, 2007). However, experiments in mice and rats studying at the effect of intracranial self-administration (ICSA) and intracranial place conditioning (ICPC) have proven that the VTA itself is a crucial site for reward processing (see McBride et al., 1999). ICSA involves implantation of a cannula into the brain which delivers a drug when the animal completes a particular response i.e. nosepoke, whilst in the ICPC paradigm, a drug is infused and the place preference measured in animals after infusion. Experiments have shown that animals will self-administer morphine into the VTA and that the effect is blocked by a morphine antagonist, demonstrating that the effect is mu receptor-mediated (Bozarth and Wise, 1981; Devine and Wise, 1994). Morphine infusion into the VTA also causes the induction of place preference (Phillips and LePiane, 1980; Bozarth, 1987).

## 2.1.3.3 Human imaging studies

Human imaging studies have confirmed an important role of the VTA in reward processing. Functional magnetic resonance imaging (fMRI) studies have shown an increase in BOLD (blood oxygenation level-dependent) signal in the VTA after administration of rewarding drugs (Breiter et al., 1997; Sell et al., 1999) and a decrease with noxious heat application (Becerra et al., 2001). This supports electrophysiological studies suggesting that the VTA processes both rewarding and aversive stimuli, and adds weight to the theory that the VTA DA system has a role in incentive salience.

Future imaging research may focus on investigating the detailed functional connectivity of the VTA and its role in reward networks (see Camara et al., 2009).

## 2.1.4 Ontogeny of the dopaminergic system

The adult dopaminergic system has been well-characterised, but the development of this system and the effect of neonatal interference on adult dopaminergic function has also been an active area of research over the last 50 years.

### 2.1.4.1 Cell differentiation

Dopamine cell differentiation and localisation is driven by specific gene pathways early in embryonic life. The transcription factors sonic hedgehog (Shh) and fibroblast growth factor 8 (Fgf8) induce mesolimbic dopamine cell differentiation in the ventral midbrain (later giving rise to the substantia nigra and VTA) at E10.5 to E13 in the mouse. The survival of the DA cells and enzymes regulating their activity (i.e. tyrosine hydroxylase) depend on transcription factors such as *Nurr1, Pitx3,* and *Lmx1b* for cell survival and maintenance throughout the lifetime of the animal (see Alavian et al., 2008).

### 2.1.4.2 Monoamine levels and receptor expression

Dopamine and its receptors appear between E10 and E15 in the rat (Voorn et al., 1988; Schambra et al., 1994) and reach adult concentrations at around P50 (Loizou and Salt, 1970). The dopamine transporter protein (DAT), which regulates the amount of dopamine present in the synaptic cleft, increases in density from E18 to P28, when it reaches adult levels and remains stable thereafter (Galineau et al., 2004). The enzymes that catalyse degradation of dopamine are present at near adult levels in the neonate (Karki et al., 1962).

Reports on the development of dopamine receptor expression have been inconsistent in the literature (see Rho and Storey, 2001; McCutcheon and Marinelli, 2009). In general however, autoradiography and immunohistochemical studies have shown that receptor expression increases during development, peaking during adolescence and then decreasing over adulthood. For example, in the rat striatum (including the VTA), DA receptor levels peak between P28 and P40 (Noisin and Thomas, 1988) and decrease after that, stabilising by 4 months of age (Andersen et al., 2000).

However, the expression of DA receptors in prefrontal areas does not peak until P60 (Tarazi and Baldessarini, 2000). This is in line with the general delayed maturation of this area, which is involved in executive functions such as planning and goal-directed behaviour in both animals and humans.

## 2.1.5 Opioids

The opium poppy, Papaver somniforum, has given rise to some of our most potent analgesic drugs, but has also been the cause of widespread drug abuse that blights many lives, due to its dual properties as pain-relieving and rewarding.

Opioids are peptides which act on the G-protein-coupled opioid receptors, mu (µ), kappa (κ) or delta (δ). Endogenous opioids are divided into three categories – endorphins, enkephalins and dynorphins. These have agonist activity at all three receptors, but each receptor displays distinct ligand sensitivity and there is much crosstalk of opioid signalling, possibly due to receptor heterodimerisation (Sora et al., 1997; Ikeda et al., 1999). The endogenous opioids arise from three precursor proteins that can code for more than 20 different endogenous opioids – pro-opiomelanocortin, prepro-enkephalin, and preprodynorphin (see Vaccarino and Kastin, 2001). Exogenous peptides are well-known as powerful analgesics and also drugs of abuse – i.e. heroin, codeine and morphine and their various analogues; all derivatives of opium.

The three types of opioid receptor, and the more recently discovered 'orphanin' receptors ('orphan' receptors that share a high structural homology to classical opioid receptors) are widespread throughout the nervous system. In the spinal cord, the mu opioid receptor (MOR) is the most numerous, accounting for ~70% of receptors seen in the substantia gelatinosa (layers I and II) of the dorsal horn of the spinal cord (Kar and Quirion, 1995).

Supraspinally, mu, kappa and delta receptors (MOR, KOR and DOR) are widespread and found in the locus coeruleus, rostroventral medulla (RVM), periaqueductal grey (PAG), ventral tegmental area (VTA), thalamus, amygdala, hippocampus and cortex (Atweh and Kuhar, 1977). Outside of the central nervous system, they are expressed by peripheral nociceptors, cardiac and skeletal muscle, the myenteric plexus (the major nerve supply to the gastrointestinal tract) and various white blood cells.

## 2.1.5.1 Morphine

So-named after Morpheus, God of Dreams, morphine is the opioid which acts on mu opioid receptors and was isolated in 1806, having been used in its natural form for hundreds of years as a painkiller. It is coded for by the Oprm gene, which codes at least six splice variants of the MOR (Pan et al., 2005).

Morphine is an important analgesic and, at the level of the spinal cord, mainly acts on presynaptic C fibre terminals, decreasing neurotransmitter release and so decreasing the ascending afferent nociceptive signal (Dickenson et al., 1987). Low doses of opioids applied directly to the spinal cord (epidurally) can produce clinically effective and localised analgesia and this fact has been exploited for pain relief during childbirth (Saeki and Yaksh, 1993).

Supraspinally, morphine acts on many areas of the brain, including the periaqueductal grey (PAG), rostroventral medulla (RVM), and VTA, and causes an increase in the descending inhibition mediated by these areas, thereby decreasing pain sensation. Injections of morphine directly into the PAG and RVM will inhibit spinally-mediated nociception, an effect which can be blocked by the MOR antagonist naloxone (Cheng et al., 1986; Moreau and Fields, 1986; Fang et al., 1989). Naloxone administration will also enhance pain sensation when given alone or after oral surgery (Levine et al., 1978a).

Once morphine has bound to the receptor, it acts by initiating signalling cascades via the G proteins that are liberated by agonist binding to the receptor. Morphine predominantly acts via the $G_{i/o}$ signalling pathway, which acts by inhibiting production of cyclic AMP (cyclic adenosine monophosphate), a second messenger molecule important in many biological processes.

In the VTA, morphine binds to MORs on inhibitory GABAergic interneurons, causing chloride ion influx and hyperpolarisation. This lifts the baseline inhibition of the DA cells and activates the mesolimbic DA pathway (Johnson and North, 1992; Bonci and Williams, 1997; Garzón and Pickel, 2001). Therefore morphine has its rewarding effects via disinhibition of the dopaminergic system.

MOR signalling is terminated by phosphorylation of the receptor by GRKs (G protein-coupled receptor kinases) which is then internalised into the cell by β arrestins. Chronic morphine use leads to cellular tolerance of the system which reduces the effect of opioid drugs by a number of mechanisms. These include shifts in coupling of the GPCR from $G_{i/o}$ to $G_s$, downregulation of the number of MORs on the cell surface, and upregulation of glutamate and other opioid-opposing pathways (see Borgland, 2001; Christie, 2008; Ueda and Ueda, 2009 for comprehensive reviews).

## 2.1.5.2 The developmental regulation of MOR expression and morphine function

### 2.1.5.2.1 Ontogeny of MOR expression

In the first week of the neonatal rat's life, mu and kappa are the predominant opioid receptors, shifting to a predominance of mu and delta receptors in the adult (Leslie et al., 1982). Delta receptor expression appears after the first postnatal week (McDowell and Kitchen, 1987) and the appearance of DOR subtypes is linked to the onset of weaning (Kitchen et al., 1995).

MOR mRNA has been found in the E13 rat and is isolated to the striatum by E18 (Georges et al., 1998). Receptors are present in the rat spinal cord from E16 (Kirby, 1981) and show an initial decline at birth, then a sharp rise over the first two postnatal weeks to adult levels (Spain et al., 1985). Endogenous opioid peptides are present in mouse brain at E11.5 and rat brain at E18 (Ruis et al., 1991; Pickel et al., 1982) and increase to adult levels by P25 (Bayon et al., 1979).

### 2.1.5.2.2 Binding to opioid receptors is seen prenatally

Opioid receptor binding, seen by *in vitro* quantitative receptor autoradiography studies, is shown from embryonic day (E) 12 onwards in the striatum, and has been shown to increase during early postnatal life to peak at 3 weeks after birth (Kent et al., 1981; McDowell and Kitchen, 1987). In the spinal cord, MOR binding sites are diffuse throughout the cord in neonatal rats, but become mainly localised to more superficial laminae by adulthood. In addition, the density of binding sites peaks at P4, and declines

to adult levels by P21 (Kar and Quirion, 1995). This shows that both the presence and functionality of MORs changes dramatically during the first few weeks of life.

### 2.1.5.2.3 Morphine analgesia in the neonate

Morphine analgesia is more efficacious in human neonates than in adults (see Nandi and Fitzgerald, 2005), and when administered epidurally, morphine has a greater analgesic effect in rat pups than adult animals (Marsh et al., 1999a; Marsh et al., 1999b). Animals lacking the MOR show no morphine-induced analgesia, but no overt behavioural abnormalities or major compensatory changes elsewhere in the opioid system, emphasising the importance of the MOR in analgesia (Matthes et al., 1996; Kitchen et al., 1997).

The effect of morphine is shown to increase with age, increasing by 40-fold between P3 and P14 (Giordano and Barr, 1987), with the analgesic potency of morphine proposed to reach adult levels by P14 (Barr and Lithgow, 1986). This changing efficacy, in addition to the proliferation of receptors that occurs during development, could also in part be due to changing ratios of morphine metabolites during development. Morphine is metabolised into morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), which are anti-analgesic, and analgesic, respectively. Therefore the analgesic efficacy of morphine partly depends on this metabolism-defined ratio, which is different in prematurely-born neonates, and matures with age (Hartley et al., 1994). The increased potency of morphine in young animals could also be partially due to a blood-brain barrier with higher permeability than that of the adult (Kupferberg and Way, 1963).

### 2.1.5.2.4 Chronic morphine administration in the neonate alters opioid signalling

Chronic morphine, administered from P1 to P4, results in a decrease in brain MOR density, which gradually recovers to control levels by P14 (Tempel et al., 1988), and alters opioid receptor numbers and sensitivity, desensitising animals to opiate analgesia throughout life (Thornton and Smith, 1998). Morphine administration during the first week of life also causes increased MOR binding and decreased hot plate latencies in adulthood (Handelmann and Quirion, 1983; Handelmann and Dow-Edwards, 1985).

Similar to the effects of exogenously applied morphine, administration of the endogenous endorphin beta-endorphin also showed a decrease in opiate receptors in the brain in adults (Zadina et al., 1985). Furthermore, prenatal morphine administration to pregnant dams caused reduced adult morphine analgesia (Johannesson and Becker, 1972; O'Callaghan and Holtzman, 1976). Together, these experiments show that prenatal and early morphine/opioid exposure to the neonatal rat causes long-term alterations in brain opiate receptors. This effect is sexually dimorphic, with opposite morphine analgesic responses seen in adult males and females after 7 days of repeated morphine exposure within the first week of postnatal life (Arjune and Bodnar, 1989).

### 2.1.5.2.5 Modulation of opioid signalling by external influences

The opioid system is sensitive to perturbance during development by factors such as stress, injury, and exposure to opioid antagonists. Footshock and recurrent testing on the hot plate test have both shown a later decrease in opioid sensitivity, as reflected by an increased latency to response on the hot plate test, and decreased naloxone binding after repeated hot plate stress (Bardo et al., 1981; Torda, 1978). Juvenile isolation between 4 and 5 weeks of life will induce stress, and alters opioid binding in the brain and opioid release after later social interactions (Van den Berg et al., 1999a; Van den Berg et al., 1999b). Blocking opioid signalling (with the opioid antagonist naltrexone) during development can affect dendritic spine development in the cortex and hippocampus, showing that opioid signalling is in part responsible for neuronal development (Hauser et al., 1989). This evidence suggests roles for opioid signalling in brain development and social behaviours, in addition to its more well-known roles in analgesia and reward.

## 2.1.5.3 Morphine is rewarding

In humans, morphine is rewarding, due to its ability to induce euphoria and relaxation and morphine and the morphine derivative, heroin, are both drugs of abuse. A large body of evidence has confirmed that morphine is rewarding in the VTA of animals. Rats will self-administer morphine and morphine agonists directly into the VTA, and MOR antagonists i.e. naloxone prevent this behaviour (Bozarth and Wise, 1981; Self and Stein, 1993; David and Cazala, 1994a). Injection of morphine and the MOR agonist DAMGO ([D-Ala2, NMPhe4, Gly(ol)5]enkephalin) into the VTA causes conditioned place preference (CPP) and morphine-induced CPP is now used as an animal model for drug-taking, withdrawal and relapse (Lin et al., 2010).

A large body of evidence accumulated over the years has shown that the mu receptors of many areas of the brain, including the nucleus accumbens, hypothalamus and amygdala, contribute to the rewarding effects of opiates (see Le Merrer et al., 2009). Knocking out the Oprm gene in mice that codes for the MOR causes alterations in both analgesia and reward behaviours. Matthes et al (1996) showed that MOR KO mice lose morphine-induced analgesia, cannot learn conditioned place preference, and do not develop physical dependence. Later papers studying genetically engineered animals that lack the MOR have shown deficits in reward behaviours, therefore confirming its essential role in reward processing (Sora et al., 1997; Kieffer and Gaveriaux-Ruff, 2002).

Morphine fails to induce rewarding effects in animals that have inflammatory or nerve injuries, and DA release in the VTA is markedly attenuated in these pain models (Suzuki et al., 1996; Ozaki et al., 2002). This is suggested to be due to a sustained reduction in the ERK signalling pathway in DA cells in neuropathic animals (Ozaki et al., 2004) and could provide a reason why patients that use morphine as an analgesic do not tend to get addicted to it (Fields, 2007).

## 2.1.5.4 Morphine and dopaminergic signalling

The rewarding effects of morphine are due to the disinhibition of the DA system. Evidence for this arises from a number of studies looking directly at the effect of one upon the other. For example, morphine's rewarding effects are blocked by dopamine antagonism in rats (Suzuki et al., 1995), while D2 receptor KO mice do not exhibit the rewarding effects of opiates (Maldonado et al., 1997) and the MOR antagonist naloxone can block the motivational effects of dopamine signalling (Shippenberg and Herz, 1988). Recent work has shown that morphine will activate the dopaminergic cells of the VTA (as seen by expression of the immediate early gene Fos) regardless of the presence of a noxious stimulus (formalin injection into the hindpaw), emphasising that morphine will activate dopaminergic circuits, even in stressful situations (Bajic and Commons, 2010).

In humans, dopamine D2 receptors are crucial in reward and addiction (see Volkow et al., 2009) and endogenous opioids can modulate DA reward systems (see Fields, 2004).

Placebo may involve endogenous opioid systems (Benedetti et al., 2005), and a PET study using a specific MOR tracer shows that placebo activates MOR-mediated neurotransmission (Zubieta et al., 2001).

## 2.1.6  Reward processing in the neonate – the role of dopamine

Dopamine also has a critical role in reward processing in the neonate. Amphetamine and cocaine, which both increase DA levels, induce place preference in the neonate rat (P3 to P10) (Barr and Lithgow, 1986) and rats in the first week of life can learn to nudge a lever to receive electrical stimulation of dopaminergic sites in the brain (Moran et al., 1981; Lithgow and Barr, 1984). Preweanling mice (<P21) can also exhibit conditioned place preference to a compartment previously paired with cocaine (Laviola et al., 1992).

D1 and D2 receptor antagonism during development shows that the receptors contribute to reward behaviours with a differing developmental profile. Using conditioned place preference as a measure of reward-related behaviour, D1 receptor antagonism blocks place preference to the same extent as it would in the adult, whereas D2 receptor antagonism affects CPP in the P10 but not the P17 rat (Pruitt et al., 1995), showing that the relative balance of dopamine receptor subtype alters during development.

## 2.1.6.1 Bonding behaviour is mediated by dopaminergic pathways

The process of an animal bonding, or attaching, to its mother is crucial for survival. John Bowlby (1958; 1978; 1988) developed modern attachment theory –the idea that an infant will learn to form a bond to its primary caregiver, who protects, feeds and supports it. This attachment has been proposed to be rewarding for both offspring and mother and it is thought that bonding behaviour is in part dopaminergically mediated (see Insel, 2003), and that attachment learning, as well as fear learning, relies on dopaminergic signalling in the amygdala (Barr et al., 2009).

Neonates can learn and show preference for a lactating over a non-lactating nipple (Kenny and Blass, 1977) and the dopamine system is critical for this (Becker and Smotherman, 1996).  Knock-out studies show that a lack of dopamine during the first two weeks of neonatal life is not fatal (Zhou et al., 1995), but it is nevertheless a critical period during which disturbances of the maternal bond can have long-lasting effects on the DA system. Maternal deprivation alters patterns of dopamine receptors, dopamine

release and dopaminergic cell number in the VTA and NAcc in adulthood (Meaney et al., 2001), and postnatal exposure to glucocorticoid stress hormones causes an increase in the number of dopaminergic cells in both the VTA and the substantia nigra pars compacta (SNpc) (McArthur et al., 2005).

Attachment behaviour also relies on activation of the DA reward pathways in the mother. Human fMRI studies have shown that the BOLD signal in the VTA is increased in mothers when they viewed video clips of their infants (Noriuchi et al., 2008) and Dbh knockout mice (that lack the gene to produce dopamine β-hydroxylase) fail to nurture their offspring (see Palmiter, 2008). Dopamine is released upon pup exposure and VTA or NAcc lesions interrupt maternal care (see Insel, 2003). Taken together, these data suggests that dopamine is important for learning attachment in the neonate and for maternal behaviour in the parent animal.

### 2.1.7 Morphine is rewarding in the neonate

Many papers have shown that morphine can induce reward behaviours in young animals, and that chronic morphine administration can cause tolerance to its analgesic effects, similar to the adult. Morphine or met-enkephalin (an opioid peptide that acts on MORs) administered directly into the VTA of the P4 and P5 rat caused odour-cued place preference, whereby the neonate associates a place where they received the morphine to a specific odour i.e. oranges, and will then return to the orange-smelling box when given the choice (Barr and Rossi, 1992; Kehoe and Blass, 1986c). Analgesic tolerance to opioids can develop in both neonatal humans, as seen by an increase in dosage needed to reduce pain behaviours and maintain sedation (Taddio and Katz, 2004) and, to a lesser extent, animals (Van Praag et al., 1993; Van Praag and Frenk, 1991a).

Studies investigating morphine withdrawal have shown that the neonatal rat exhibits withdrawal behaviours after chronic morphine treatment (>5 days). Shaking, vocalisations, escape behaviour and hypersensitivity to noxious stimuli are seen, which are the same as those seen in the adult (Ceger and Kuhn, 2000; Jones and Barr, 1995; McPhie and Barr, 2000; Gong et al., 2010; Zhang and Sweitzer, 2008). Furthermore, withdrawal from chronic morphine in the neonate sees activation (as measured by c-Fos expression) of the same brain regions that are active during withdrawal in the adult,

including the periaqueductal grey, locus coeruleus, and nucleus accumbens (McPhie and Barr, 2009).

Related to the rewarding effects of maternal attachment, mu opioid receptor KO mice show deficits in attachment behaviours. They do not show a preference towards their mother's cues and do not exhibit distress responses to maternal deprivation (Moles et al., 2004), lacking the motivational drive to seek maternal care. The authors suggest that the rewarding effect of attachment is lost in these animals.

## 2.2 Hypotheses

The evidence presented above shows that morphine activates dopamine pathways, and it has therefore been chosen as a trigger for the DA system in this set of experiments. While it is evident that dopaminergic pathways are active in the first days of life in learning and maternal attachment, we wished to explore the integrity of dopaminergic reward pathways in the newborn. This is an important predicate of the hypothesis that early injury can affect reward processing in the adult, due to the overlap of the pain and reward systems. The aim here was to perform a quantitative comparison of the activation of the dopaminergic system in young and adult rats with morphine. The activation of dopaminergic cells in the VTA was measured using pERK expression following systemic morphine administration.

We hypothesised that:

- The dopaminergic system in the VTA is functional in the newborn rat.
- Administration of a rewarding stimulus, morphine, will activate the DA cells of the VTA in the newborn.
- The effect of morphine on DA cell activation in the VTA will not alter significantly with postnatal age.

## 2.3 Methods

Sprague-Dawley male and female rats were used at postnatal days (P) 7, P21 and adult (>P50). Five groups were designated (see Table 2-1):

| Treatment group | Number per group |
|---|---|
| Baseline (no injection) | 3-4 |
| Single subcutaneous (s.c.) saline dose | 3-4 |
| Repeated s.c. saline dose | 4 |
| Single s.c. morphine dose | 4 |
| Repeated s.c. morphine dose | 4 |

**Table 2-1 – A table to show the treatment groups and numbers of animals per group.**

## 2.3.1 Timeline

The diagram below is a schematic of the experimental design.
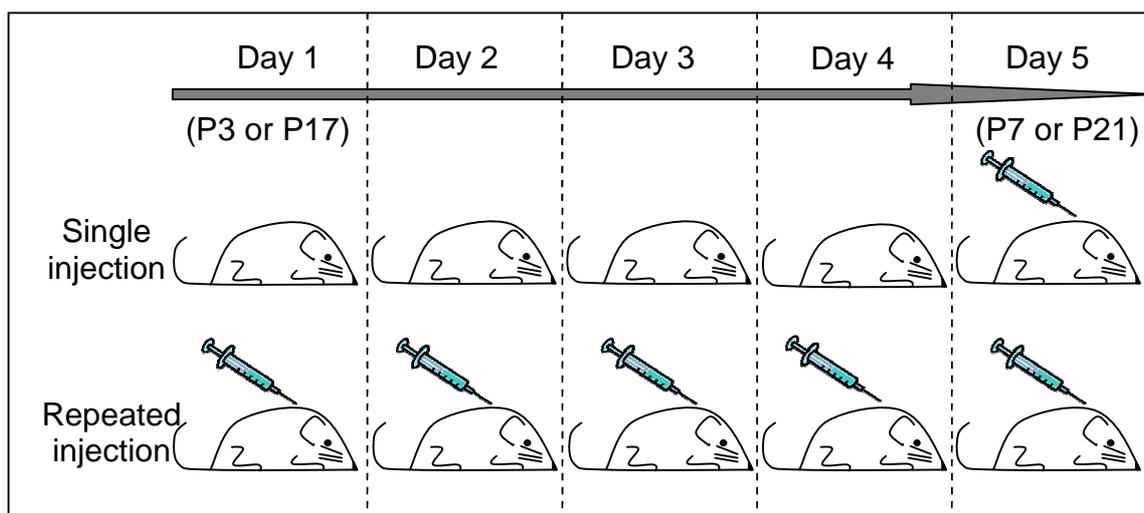


**Figure 2-3 – A schematic diagram to illustrate the time-line of the experiment. Animals either received a single (top row) or repeated dose (lower row) of morphine or saline. The single dose was administered on one day only. The repeated dose regime began 5 days prior to sacrifice so that the animals in each age group were sacrificed on the same day.**

Animals were killed on day 5, 30 minutes after the final morphine/saline injection, by intraperitoneal (i.p.) injection of an overdose of pentobarbital, and transcardially perfused with 4% paraformaldehyde (PFA). Brains were dissected out, post-fixed in 4% PFA for 4 hours, then transferred to a 30% sucrose and azide solution (see Solutions) and stored at 4°C.

## 2.3.2 Morphine administration

### 2.3.2.1 Ages

- Morphine was administered subcutaneously (s.c.) in 0.9% saline solution to the neonate (<P7), preweanling (P21) and adult (>P50, >180g). Control animals received saline only injections.

- The ages were chosen to reflect key periods of development for the animal. The first three weeks of life are a time of great developmental plasticity in the rat CNS (see Fitzgerald, 2005) and the literature on dopaminergic development during this time suggests that changes in receptor expression and subtype as well as maturation of functional reward pathways are taking place over this period (see McCutcheon and Marinelli, 2009).

- These time points also correspond to the ages used later in this thesis to test the effect of pain in early life on later reward processing (see Chapters 3 and 4).

### 2.3.2.2 Single dose of morphine:

- In this group of experiments, animals received one s.c. morphine injection only, as shown in Figure 2-3.

- The dosage and volume used are detailed in Table 2-2:

| Age | Dose | Volume of injectate |
|---|---|---|
| P7 | 5mg/kg | 100µl |
| P21 | 10mg/kg | 200µl |
| Adult | 10mg/kg | 200µl |

**Table 2-2 - A table to show the ages, doses and volume of injectate in the single morphine injection group**

### 2.3.2.3 Repeated dose:

- In this group of experiments, animals were injected s.c. once daily for 5 days, as shown in Figure 2-3.

- Repeated injections over 5 days was chosen as the literature suggests that this is long enough to allow tolerance to morphine to occur (see Ueda and Ueda, 2009; Christie, 2008).

- For repeated injection, dosage and volume used are detailed in Table 2-3 (below):

| Age | Dose | Volume of injectate |
|---|---|---|
| P3 to P7 | 5mg/kg | 100µl |
| P17 to P21 | 10mg/kg | 200µl |
| Adult | 10mg/kg | 200µl |

Table 2-3 - a table to show ages, doses and volume of injectate in the repeated morphine injection groups

## 2.3.2.4 Rationale for dosage

- As this experiment was to investigate reward processing, it was important to choose a dosage that was known to activate the reward pathways.
- Analgesic doses are lower than those used in the reward/addiction literature and in the neonate range from 0.1mg/kg to 5mg/kg (McKelvy and Sweitzer, 2009; Abbott and Guy, 1995; Marsh et al., 1997).
- The reward literature uses higher doses of opioids to stimulate the supraspinal dopaminergic system. Doses range from 5mg/kg up to 20mg/kg (Barr and Wang, 1992; Narita et al., 2004a; Jones and Barr, 1995; Ceger and Kuhn, 2000). For the purposes of this experiment, a dose of 10mg/kg was chosen. As morphine causes respiratory depression, this dose was halved for animals <P7, to 5mg/kg. This prevented respiratory depression or hypothermia.
- Behavioural responses (sedation and analgesia) were tested following morphine administration using loss of righting reflex and increased threshold to noxious mechanical stimulation (using von Frey hair).

## 2.3.3 Immunohistochemistry

Tyrosine hydroxylase (TH) is present in all dopaminergic cells and so was chosen as a DA cell marker in this experiment. Phosphorylated ERK (pERK) was chosen as a marker of cell activation in the dopaminergic cells. Other studies have shown the activation of the DA system by co-localisation of TH and pERK in the VTA (Ozaki et al., 2004), and shown that morphine specifically upregulates pERK in the DA cells of the VTA (Berhow et al., 1996).

The following protocol was used for immunostaining:

1. 40µm sections of the ventral tegmental area were cut on the freezing microtome and placed into wells containing 5% sucrose in azide (see Appendix 1: Solutions).

2. Sections were transferred to Röhren tubes (Sarstedt, Leicester, UK) 6 sections per tube, each at a distance of 240µm from each other, and blocked in 3% goat serum for 1 hour (see Appendix 1).

3. Phosphorylated ERK antibody (Cell Signaling Technology, MA, USA) was placed onto the sections at a 1:250 dilution (4µl per ml) in TTBS (see Appendix 1). The tubes were incubated overnight at room temperature.

4. Sections were washed with 0.1M PB (see Appendix 1), 3 times, for 10 minutes per wash.

5. The pERK secondary antibody was placed onto the sections. This was biotinylated anti-rabbit, raised in goat (Vector Laboratories Inc., CA, USA), at a 1:500 dilution (2µl per ml) in TTBS. Sections were incubated for 90 minutes at room temperature.

6. Sections were washed as above.

7. The avidin-biotin protocol was used to amplify the signal. Using the Vectastain ABC kit (Vector Laboratories Inc., CA, USA), 4µl of 'A' and 4µl of 'B' per ml TTBS were stirred for 30 minutes.

8. Avidin-biotin solution was placed onto the sections for 30 minutes at room temperature.

9. Sections were washed, as above.

10. Biotinylated tyramide (Perkin Elmer Life Sciences, MA, USA) was diluted with the amplification diluent 1:75 (13.3µl per ml) and placed onto the tissue for 7 minutes.

11. Sections were washed.

12. FITC avidin (Vector Laboratories Inc., CA, USA) was placed onto the tissue at a concentration of 1:600 (1.67µl per ml) in TTBS for two hours. Sections were kept in the dark to prevent bleaching of the fluorophore.

13. Sections were washed.

14. The tyrosine hydroxylase primary antibody (Chemicon, MA, USA) was placed onto the tissue at a concentration of 1:1000 (1µl per ml) in TTBS and was kept in the dark to incubate overnight at room temperature.

15. Sections were washed.

16. The tyrosine hydroxylase secondary antibody was placed onto the sections. This was the fluorophore 594-alexa anti-rabbit, raised in chicken (Invitrogen Ltd., Paisley, UK) at a dilution of 1:500 (2µl per ml) in TTBS. Sections were incubated in the dark for 2 hours at room temperature.

17. Sections were washed and transferred to 0.01M PB and mounted onto gelatinised slides, kept in the dark as much as possible to prevent bleaching of the fluorophores.

18. Slides were dried in the dark overnight at room temperature and coverslipped with Fluoromount (Sigma-Aldrich, Dorset, UK).

## 2.3.3.1 Immunohistochemistry controls

Four control tubes of sections were prepared to check for the effects of non-specific binding of the antibodies (where the antibody binds to the tissue at random instead of to the protein of interest). The control tubes underwent the same washing and staining as the experimental tubes at all stages of the protocol, except the sections were not exposed to the antibody at the relevant stage of the protocol,  and were placed into 0.1M PB instead.

The tubes were prepared under one of the four following conditions:

1. The pERK primary antibody was absent
2. The pERK secondary antibody was absent
3. The TH primary antibody was absent
4. The TH secondary antibody was absent

These sections are not shown as the background levels of fluorescence were so low that the tissue is not visible.

## 2.3.4 Definition of the ventral tegmental area

- The co-ordinates of the VTA, as defined by the stereotaxic atlas of Paxinos and Watson (2004) are as follows:

From Bregma:

Anteroposterior: -5.5mm

Dorsoventral: -9.6mm

Mediolateral: +0.5mm

- However, the neonatal brain is smaller than the adult brain, so these co-ordinates need to be scaled accordingly and change during development. To overcome this problem and enable consistent identification of the VTA for counting purposes, the entire ventral part (see black dotted box, figure below) of sections of adult and neonate tyrosine hydroxylase staining (see bottom panel) were compared to identify common cytoarchitectural features.
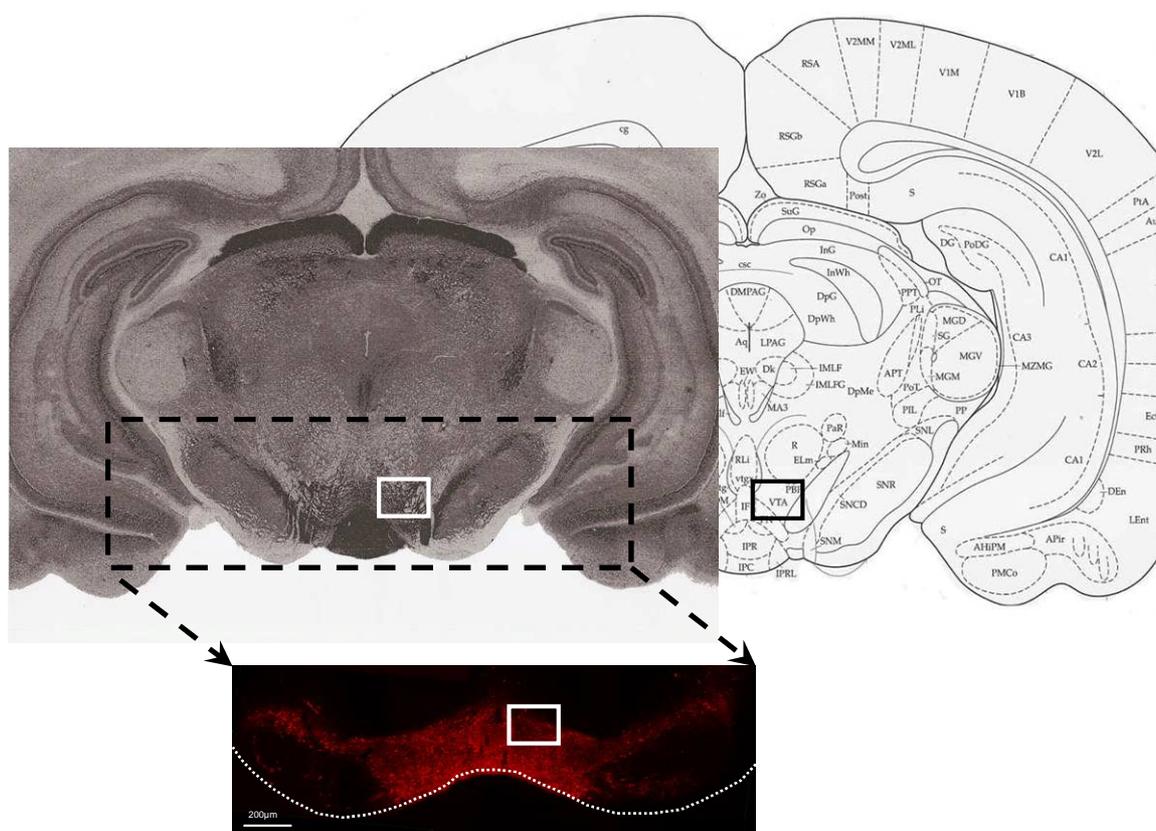


**Figure 2-4 - A diagram to show the VTA as defined by the atlas of Paxinos and Watson (2004), highlighted by the white and black oblongs in the top two panels. The black dotted section on the left panel represents the same area that is shown in the bottom panel. The bottom panel illustrates the pattern of tyrosine hydroxylase staining (red fluorescence) in the adult rat. The dotted white line delineates the ventral boundary of the tissue. The white oblong highlights the area defined as the VTA.**

- As there was no difference in the characteristic pattern of tyrosine hydroxylase staining over the three ages, the same features of TH staining were used as landmarks across all three age groups.

### 2.3.5 Visualisation of sections

- Slides were visualised using a fluorescent microscope (Leica DMR). The tyrosine hydroxylase secondary antibody (alexa-594) fluoresces at an optimal wavelength of 594nm and appears red under the appropriate filter, and the phosphorylated ERK tertiary antibody (FITC) fluoresces optimally at 488nm and appears green under the appropriate filter.

- The left VTA of each section was visualised first under the relevant wavelength filter to see fluorophore excitation and a photograph taken of the VTA at x20 magnification. The filter was then changed to visualise pERK staining and another image of the same area captured. Photographs were taken using a Hamamatsu camera linked to the microscope. Images were visualised using OpenLab 4.0.4 software (Improvision Openlab).

### 2.3.6 Cell counting

- Once images were captured, the number of TH cells and then the number of pERK cells per image were counted within the defined region. The images were then merged using the image software and the number of co-expressing cells (appearing yellow) per image counted.

- The VTA was photographed bilaterally in each section.

- 5 sections were analysed per animal, with 4 animals in each group.

### 2.3.7 Analysis of cell counts

- From raw cell counts, the percentage of tyrosine hydroxylase-positive cells that co-expressed pERK was calculated.

- To control for the change in cell density with age, which could confound the comparisons of cell counts across age groups, estimates of cell density were performed in the VTA at each age from Nissl stained sections.

- Briefly, tissue was collected and slides produced as previously described. Once slides were dry, they were placed into a toluidine blue (Nissl) stain to mark all cells for 10 minutes, then dehydrated and coverslipped with DPX.

- Nissl stained slides were visualised with light microscopy and all stained cells (neurons and glia – see Figure 2-5) counted under the same conditions as the fluorescent sections at the three ages studied (P7, P21 and adult) to give total cell counts per unit area.
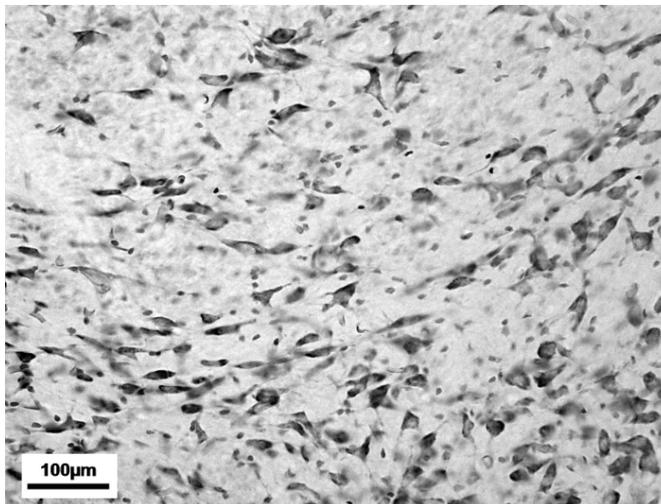


**Figure 2-5 – A representative picture of adult Nissl cell staining of the VTA, showing neurons and glia.**

- These counts were then multiplied by the area counted (calculated as $0.53mm^2$) to give cell density/$mm^2$.

- Once the total cell density was calculated, a one-way ANOVA (GraphPad Prism v.4) compared total cell numbers across ages, and a comparative ratio defining the adult as the standard of '1' was calculated (see Results). Cell counts from P7 and P21 animals were then scaled accordingly.

- Using the scaled data, averages within animal were calculated, and then group averages calculated.

- One- or two-way ANOVAs were performed (using GraphPad Prism v4) to check for group differences, depending on which test was suitable. For one-way ANOVA, Tukey's Multiple Comparisons post-hoc tests were performed to find the direction of any differences. For two-way ANOVA, Bonferroni's post-tests were performed for the same reason.

- ANOVA is a parametric method of analysis, so all data was checked for a normal distribution (D'Agostino and Pearson omnibus normality test) prior to analysis. Any non-normal data was analysed using the appropriate non-parametric test i.e. the Kruskal-Wallis test for a one-way ANOVA.

## 2.4 Results

### 2.4.1 Baseline data

The data presented in this chapter is the result of counts of subpopulations of cells in the ventral tegmental area after various treatments at different ages. Therefore to begin, data are presented on the change in cell density in the VTA with age from Nissl stained sections, and the change in relative populations of dopaminergic and pERK-positive cells.

### 2.4.1.1 Total cell density in the VTA changes with postnatal age

The number of cells per unit area in the VTA decreases during development. Cell density was calculated by counting the total number of cells per unit area ($0.53mm^2$) in VTA sections from the Baseline treatment group (n=3) per age to provide cells per $mm^2$ (see Analysis of cell counts section in Methods). Table 2-4 and Chart 2-1 (below) show the total number of cells per $mm^2$ at each age and the bar chart of this data.

|  | P7 (n=3) | P21 (n=3) | Adult (n=3) |
|---|---|---|---|
| Mean | 998.9 | 896.5 | 755 |
| SD | 63.6 | 100.6 | 23.1 |
| SEM | 36.7 | 58.1 | 13.4 |

**Table 2-4 – A table to show the average total cell counts at each age. 5 sections were counted from 3 different animals in the Baseline group at each age. Animal, then group, averages were calculated.**
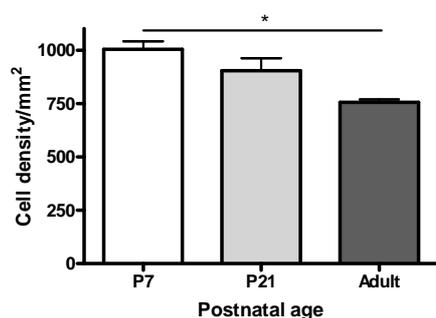


**Chart 2-1 – A bar chart to illustrate the total cell density at each age point. *p<0.05; One-way ANOVA with Tukey's Multiple Comparisons post-tests.**

Chart 2-1 shows that there is a statistically significant fall in cell density in the VTA with age (p<0.05; One-way ANOVA, Tukey's Multiple Comparisons post-tests). The density of cells is greater in the P7 (at ~$1000/mm^2$), and declines over development to a density of $755/mm^2$ in the adult rat.

## 2.4.1.2 Ratio of total cell density

To adjust for this fall in density with age, a ratio was calculated that could be applied to all DA cell counts. Using the adult data as the baseline of '1', the comparative ratio below was calculated:

| P7 | P21 | Adult |
|-------|-------|-------|
| 1.323 | 1.187 | 1 |

**Table 2-5 – A table to show the comparative ratio calculated with Adult data as the baseline of '1'.**

To allow for the change in density with age, cell counts were adjusted using the above ratio.

## 2.4.2 The baseline relative number of dopamine cells in the VTA does not differ across different developmental ages

Average dopamine (i.e. TH-positive) cell counts from each animal in all groups (n=3-4 animals in 5 treatment groups giving an n of 17-20 at each age) were calculated. The P7 and P21 counts were scaled to allow for the overall change in cell density that occurs with age. Table 2-6 and Chart 2-2 illustrate the data:

|  | P7 (n=20) | P21 (n=19) | Adult (n=17) |
|------|-----------|------------|--------------|
| **Mean** | 60.7 | 53.3 | 61.2 |
| **SD** | 10.2 | 12.2 | 9.6 |
| **SEM** | 2.2 | 2.8 | 2.3 |

**Table 2-6 – a table to show the adjusted mean TH+ cell counts in each age group.**
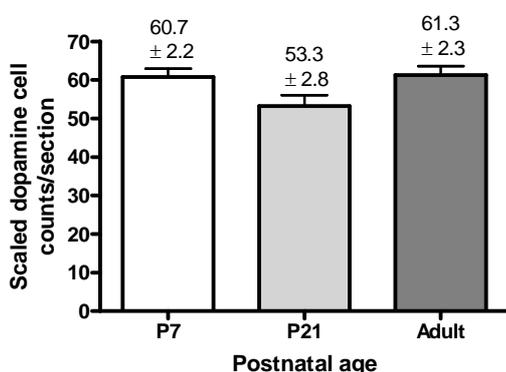


**Chart 2-2 – a bar chart to illustrate the adjusted mean number of dopaminergic cells across ages.**

Chart 2-2 shows that the relative density of dopaminergic tyrosine hydroxylase-positive cells per mm$^2$ does not differ significantly with age (p>0.05; One-way ANOVA).

## 2.4.2.1 The overall percentage of dopamine cells in the VTA at baseline is between 13 and 15%

The dopaminergic (TH+) counts were also expressed as a percentage of total cells counted and in this case no adjustment for cell density was required. Figure 2-6 illustrates how calculating percentages can circumvent (and hide) underlying developmental changes in cell density.
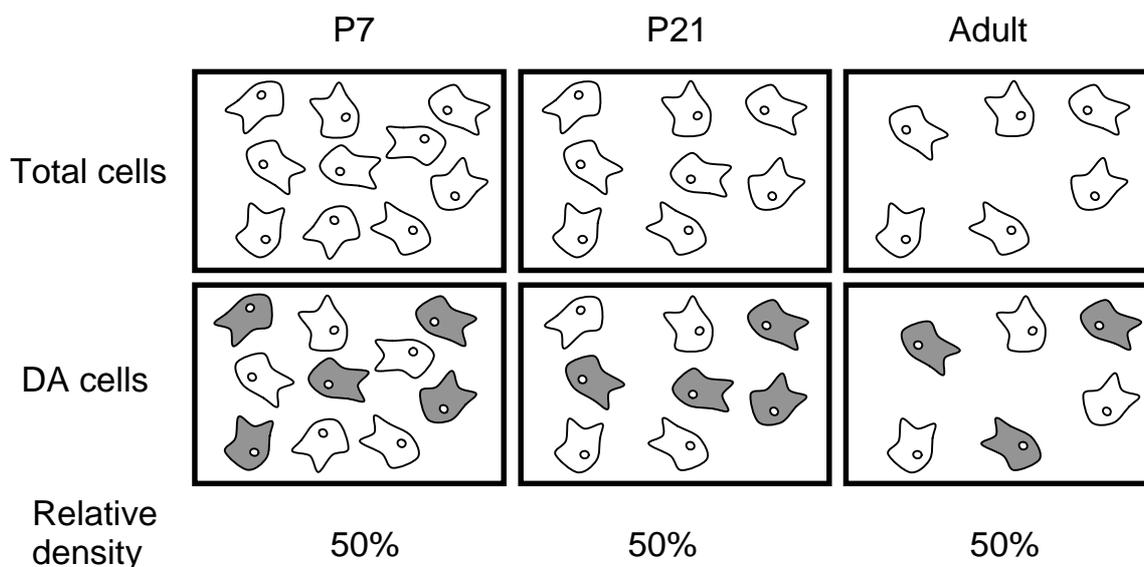


**Figure 2-6 – A figure to illustrate how cell density may decrease with age, but relative density of cells of interest i.e. DA cells, does not. The percentage of DA cells stained in the lower panel is the same across all three ages – 50% - therefore the relative density remains the same.**

The percentage of DA cells did not change with age, remaining between 13.3 and 15.31% of the total cell population (one-way ANOVA). Table 2-7 and Chart 2-3 (below) show this data:

|  | P7 (n=20) | P21 (n=19) | Adult (n=17) |
|---|---|---|---|
| **Mean** | 15.2 | 13.3 | 15.3 |
| **SD** | 2.5 | 3.0 | 2.4 |
| **SEM** | 0.6 | 0.7 | 0.6 |

**Table 2-7 – a table to show the average percentage of DA cells out of the total number of cells at each age point.**
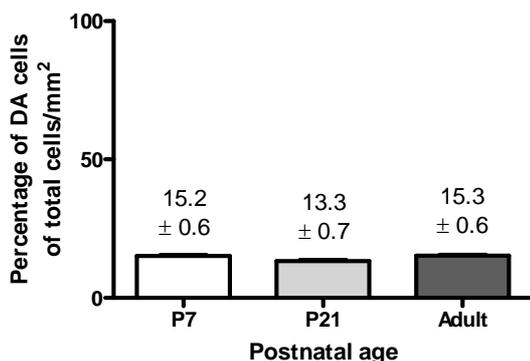
**Chart 2-3 – A bar chart to illustrate the percentage of dopamine cells of the total cell number at each age.**

Whilst the total cell density may decrease with age, the relative percentage of dopaminergic cells does not differ.

### 2.4.3  The baseline relative density of pERK-positive cells decreases in the VTA during development

Average pERK-positive cell counts from each animal in all groups (n=3-4 animals in 5 treatment groups giving an n of 17-20 at each age) were calculated. Data was then converted to cell density (per $mm^2$) and the P7 and P21 data scaled according to the ratio of cell density. Table 2-8 and Chart 2-4 illustrate these data:

|  | P7 (n=20) | P21 (n=19) | Adult (n=17) |
|---|---|---|---|
| **Mean** | 52.9 | 47.5 | 29.9 |
| **SD** | 26.1 | 24.8 | 12.5 |
| **SEM** | 5.7 | 5.7 | 3.0 |

**Table 2-8 – a table to show the average pERK-positive cell density at each age point**
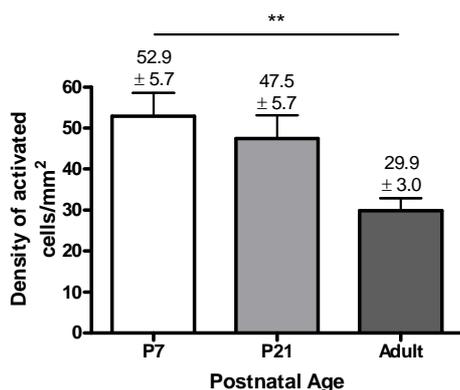


**Chart 2-4 – A bar chart to illustrate the density of pERK-positive cells in each age group.**

Chart 2-4 shows that the relative density of pERK-positive cells at baseline in the VTA decreases during development from 52.9/$mm^2$ in the P7 animals to 29.9/$mm^2$ in the adult.  This is a significant decrease (p<0.01; One-way ANOVA, Tukey's Multiple Comparison's post-tests).

## 2.4.3.1 The percentage of pERK-positive cells in the VTA at baseline does not change with age

This reduction in density of pERK cells with age is reflected in the percentage calculations but in this case, it is not significant. The percentage of pERK-positive cells of the total cell number in the VTA is 5.3% in both the P7 and P21 animals, and 4% in the adult, as shown in Chart 2-5 (below). There is no significant difference between ages ($p>0.05$, One-way ANOVA).

|  | P7 (n=20) | P21 (n=19) | Adult (n=17) |
|---|---|---|---|
| **Mean** | 5.3 | 5.3 | 4.0 |
| **SD** | 2.6 | 2.8 | 1.7 |
| **SEM** | 0.6 | 0.6 | 0.4 |

**Table 2-9 – a table to show the mean percentage of pERK-positive cells of the total number of cells at each age point.**
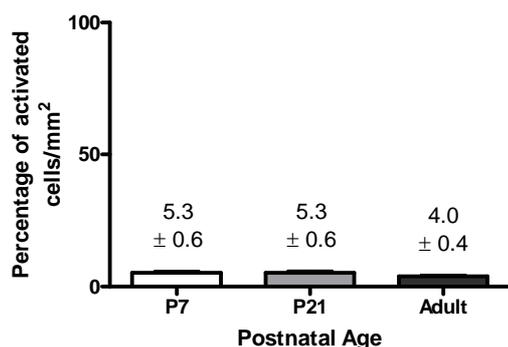


**Chart 2-5 – A bar chart to show the percentage of pERK-positive cells out of the total number of cells at each age.**

To summarise, the data presented above show that, at baseline, the cell density of the VTA decreases over development but the total percentage of DA and pERK-positive cells does not change. Adjusting cell counts to give a relative density at each age shows that the density of DA cells does not change with age, but the density of pERK-positive cells decreases over development.

## 2.4.4 The effect of morphine on activation of dopamine cells at different ages

The aim here was to investigate the activation of DA cells in the VTA in response to morphine at different ages. Activation of DA cells was measured by pERK expression. Therefore in control animals (baseline and saline-injected) and in morphine-treated animals, the adjusted cell counts of TH+, pERK+ and the percentage of co-expressing cells were calculated and the results compared across different treatment groups. The

data are initially presented within each age group. Representative pictures of cell staining at each age are presented at the end of the section. Table 2-10 is a reminder of the five treatment groups in each age group:

| Treatment group | Number per group |
|---|---|
| Baseline (no injection) | 3-4 |
| Single subcutaneous (s.c.) saline dose | 3-4 |
| Repeated s.c. saline dose | 3-4 |
| Single s.c. morphine dose | 4 |
| Repeated s.c. morphine dose | 4 |

**Table 2-10 – A table to show the treatment groups and number of animals per group.**

## 2.4.4.1 Morphine activation of dopamine cells at P7

All results presented below have been adjusted to account for the differences in total cell density across age groups. Table 2-11 (below) gives the adjusted average cell counts of dopamine (TH+), activated (pERK+) and co-expressing cells in each of the 5 treatment groups. The final column on the right shows the percentage of TH+ cells that also express pERK. Chart 2-6 illustrates the data presented in the table.

| P7 | TH+ | pERK+ | Co-expressing | Percentage of TH+ cells expressing pERK |
|---|---|---|---|---|
| Baseline (n=4) | 69 ± 6.0 | 32 ± 10.4 | 7 ± 1.6 | 11.5 ± 2.8 |
| Single saline (n=4) | 75 ± 6.3 | 22 ± 3.0 | 2 ± 0.5 | 3.3 ± 1.0 |
| Repeated saline (n=4) | 82 ± 8.5 | 37 ± 6.8 | 7 ± 1.0 | 9.3 ± 2.3 |
| Single morphine (n=4) | 82 ± 3.6 | 46 ± 8.7 | 24 ± 6.1 | 30.0 ± 8.1 |
| Repeated morphine (n=4) | 94 ± 4.8 | 47 ± 11.2 | 23 ± 7.5 | 23.0 ± 6.3 |

**Table 2-11 – A table to show the average cell counts (±SEM) in each treatment group in the P7 age group.**
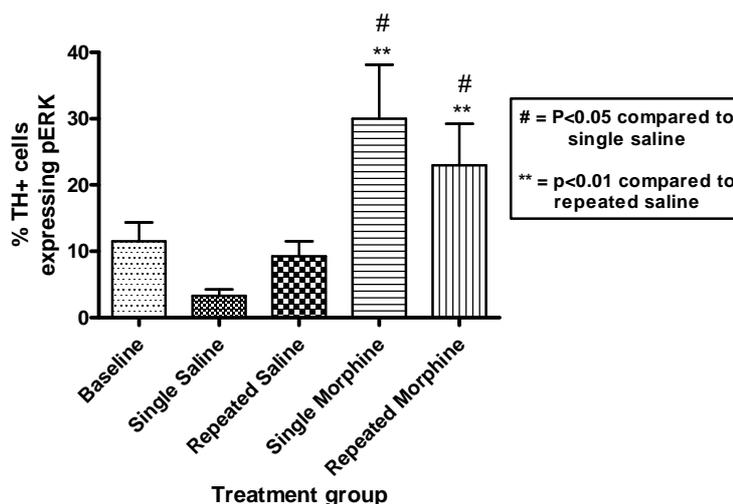
**Chart 2-6 – A bar chart to illustrate the percentage of TH+ cells co-expressing pERK in each treatment group in the P7 age group.**

These results show that both single and repeated morphine injection significantly ($p<0.05$) increase TH+ cell activation (as measured by pERK expression) above saline control group levels. A single morphine injection causes an increase ($p<0.05$) in activation from 3.31% in the single saline group to 30% in the single morphine group. There is a significant ($p<0.01$) increase in activation between the repeated saline group (9.25%) and both the single (30%) and repeated morphine (23%) groups (One-way ANOVA, Tukey's Multiple Comparison's post-tests). Therefore morphine activates the dopaminergic cells in the P7 animal when administered both singly and repeatedly over 5 days.

## 2.4.4.2 Morphine activation of dopamine cells at P21

All results presented below have been adjusted to account for change in total cell density across age group. Table 2-12 (below) gives the adjusted average cell counts of dopamine (TH+), activated (pERK+) and co-expressing cells in each of the 5 treatment groups. The final column on the right shows the percentage of TH+ cells that also express pERK.

| P21 | TH+ | pERK+ | Co-expressing | Percentage of TH+ cells expressing pERK |
|---|---|---|---|---|
| Baseline (n=3) | 45 ± 4.3 | 18 ± 1.3 | 4 ± 1.0 | 9.3 ± 2.1 |
| Single saline (n=4) | 68 ± 7.8 | 25 ± 11.0 | 4 ± 1.3 | 5.5 ± 1.6 |
| Repeated saline (n=4) | 74 ± 4.8 | 37 ± 13.3 | 3 ± 1.1 | 4.3 ± 1.2 |
| Single morphine (n=4) | 60 ± 5.8 | 35 ± 6.5 | 15 ± 4.4 | 24.3 ± 5.5 |
| Repeated morphine (n=4) | 72 ± 4.0 | 36 ± 4.4 | 13 ± 3.6 | 19.8 ± 4.5 |

**Table 2-12 – a table to show the average cell counts (±SEM) in each treatment group in the P21 age group.**
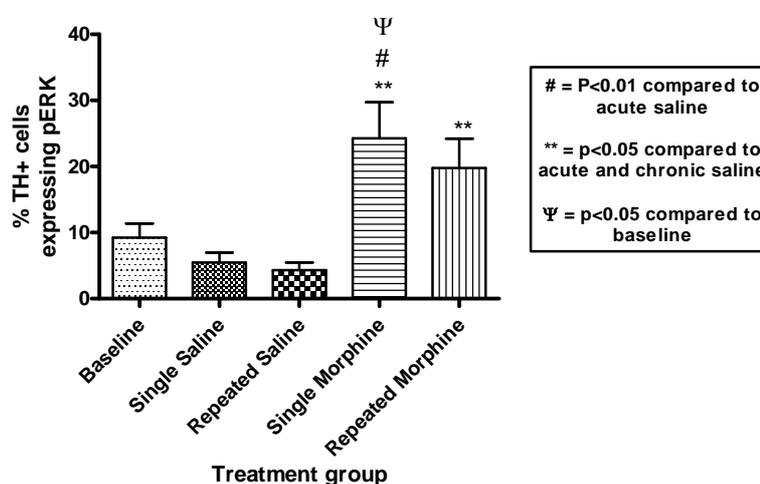


**Chart 2-7 – A bar chart to illustrate the percentage of TH+ cells expressing pERK in each treatment group in the P21 age group.**

Chart 2-7 shows that a single morphine injection significantly increases the percentage of TH+ cells that also express pERK above baseline and saline control group levels ($p < 0.05$; One-way ANOVA, Tukey's Multiple Comparison's post-tests).

A repeated morphine injection also significantly increases the percentage of TH+ cells that express pERK above saline control group levels ($p < 0.05$; One-way ANOVA, Tukey's Multiple Comparison's post-tests). These data show that morphine increases the activation of the dopaminergic cells in the P21 animal when administered both singly and repeatedly over 5 days.

These data show that of the five treatment groups, only a single morphine injection activates dopaminergic cells in the adult animal, and that in contrast to the effects at P7 and P21, repeated morphine injection does not activate dopamine cells above control group levels.

### 2.4.5 Comparing control groups across ages

## 2.4.5.1 Control group data does not differ between groups or across the ages

Comparing control groups to each other and across the ages shows that the percentage of TH+ cells expressing pERK does not change significantly with age (2-way ANOVA with Bonferroni post-tests, all values $p>0.05$). These data are shown in Chart 2-9 (below).
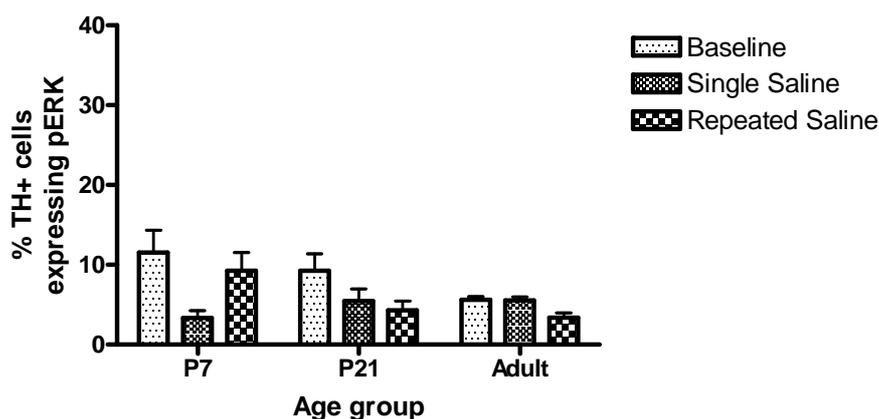


**Chart 2-9 – A bar chart to illustrate that the percentage of TH+ cells expressing pERK in the three control groups across ages.**

These data also show that injection of saline does not increase the pERK expression in TH+ cells above baseline levels, and also that an injection *per se* does not affect the percentage of TH+ cells expressing pERK.

### 2.4.6 Comparing the effects of morphine across age groups

Chart 2-10 shows the percentage of TH+ cells that express pERK after either single or repeated morphine, plus control data, at each age. Control data was pooled from the baseline, single and repeat saline injection groups.
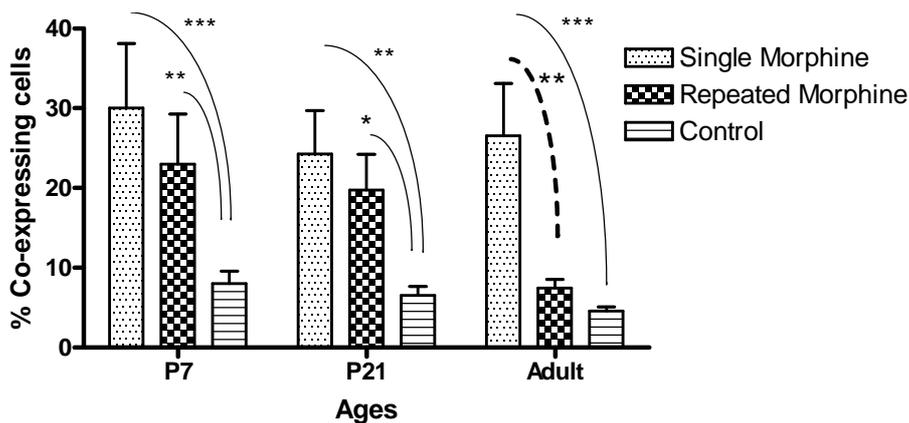
**Chart 2-10 – a bar chart to show the percentage of TH+ cells expressing pERK after single and repeated morphine injection in each age group. Both single and repeat morphine injections are above control levels in the P7 and P21 groups (solid curves). Only the single morphine injection produces an increase in activation in the adult (dashed curve). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001; 2-way ANOVA with Bonferroni's post-tests.**

## 2.4.6.1 Single morphine injection

Chart 2-10 (above) illustrates that a single morphine injection activates dopaminergic cells to 25-30% at P7, P21 and in the adult, significantly above control levels (p<0.01 or p<0.001; 2-way ANOVA with Bonferroni's post-tests). There is no significant difference between ages (p>0.05; Two-way ANOVA with Bonferroni's post-tests). These data show therefore that the DA systems of young animals react to a single morphine challenge to the same extent as that of the adults.

## 2.4.6.2 Repeated morphine injection

In the P7 and P21 animals, a repeated morphine injection activates DA cells significantly above control levels (p<0.05 or p<0.01; 2-way ANOVA with Bonferroni's post-tests). In the adult only, there is significantly less DA cell activation after repeated morphine injections compared to a single morphine injection (p<0.01; Two-way ANOVA with Bonferroni's post-tests – dashed curve in Chart 2-10) and no difference compared to control levels (p>0.05; 2-way ANOVA with Bonferroni's post-tests). There is also a trend for cell activation in the chronic morphine condition to decrease over development (p=0.08; Two-way ANOVA, Bonferroni's post-tests).

Taken together, this suggests that repeated morphine injections given over 5 days have a different effect upon dopaminergic cell activation in the adult compared to that of the younger animals.

## 2.4.7  Representative images

### 2.4.7.1 Baseline control
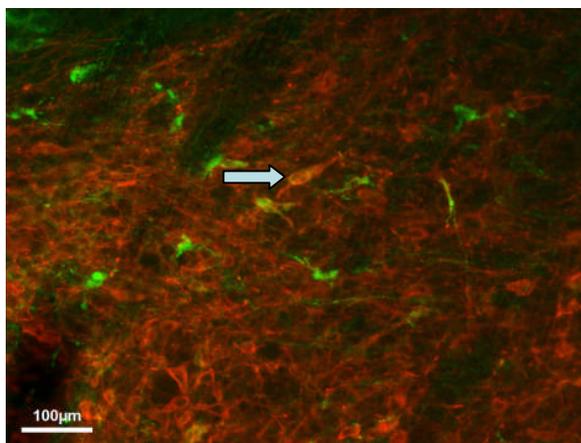


**Figure 2-7 - A representative image of tyrosine hydroxylase (red) and phosphorylated ERK (green) staining of an adult VTA section in the baseline treatment group. A single co-expressing cell, marked by a blue arrow, appears orange/yellow.**

### 2.4.7.2 Single saline control
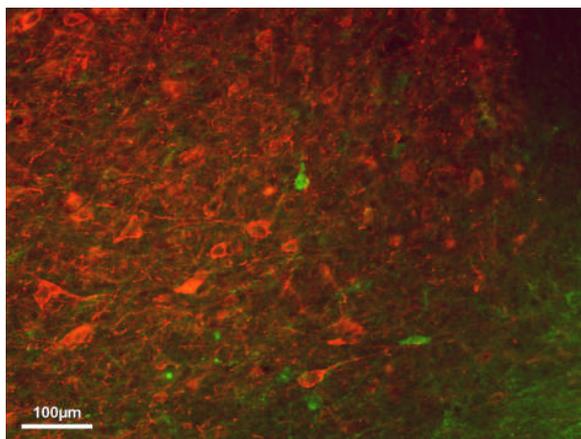


**Figure 2-8 – A representative image of TH (red) and pERK (green) staining of an adult VTA section in the single saline injection treatment group. There are no co-expressing cells.**

### 2.4.7.3 Repeated saline control
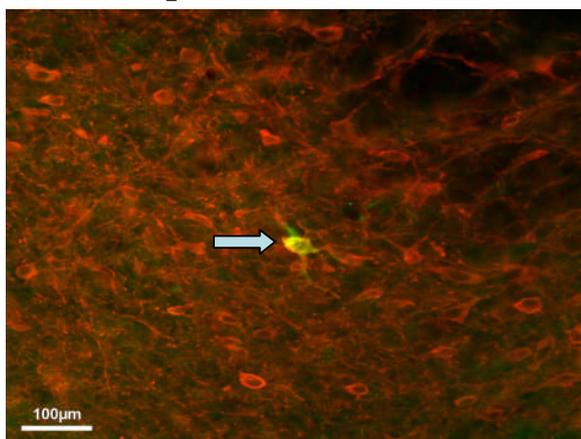


**Figure 2-9 – A representative image of TH (red) and pERK (green) staining of an adult VTA section in the repeated saline injection group. A single co-expressing cell, marked by a blue arrow, appears yellow.**

## 2.4.7.4 Single morphine injection at P7, P21 and adult

### 2.4.7.4.1 P7
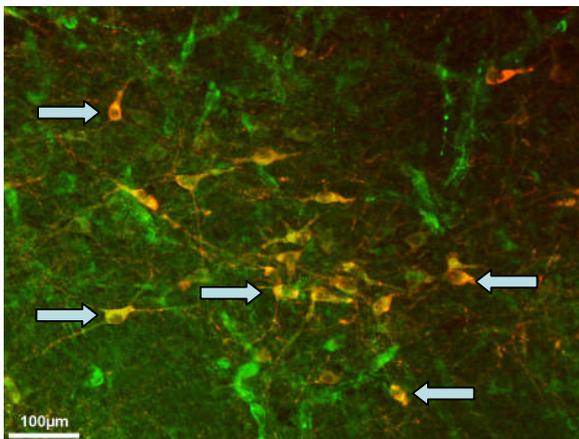


**Figure 2-10 – A representative image of TH (red) and pERK (green) staining in VTA of the P7 animal. Co-expressing cells, examples indicated by blue arrows, appear orange/yellow.**

### 2.4.7.4.2 P21
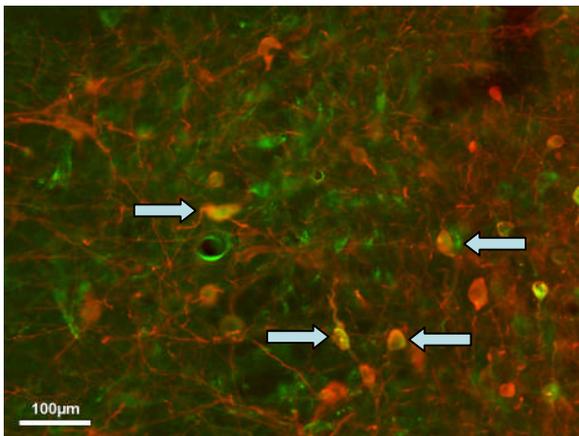


**Figure 2-11 – A representative image of TH (red) and pERK (green) staining in the VTA of the P21 animal. Co-expressing cells, examples indicated by blue arrows, appear orange/yellow.**

### 2.4.7.4.3 Adult
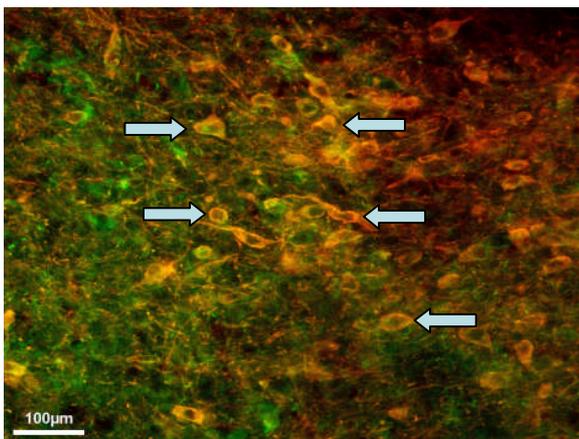


**Figure 2-12 - A representative image of TH (red) and pERK (green) staining in the VTA of the adult animal. Co-expressing cells, examples indicated by blue arrows, appear orange/yellow.**

## *2.5 Discussion*

### 2.5.1 Summary of results

### 2.5.1.1 Cell density in the VTA decreases over postnatal development

The results show that the density of total cells per $mm^2$ decreases over development. This is not surprising in view of the increase in cell size, length, and complexity of dendritic arbors over development (see McAllister, 2000), the proliferation of glial cells (Nixdorf-Bergweiler et al., 1994), and also the increase in myelination of nerve fibres that occurs during the first few weeks of an animal's life (Butt and Berry, 2000), pushing cell bodies further apart. This decrease in density means that raw cell counts of populations of neurons in a fixed area of brain tissue are potentially misleading. What might appear as a decrease in TH+ve cell number, for example, may simply be reflecting the 'spreading out' of all cells in this region. To circumvent this problem two approaches were used. One was to calculate the change in overall cell density, and thus calculate a ratio to adjust TH+ve and pERK+ve counts at each age. The other was to express the counts as a percentage of the total cell count in the area at each age. These calculations gave consistent data that could be compared across ages.

### 2.5.1.2 Single morphine injection

A single morphine injection caused dopamine cell activation in the VTA above control group levels at all ages, raising the percentage of activated DA cells from 5-10% in control animals to 26-30% in morphine-injected animals. This shows that the P7 and P21 dopaminergic system responds to a single morphine injection in much the same way as the adult, fulfilling one hypothesis of this experiment.

The two other hypotheses of this chapter are also fulfilled – that the dopaminergic system of the VTA is functional in the newborn rat, and that morphine, a rewarding stimulus, will activate this system. Overall, these results are consistent with the literature which has shown that morphine activates reward pathways in the neonate to the same extent as the adult (Kehoe and Blass, 1986a; Kehoe and Blass, 1986b; McPhie and Barr, 2000).

## 2.5.1.3 Repeated morphine injection

Repeatedly injecting morphine over a 5 day period causes different effects in the preweanling animals compared to the adults. Adults appear to develop neurobiological tolerance over five days of morphine treatment, shown by a decrease of DA cell activation to baseline levels.

In the P7 and P21 groups, however, dopamine cell activation is still high compared to saline control groups after 5 days. Indeed, there are no differences in DA cell activation between single and repeated morphine injections at these ages, suggesting that the preweanling animals are not developing tolerance to morphine. Other investigators have shown that the neonate does not develop analgesic tolerance to morphine (Rozisky et al., 2008; Van Praag and Frenk, 1991a). These results are not in line with the hypothesis that the effect of morphine will not differ significantly with age.

### 2.5.2 Technical considerations

## 2.5.2.1 Immunohistochemical staining

Whilst every effort is taken when processing and counting immunohistochemically-treated slides, there are a number of points at which methodological processes can potentially confound the results. A perfusion that does not circulate the paraformaldehyde as successfully as another, due to an incorrect or less-than-optimal placement of the needle into the heart, can affect the quality of tissue that is later visualised under the microscope. For example, blood vessels can also be stained with the pERK antibody in badly fixed tissue, making cell counting more difficult. To partly overcome this problem, the shape and size of every spot of staining had to be carefully considered to ensure neurons only were being counted. Using the TSA (tyramide signal amplification) method to amplify the pERK signal also reduced the signal-to-noise ratio and reduced the risk of identifying false positives.

The immunohistochemical process itself is not without problems. Antibodies may bind more or less specifically depending on the quality of the tissue or on the amount of washing comparative between experiments, and the background levels of antibody binding may confound later cell counts. To control for these confounds, control slides are produced that give images of the background levels of staining that prove the

antibody is binding correctly. For these experiments, negative control slides produced without the primary or secondary antibodies proved that the antibodies used were specific and showed low background levels of staining.

## 2.5.2.2 Gender

A potential confound in these experiments was the lack of separation for gender in the preweanling animals. Males alone were used in adulthood. Despite research showing gender variations in response to morphine (Cicero et al., 1996; McKelvy and Sweitzer, 2009), we hypothesised that as the preweanling animals had not yet begun to enter sexual maturity, there should not be any differences in morphine response due to hormonal variation between genders.

## 2.5.2.3 Morphine dosage

The dosage of morphine was different between the youngest P7 group and the P21/adult animals. A dose of 10mg/kg was originally chosen, as this dosage had been used in the literature when investigating morphine reward (Jones and Barr, 1995; Barr and Goodwin, 1997). However, to check the effects of this high dosage (which is higher than that needed for analgesia) on young animals, a pilot study was carried out to determine the effect of a 10mg/kg dose on a single P6 animal. Morphine is well-known as a respiratory depressant (Takita et al., 1997), and morphine at this dosage produced considerable respiratory depression and hypothermia in the P6 animal, which, if applied to all groups of <P7 animals in this experiment, would have meant continuous monitoring of animals was necessary over the 30 minute post-injection period before sacrifice to prevent suffocation or trampling by the dam. A dose that produced sedation and analgesia, but not significant hypothermia or respiratory depression, was found to be 5mg/kg, so this dosage was selected as appropriate for the youngest animals. All pups displayed clear sedation (as seen by loss of righting reflex) and analgesia (as seen by lack of response to tail pinch).

This need for a lower dosage in younger animals is in line with the literature showing that morphine is more efficacious in the neonate, due to higher MOR levels, an increased permeability of the blood-brain barrier, and slower metabolism of morphine (Giordano and Barr, 1987; Nandi et al., 2004; Hartley et al., 1994).

Despite the technical considerations discussed, overall the methods are sufficiently robust to enable significant differences and firm conclusions to be drawn.

## 2.5.3 Development of tolerance to morphine

Tolerance to morphine is defined as the need for ever-increasing concentrations over a period of chronic treatment to produce the original effect, and can occur at a cellular and systems level. The mechanisms of many aspects of tolerance are not yet fully understood, but there are a number of molecules and signalling pathways implicated in tolerance to chronic opioids.

### 2.5.3.1 Analgesic tolerance is not seen in young neonates

Analgesic tolerance to morphine is seen in adults, but not seen in neonates until at least P8 (Barr and Lithgow, 1986; Van Praag and Frenk, 1991a; Rozisky et al., 2008). This is earlier than previous studies had reported, and does not mirror the increase in number of receptors that occurs up to P15 (Zhang and Pasternak, 1981; Auguy-Valette et al., 1978), but could be due to masking effects of rapidly proliferating opioid receptors during the first two postnatal weeks, which will see an increase in receptor expression during the time that repeated morphine dosages induce cellular tolerance mechanisms (Van Praag and Frenk, 1991a). It is unlikely that a lack of receptors causes the lack of tolerance seen in the neonates and P21 animals, as MOR expression has reached adult levels by P15, so it would be expected that P21 animals would display the same tolerance as adults.

However, the dosages used in this experiment were significantly higher than the dosages used to induce analgesia, and analgesic tolerance was not specifically measured in these animals. Tail pinch or suprathreshold von Frey hair application qualitatively showed that analgesia was still apparent after 5 days of repeated morphine injection in all ages. Perhaps if sensitive quantitative tests of nociceptive withdrawal thresholds had been measured, analgesic tolerance would have been clear.

## 2.5.3.2 Acute receptor desensitisation and internalisation after morphine binding does not explain the lack of tolerance in neonates

Agonist binding causes the MOR to become activated, by dissociation of the Gα and Gβγ subunits, which both trigger the $G_{i/o}$ inhibitory intracellular signalling cascades. Desensitisation of the receptor by phosphorylation occurs rapidly after the agonist has bound, and can occur via a number of kinases i.e. G-protein receptor kinase (GRK), protein kinases A and C (PKA/PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII), all of which have been implicated in morphine tolerance (see Ueda and Ueda, 2009; Zhang et al., 2009). After phosphorylation, β arrestin binds to the MOR and uncouples the MOR from its signalling pathways. Endocytosis of the MOR from the cell surface then occurs via the scaffold protein clathrin, internalising the MOR. Once the MORs are internalised into the cell, 20% are degraded by lysosomes, and 80% de-phosphorylated then recycled back onto the cell surface membrane. This process occurs rapidly within ~30 minutes (see Christie, 2008), resulting in a fast turnover of receptors.

MOR agonists such as DAMGO are efficient at the internalisation and resensitisation of the MOR as described above, but morphine itself does not cause receptor internalisation to the same extent (see Borgland, 2001; Bailey and Connor, 2005). Therefore tolerance to chronic morphine may occur due to receptor desensitisation rather than via internalisation, perhaps via an alteration in the activity of the regulatory proteins that would normally internalise and recycle the receptor, i.e. PKC, GRK, or β arrestin (see Christie, 2008). Finn and Whistler (2001) propose that the MOR may remain phosphorylated at the cell surface membrane, is not internalised and recycled, and may continue to signal in a chronic morphine condition, providing an explanation as to the continuing action of morphine in younger animals.

Dang and Williams (2004) showed that desensitisation by phosphorylation of the MOR is faster, and receptor internalisation is slower after chronic morphine treatment (5 days), suggesting that chronic morphine can influence the kinetics of the rapid effects described above, but this alone is not likely to account for the long-term changes seen over days and weeks of morphine administration.

## 2.5.3.3 Chronic morphine treatment leads to cellular tolerance

Chronic and excessive stimulation of MORs over a long time period can lead to homeostatic ('allostatic') adjustment of downstream signalling systems (see Le Moal and Koob, 2005) and these signals may directly affect neural excitability, potentially contributing to tolerance. An example of this is the adenylyl cyclase (AC)-cyclic AMP-PKA cascade.

Chronic morphine treatment causes 'superactivation' of the AC signalling cascade (see Bailey and Connor, 2005; Martini and Whistler, 2007). Acutely, opioids inhibit AC activity, which causes a decrease in cAMP (3',5'-cyclic adenosine monophosphate) levels. However, after chronic morphine treatment, there is an increase in AC and therefore cAMP in the continued presence of the agonist, resulting in a compensatory homeostatic recovery to baseline levels of cAMP, and therefore tolerance of the system to morphine (Avidor-Reiss et al., 1996). This changing level of cAMP may affect transcription factors such as CREB (cAMP response element binding protein) and ΔfosB further down the signalling pathway, having a knock-on effect on cellular responses. Both CREB and ΔfosB have been heavily implicated in the molecular mechanisms of drug addiction (see Nestler, 2004).

Other molecules that couple to signalling cascades and are directly affected by the MOR include the MAP kinases (mitogen-activated protein kinases) and PI3 (phosphoinositide-3) kinase. One MAPK, ERK (extracellular-signal-regulated kinase) is activated by the liberation of the MOR Gβγ subunit and has been implicated in receptor desensitisation (see Christie, 2008). PI3 kinase is also activated by Gβγ subunits, and could stimulate PKC signalling, which itself may have a role in receptor desensitisation, as described above.

## 2.5.3.4 Tolerance of neural systems results from chronic morphine treatment

Changes in neuronal firing, synaptic strength and chemical balance may all be altered by chronic morphine, causing knock-on effects within the whole network. These kinds of changes are complex and not well understood at this time.

### 2.5.3.4.1 'Anti-opioid' systems

Networks that are functionally antagonistic to endogenous opioid signalling have been proposed that may be disturbed under chronic morphine conditions. The κ-opioidergic system and its ligands (the dynorphins) may be organised in a functionally antagonistic manner to μ opioid signalling (see Shippenberg et al., 2007) and other suggested anti-opioid systems include the nociceptin, orphanin, neurokinin-1, neuropeptide FF and cholecystokinin-mediated systems (see Ueda and Ueda, 2009; King et al., 2001).

A further proposed anti-opioid system revolves around NMDA receptor signalling (Mao et al., 1995; Mao, 1999). Antagonising the NMDA-R blocks opioid tolerance and dependence (Trujillo and Akil, 1991; Mendez and Trujillo, 2008), and mice lacking the NMDA receptor (NR)2A subunit do not develop analgesic tolerance. Electroporation of the receptor back into the VTA restores analgesic tolerance in these animals (Inoue et al., 2003). These data suggest that the NMDA receptor may be involved in the development of tolerance at the systems level, perhaps through synaptic plasticity and long-term potentiation, as mentioned below.

### 2.5.3.4.2 Glial function and tolerance

Astrocytes and microglia activate in response to chronic morphine treatment, and there is evidence that this activation may influence neural excitability and synaptic plasticity, perhaps playing an important role in tolerance and dependence to opioids (Narita et al., 2004b; Watkins et al., 2005). Glia also produce growth factors and cytokines which can affect synaptic plasticity i.e. via cAMP/CREB activation in the locus coeruleus (Akbarian et al., 2002).

### 2.5.3.4.3 Synaptic plasticity

Opioids can affect synaptic plasticity after a single morphine injection, so it is likely that chronic opioids will also affect the balance of cell signalling, perhaps via long-term potentiation (LTP) and long-term depression (LTD) mechanisms (Ungless et al., 2001; Christie, 2008). Acute morphine administration causes imbalances in excitatory AMPA and NMDA receptors in the dopaminergic cells of the VTA (Ungless et al., 2001) and also causes decreased LTP induction at GABAergic synapses onto these dopaminergic cells (Nugent et al., 2007). This strongly suggests that long-term changes would occur in synaptic plasticity with chronic morphine treatment.

## 2.5.4 Potential explanations for the lack of tolerance in the neonate as compared to the adult

The results of the experiments in this chapter showed that neonates do not develop cellular tolerance to repeated morphine injection, as seen by the fact that the activation of the DA cells did not decrease to baseline levels after 5 days of injection, unlike the adults. This is unlikely to be due to a lack of functional morphine receptors, as these have been found in the newborn animals in both spinal cord and brain, and both analgesic and addictive behaviours are seen in animals in the first week of postnatal life (Abbott and Guy, 1995; Jones and Barr, 1995; Barr and Goodwin, 1997; Barr et al., 2003).

The delay in development of tolerance is also unlikely to be due to a lack of functioning dopamine neurons, as these are present in the VTA at birth (see Alavian et al., 2008), and the DA neurons of the VTA will respond to morphine challenge in the P4 rat (Barr and Rossi, 1992). However, the numbers of DA receptors in the VTA do not peak until P28 (Noisin and Thomas, 1988), and the relative importance of D1 and D2 receptor signalling changes during the first 17 days of life (Pruitt et al., 1995). Dopamine receptors in the prefrontal cortices, which are reciprocally connected to the VTA, do not reach adult levels until around P60 (Tarazi and Baldessarini, 2000). This suggests that within the first two weeks of life, dopaminergic signalling within the VTA and its projections to prefrontal areas is not yet mature, and the lack of tolerance seen may be partly due to this immaturity.

To illustrate, in the adult the VTA is reciprocally connected to the medial prefrontal cortex (mPFC), which sends excitatory glutamatergic output to the DA cells of the VTA, acting preferentially on NMDA receptors (Taber and Das, 1995; Wang and French, 1993). Lesions of the mPFC deprive DA cells of one of their major sources of excitation, and the mPFC is implicated in the induction of the behavioural sensitisation seen after chronic morphine (Hao et al., 2007). If this single area is implicated in addictive behaviours, it is likely that other prefrontal areas are also involved in the response to chronic drug administration in the adult.

However, in the neonate, this connectivity may not be functional. Studies have shown that the prefrontal cortex shows a delayed maturation in comparison to midbrain areas, and that dopaminergic signalling in the adult can be qualitatively different to the neonate (see McCutcheon and Marinelli, 2009). Therefore the lack of tolerance seen in the neonates could be due to immature connectivity to brain areas involved in the expression of tolerance in the adult. This immature connectivity could perhaps be due to a lack of a number of factors: mature neurons in prefrontal areas; interneurons to connect neurons in different areas; functional synapses between the areas; and/or white matter to connect the areas. This explanation would fit with the result that the P21 animals did not develop tolerance, as the prefrontal cortex is not functionally mature until at least P45 (Tseng and O'Donnell, 2007).

A further hypothesis to account for a lack of tolerance in the neonate could involve the NMDA receptor and its role in synaptic plasticity. As described above, systems tolerance could be partly controlled by action of an 'anti-opioid' NMDA-mediated system (see Mao, 1999; Ueda and Ueda, 2009). In line with data from the adult, Zhu and Barr (2003) found that NMDA receptor antagonists block the development of morphine tolerance. However, this only occurs after 14 days of age and not at all in animals in the first week of life, which the authors speculate is due to an immature composition of NMDA receptor subunits that does not mature until the third week of life. This would suggest that the neonate cannot develop cellular tolerance, which is in line with the data presented in this chapter.

## *2.6 Conclusions*

The results presented in this chapter suggest that the reward pathways in the VTA of the neonate are functionally mature, as morphine will activate the mesolimbic dopaminergic pathways in the youngest animals, as measured by pERK expression in DA cells. This is in line with literature showing that morphine is rewarding in the neonate, i.e. it will induce conditioned place preference (Kehoe and Blass, 1989), and symptoms of withdrawal will appear on cessation of morphine administration (Jones and Barr, 1995; Ceger and Kuhn, 2000).

The results also show that P7 and P21 animals do not develop cellular tolerance to 5 days of morphine administration, but that the adult does. These results are in line with the literature that shows that adults develop cellular tolerance, but that neonates do not (Rozisky et al., 2008). These animals may not develop cellular tolerance in the dopaminergic cells studied due to an immaturity of NMDA receptor composition, and/or a lack of functional connectivity to the medial prefrontal cortex. The lack of tolerance is unlikely to be due to immature opioid receptor expression, as the levels of opioid receptors are mature by the end of the second postnatal week (Auguy-Valette et al., 1978). Nor are the morphine or dopaminergic systems immature at P21, as both are sensitive to reward in the neonate (Lithgow and Barr, 1984; Barr and Rossi, 1992).

These results are encouraging for rest of this thesis, as they provide a solid basis for the further experiments in this thesis which will look at the long-term effects of injury during the neonatal period on adult reward processing. The results presented here prove that the reward pathways during the neonatal period are viable, and so it is possible that pain during this period has the potential to impact upon reward processing, much as pain in adulthood can affect reward processing.

# 3 Chapter 3 – The effect of neonatal injury on adult reward behaviours

## 3.1 Introduction

This chapter addresses the fundamental question of this thesis: if pain processing can be affected by early life injury, then can other modalities of cognitive processing also be affected? The hypothesis presented in this thesis is that interfering with the developing nociceptive system will shift processing in the reward systems of the brain in adulthood. The experiments presented in this chapter are aimed at investigating the long-term effects of neonatal experience of foot wound on reward-related behaviour in adult life. To do this, preweanling animals were repeatedly injured, and the sensory and affective alterations in behaviour studied when the animals were mature.

### 3.1.1 Injury during early life

As discussed in the introduction to this thesis, there is much evidence in the literature that neonatal pain can cause long-term alterations in pain processing in the older and adult organism (Ren et al., 2004; Lidow et al., 2001; Walker et al., 2009b). This is particularly relevant for informing clinical practice in human neonates, as many of the procedures performed that are necessary for survival can cause long term changes in nociceptive processing that can affect the child later in life (Buskila et al., 2003; Grunau et al., 2006; Walker et al., 2009a).

#### 3.1.1.1 Modelling the heel lance in humans

In this chapter, rats that were repeatedly injured as neonates are to be studied in adulthood for sensory changes, and also for subtle changes in behaviour towards a reward. To make this investigation as relevant to human clinical practice as possible, the model of injury chosen was designed to reflect the heel lance procedure routinely performed in the neonatal intensive care unit (NICU): the plantar skin incision model.

On the NICU, blood must be gathered from infants to monitor blood oxygenation levels. However, particularly in preterm infants, veins are not mature enough to cope with repeated venepuncture, so to overcome this problem, blood is collected via heel lance.

In this procedure, the foot is warmed to stimulate blood flow, the heel is punctured with a small blade, and the foot squeezed to cause bleeding and allow blood collection.

Whilst neonates on the NICU do not experience only heel lance, it is the most common invasive procedure performed in the NICU (Johnston et al., 1997), causes behavioural responses i.e. facial distortion and limb withdrawal, and activates cortical somatosensory processing areas (Slater et al., 2006). The fact that heel lance is processed by both the spinal cord and brain in infants makes it a relevant procedure to model in animals where adult cognitive processing is being investigated. Other papers have modelled heel lance by repeated needle prick (Walker et al., 2003a) or insertion of a needle completely through the paw (Anand et al., 1999), but in this chapter, the plantar skin incision model of post-operative pain was chosen to mimic the neonatal repeated heel lance procedure.

The plantar skin incision model, or 'foot wound' as it will be referred to hereafter, was developed by Brennan and colleagues (1996). It was developed to model surgical injury and its effect both peripherally and centrally has since been thoroughly described (Brennan et al., 2005). It has also been used in neonates and shown to produce acute thermal and mechanical hypersensitivity (Ririe et al., 2003) and to alter spinal nociceptive processing (Ririe et al., 2008).

Recently, Walker and colleagues (2009b) have shown that a single neonatal foot wound causes enhanced sensitivity to mechanical stimuli upon re-injury two weeks later, compared to animals with no neonatal foot wound. Furthermore, this effect is not apparent when the original foot wound was performed in older animals (postnatal days (P)10, 21 or 40), suggesting a critical period within the first week of postnatal life for the development of heightened sensitivity after re-injury. This paper is important for the experiments in this chapter, as it shows that a single neonatal foot wound can have long-lasting effects on pain sensitivity after the wound has healed, and that re-injury to the same site can exacerbate the resulting hypersensitivity.

Methodologically, the foot wound model has a number of advantages. Firstly, it is a short, simple, non-invasive surgical procedure. Therefore, animals are only briefly anaesthetised and possible confounding effects upon adult behaviour due to anaesthetic

administration are minimised. Also, like the heel lance, an incision is made into the foot/paw, meaning similar physiological response mechanisms are involved in both i.e. inflammatory responses in a particular dermatome and the associated peripheral and central nervous system changes. Another advantage is the reproducibility of the foot wound surgery, allowing accurate comparisons at different ages after the same stimulus. As Figure 3-1 shows, the anatomical landmarks of the rat hindpaw are clear, and these do not change over development. This reproducibility is important when comparing animals which will all display different patterns of individual differences.



**Figure 3-1- A schematic diagram of the rat hindpaw, showing footpads. The relative location of these on the paw does not change throughout the lifetime of the animal. Adapted from Walker et al (2009b).**

## 3.1.2 Measuring reward behaviours

A reward is something that inspires the motivational incentive to seek it. In simple terms, it is something that an organism will work for. Rewards can be almost anything, from basic reinforcers such as food, sex and drugs, to complex social behaviours, such as friendship and teamwork. Reward responses in animals and humans have been a massive area of research over the past 100 years – to help understand the motivations that drive drug abuse, and also to help ameliorate the symptoms of withdrawal.

In animals, reward behaviour can be measured in a variety of ways. Some paradigms are relatively simple, and based on operant conditioning principles, where an animal learns to perform a behaviour to receive a conditioned reward. Other paradigms are more complex, and the animal has to learn to perform, or withhold, a particular set of responses within a specific time-frame to receive the reward. These more complex paradigms can often take into account other outcome measures, such as anxiety behaviours, impulsivity and anhedonia (the loss of ability to experience pleasure).

To investigate reward behaviours in animals, a number of considerations have to be taken into account. Which paradigm would be most appropriate to use? Should an existing paradigm be modified to make it appropriate for these sets of experiments?

Should a pharmacological drug or something more naturalistic be used as the rewarding stimulus? These questions are considered below.

## 3.1.2.1 Classical conditioning

Many reward paradigms are based on extensions of classical conditioning principles, first detailed by Pavlov in his experiments with salivating dogs (1927). In a classical conditioning paradigm, at baseline an animal naturally associates an unconditioned stimulus (UCS) i.e. food, with an unconditioned response (UCR) i.e. salivation. During conditioning, another stimulus presented with the food (i.e. a bell) becomes associated with the salivatory response. After conditioning, the bell becomes a conditioned stimulus (CS), where the sound of it alone can cause salivation, now termed as the conditioned response (CR).

## 3.1.2.2 Operant conditioning

Operant conditioning is an extension of classical conditioning, and is learning that occurs through rewards and punishments, as the animal learns to associate performance of a particular behaviour with a certain outcome. Skinner first used the term 'operant' to refer to any "active behaviour that operates upon the environment to generate consequences" (Skinner, 1953) and his behaviourist ideas have shaped the study of animal behaviour over the last 5 decades. Operant conditioning takes into account both reward and punishment, the former increasing the frequency of the specific behaviour and the latter decreasing the frequency.

### 3.1.2.2.1 Progressive ratio measurement

Progressive ratio testing is a reward-sensitive procedure based on operant conditioning principles. In this procedure, an animal receives a reward i.e. sweetened condensed milk, upon lever press or nose-poke. The number of lever presses/pokes required to produce the reward continually increases until the animal stops responding, at which point the 'break point' is determined. Weaker rewards are shown to have lower break points (Hodos, 1961). A brief search of the literature shows that within the last year, rewards given on a progressive ratio schedule have included ethanol (Li et al., 2010b), heroin (Wang et al., 2009b), cocaine (Wang et al., 2009a), and food (Sharf et al., 2010) amongst many others, showing how it is a widely used experimental tool.

## 3.1.2.2.2 Conditioned place preference

### 3.1.2.2.2.1 Principles of Conditioned Place Preference

Conditioned place preference (CPP) is another behavioural paradigm based on operant conditioning principles and has been used hundreds of times in rodents over the course of the last 20 years, generally to investigate the rewarding properties of drugs of abuse (see Tzschentke, 1998).

CPP paradigms normally consist of two chambers with differing characteristics i.e. vertical versus horizontal stripes, different odours etc., although square open fields with differing characteristics between each quadrant have also been used successfully (Vezina and Stewart, 1987).

To run the experiment, the animal is placed into one of the two chambers and either administered with the drug under investigation or exposed to the reward in question (in the case of drug injection, administration occurs immediately prior to placement in the chamber) on a certain number of conditioning days prior to testing. On the test day, the animal is placed into either the non-conditioned chamber, or a neutral chamber attached to both conditioning chambers, and the amount of time the animal spends in the conditioned chamber is measured. The animal will spend more time in the chamber with which it associates the rewarding stimulus. It is said that if a stimulus has been rewarding, it will demonstrate CPP (see Tzschentke, 1998).

### 3.1.2.2.2.2 Applications of conditioned place preference

The literature overwhelmingly shows that CPP is a reliable indicator of a reward's motivational properties (see Tzschentke (1998) and (2007) for comprehensive reviews).

CPP has been shown to result from a huge array of drugs, often mediated by the mesolimbic dopaminergic system; Amphetamine, cocaine, heroin and morphine, which all increase DA levels, and various combinations of these with other drugs, all induce CPP. Conversely, CPP is blocked by dopaminergic antagonists. Other rewarding drugs that induce CPP have included ethanol; nicotine; benzodiazepines such as diazepam which enhance GABAergic transmission; MDMA (3,4-methylenedioxymethamphetamine, commonly known as Ecstasy); LSD (lysergic acid

diethylamide); phencyclidine (PCP, or 'angel dust'); various cannabinoids; and some hormones, amongst many others. Natural reinforcers such as food, sucrose-sweetened water, social play, home cage odours and the presence of pups in maternal rats have also been shown to induce CPP. Clearly, it is a powerful test of whether or not a stimulus is rewarding.

CPP has also been used to investigate the effect of lesions on reward behaviours, helping to show which specific areas of the brain are involved in reward processing. Lesions by 6-hydroxydopamine (6-OHDA), which selectively ablates dopaminergic and noradrenergic neurons, have shown that the nucleus accumbens shell and ventral pallidum are necessary for morphine-induced, diazepam-induced and novelty-induced CPP (Shippenberg et al., 1993; Spyraki and Fibiger, 1988; Pierce et al., 1990) Excitotoxic lesions (by ibotenic acid or kainic acid) of the NAcc, ventral pallidum, amygdala and medial PFC, amongst many others, have been shown to be necessary for induction and learning of CPP (Olmstead and Franklin, 1996; Hiroi and White, 1993; Brown and Fibiger, 1993; Tzschentke and Schmidt, 1998). Finally, electrolytic lesions, caused by driving a large current through brain tissue and destroying it, cause CPP loss, as do radiofrequency lesions and knife-cut lesions (see Tzschentke, 1998).

Studies of different strains of rats and mice have shown inherent differences in drug response, as shown by CPP. Conditioned place preference has even been used in non-mutated animals to breed separate strains. Rats that showed naturally strong or weak cocaine-induced CPP, paired over three generations, produce discrete strains with differences in cocaine sensitivity (Schechter, 1992). Testing of genetically manipulated mice has also confirmed the role of a number of molecules in drug reward i.e. mu opioid receptor (MOR) and dopamine D2 receptor knock-out mice will not develop morphine-induced CPP (Matthes et al., 1996; Maldonado et al., 1997).

### 3.1.2.2.2.3 Conditioned Place Avoidance

A modification of the CPP paradigm, conditioned place avoidance (or aversion), is based on the same principles as CPP, but is used as a way to measure the aversive, or punishing properties of a stimulus. Animals are exposed to an aversive stimulus (e.g. footshock or a dopamine antagonist) that is paired to one of the two conditioning chambers, and time spent in that chamber upon post-conditioning testing is lower

compared to pre-conditioning baselines. Therefore the animal has learnt to avoid the chamber in which the aversive stimulus occurred.

Pain is both an aversive stimulus and state for an animal to be in, and pain research is beginning to use CPA as a way of modelling the affective components of pain. For example, Hummel et al (2008) showed that animals in neuropathic and inflammatory pain states will show aversion to a previously neutral chamber in which the injured paw was repeatedly mechanically stimulated during 5 days of conditioning. Other studies have shown that injection of inflammatory agents such as carrageenan and complete Freud's adjuvant (CFA) into the hindpaw, and also nerve injury, will induce CPA when the injured paw/nerve territory is mechanically stimulated during conditioning (LaBuda and Fuchs, 2000; van der Kam et al., 2008). In the studies above, morphine analgesia delivered concomitantly to conditioning either attenuated or abolished CPA induction.

### 3.1.2.2.2.4 Intracranial place conditioning

Another modification of the classic CPP paradigm, intracranial place conditioning (ICPC), is a useful paradigm for investigating the brain regions that are involved in signalling reward. By implanting cannulae into different regions implicated in reward processing, the effects of drug agonists and antagonists can be closely linked to specific areas.

Intracranial administration of the drug is given in association with one of the CPP chambers during conditioning, and place preference measured on test days. This is a non-operant test with no learning of behaviour needed to elicit drug reward.

ICPC studies have shown that dopaminergic drugs in a variety of brain regions will induce CPP. Morphine injection into the VTA induces CPP, as do endogenous opioid peptides and mu and delta opioid receptor agonists (Phillips and LePiane, 1980; Phillips and LePiane, 1982; Bals-Kubik et al., 1993). Neurotensin, which modulates dopaminergic signalling, injected into VTA also causes CPP (Glimcher et al., 1984). Other areas implicated after dopaminergic drug injection include the nucleus accumbens (NAcc), in particular the shell section, medial prefrontal cortex (mPFC), dorsal hippocampus, periaqueductal grey, and lateral hypothalamus (see McBride et al., 1999).

All the above examples rely on DA signalling, but ICPC has shown other neurotransmitter systems are involved in reward processing. For example, injection of the cholinergic agonist cytosine into the VTA causes CPP (Museo and Wise, 1994) and substance P, which is closely implicated in serotonergic signalling, injected into the ventral pallidum and nucleus basalis magnocellularis (NBM) induces CPP (Hasenohrl et al., 1998). Taken together, ICPC can show a myriad of regions responsible in part for reward signalling.

ICPC can be used in conditioned place avoidance paradigms as well. Johansen and Fields (2004) showed that the anterior cingulate cortex (ACC) is involved in learning the aversive component of pain. Excitotoxic lesions, performed by microinjection of ibotenic acid, prevented the expression of formalin-induced CPA if the area was lesioned before, but not after, conditioning. Furthermore, stimulation of the ACC with excitatory amino acids, in the absence of pain, also produced CPA. These data, together with an earlier paper from the same laboratory (Johansen et al., 2001) show that the ACC encodes a teaching signal for aversive stimuli.

However, ICPC can be problematic. The implantation of a cannula into the brain is an invasive procedure, and post-surgery animal recovery must be carefully monitored. The diffusion extent of drugs that are being infused into the brain must be well characterised to minimise spread into other nearby structures, and the neurotoxicity of the drug in question must also be well known to prevent local damage that could influence results for confounding reasons. Finally, localisation of the cannula must be scrutinized after the experiments are concluded to check that it has been placed in the correct area – incorrect placement of cannula could negate any results from that particular animal. In brain regions such as the ventral tegmental area (VTA), this is particularly important, as the VTA is small and bordered by many areas such as the substantia nigra that could affect experimental results greatly (see Figure 3-2).
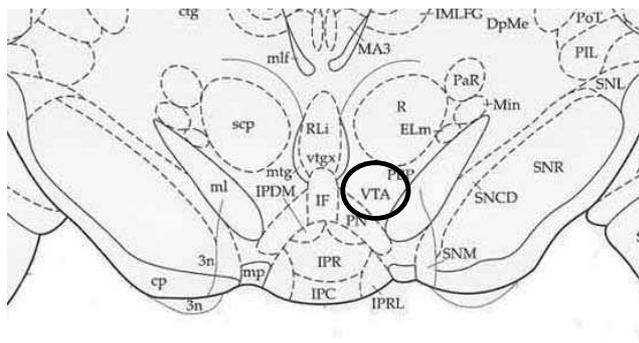
**Figure 3-2 – A schematic diagram of a coronal section of the brain, with the ventral tegmental area highlighted by the black circle. It is small and has borders with many other areas. Adapted from Paxinos and Watson (2004).**

A major weakness of the ICPC procedure is that the animal has no control over the administration of the agent, meaning that whilst associative learning is displayed, the motivational aspect of drug-seeking is largely lost (see McBride et al., 1999).

### 3.1.2.2.3 Summary of place preference paradigms

CPP has been popular with researchers for a number of reasons: it is cheap and easy to set up, does not require large amounts of training, and it can produce reliable and reproducible drug effects.

However, the lack of CPP induction with a certain stimulus does not automatically mean that a drug will not be rewarding or potentially addictive. The testing apparatus is a highly artificial construct, especially where external drug administration is concerned, and it can be argued that, whilst sensitive to pharmacological manipulation, CPP is not representative of an environment that an animal may encounter in its natural habitat.

### 3.1.2.2.4 Intracranial self-stimulation (ICSS) and intracranial self-administration (ICSA)

Intracranial self-stimulation or self-administration are other useful methods of investigating drug reward in animals, delivering rewarding stimuli directly into the brain. They involve placement of an electrode or cannula into a specific brain region, and the animal will learn to perform certain behaviours i.e. lever pressing or nose-poke, to receive electrical stimulation or a drug infusion in response. The main advantage of ICSS/ICSA is that the animal is in charge of its own electrical impulse/drug

administration, and in this way it is an operant conditioning paradigm, whereas in CPP the animal has little or no control over access to the rewarding stimuli and displays simple associative learning to an environment. Rewards can be administered in a linear fashion, with a single lever press/nose-poke resulting in a single 'shot' of the reward, or on a progressive ratio schedule to examine reward magnitude, break points, and the effects of withdrawal directly in the brain regions implanted with the electrode or cannula.

**3.1.2.2.4.1   Electrical intracranial self-stimulation (ICSS)**

Olds and Milner (1954) first found that animals will keep returning to an area of testing apparatus in which they had received electrical stimulation of certain brain regions, and that the animals could then be trained to self-stimulate by pressing a lever. Olds and Milner concluded that electrical stimulation could serve as a positive reward in operant conditioning. Since these discoveries, ICSS has been demonstrated in all species that have been tested, and even in humans, with electrodes in the caudate nucleus, amygdala, intralaminar thalamic nuclei and middle hypothalamus mapping intensities of stimulation necessary for the sensation of 'rewarding' and 'aversive' properties (Bishop et al., 1963). Both animals and humans will choose to forgo food, even to the point of starvation or exhaustion in animals, in order to continue the lever-pressing that delivers the electrical stimulation. Aversive stimuli, such as footshock, will also be better tolerated or ignored altogether when there is the option of self-stimulation (Olds, 1958).

Electrical ICSS paradigms can be carefully manipulated to deliver controlled and reproducible stimuli. The magnitude of the reward, and therefore how motivated the animal is to work for it, can be controlled by altering the duration and frequency of the pulse, therefore altering the intensity of the stimulus.

The effects of ICSS can be potentiated by concomitant drug administration. For example, drugs of abuse such as cocaine, morphine, heroin, amphetamine and nicotine, amongst others, show synergistic effects with ICSS, potentiating ICSS by lowering the threshold for stimulation, and elevating the dopamine concentrations within the brain (see McBride et al., 1999).

The converse of reward is anhedonia – a loss of sensitivity to reward – and can be quantified and explored using ICSS. An increase in the levels of stimulation required to maintain a particular level of bar pressing/nose-pokes is seen as a decreased sensitivity to brain reward. Increases in self-stimulation are also seen in drug withdrawal states, making ICSS a useful tool for modelling for drug withdrawal symptoms in humans.

#### 3.1.2.2.4.2 Intracranial self-administration (ICSA)

Drug administration via implantation of a cannula into brain sites has also provided valuable insights into the actions of rewarding drugs on brain processing. ICSA studies in rodents have shown that, like CPP and ICSS, dopaminergic signalling in the mesolimbic pathway, originating from the ventral tegmental area, is crucial for reward processing (see McBride et al., 1999). Also like ICPC and ICSS, ICSA has confirmed the brain regions involved in reward processing, again mainly those linked to or explicitly part of the mesolimbic dopaminergic pathway: the VTA (Bozarth and Wise, 1981; Devine and Wise, 1994; Wise and Hoffman, 1992), the NAcc shell (Roberts et al., 1980; Barak Caine et al., 1995), the medial PFC (Goeders and Smith, 1993) and the lateral hypothalamus (David and Cazala, 1994b; Olds and Williams, 1980), amongst others. All of these regions will respond to a wide range of drugs of abuse.

### 3.1.2.2.5 Summary of intracranial electrical stimulation and self-administration

Like ICPC, both ICSS and ICSA provide useful tools for understanding how rewards are processed in the brain, as well as giving insight into the anatomical structures and associated neurochemistry. Also, the fact that self-stimulation has been shown in humans to demonstrate reward has proven hugely useful in drawing trans-species parallels, a rare occurrence in many areas of research.

However, as with ICPC, self-stimulation paradigms can be problematic. Implantation of an electrode or cannula is invasive and must be properly verified, and the situation in itself is highly artificial – there are no natural environmental circumstances under which an animal would experience the same events. Self-stimulation bypasses the peripheral nervous system and the senses such as touch, smell and taste that would normally accompany a reward (see Wise, 2002). Whilst enabling specific brain areas and

neurotransmitters to be identified and manipulated, it is not an ethologically valid method of investigating reward.

### 3.1.3 Novelty, exploration and the approach/avoidance conflict

It is widely accepted that novelty is rewarding. In animals, novel objects are preferentially investigated (Bevins and Besheer, 2005; Harris et al., 2007a), and novelty preference (neophilia) for faces is apparent in young human infants (<6 months) (Rhodes et al., 2002).

Under CPP testing conditions, Bevins and Bardo (1999) showed that conditioning with novel objects can elicit place preference on test days, and that expression of this CPP relied on dopaminergic signalling, as antagonism of both D1 and D2/D3 receptors blocked the CPP. Novelty preference in animals has been argued to be a more subtle and valid test of reward than conventional drug rewards, mimicking regular, everyday human rewards, such as enjoying a food treat (see Bevins and Besheer, 2005).

It is suggested that intense novelty may, however, elicit avoidance rather than approach, producing a curvilinear relationship between novelty and approach – see Figure 3-3 (see Hughes, 2007).
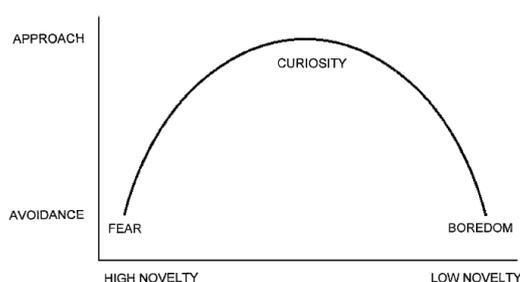


**Figure 3-3 – A schematic graph showing the proposed curvilinear relationship between novelty and avoidance. High novelty can produce avoidance behaviours, whereas low novelty can result in behavioural 'boredom'. Curiosity is a pay-off between approach/avoidance at non-extreme levels of novelty. Adapted from Hughes (2007).**

Exploration of a novel environment will, based on the novelty-as-reward assumption, be rewarding; however, it will be tempered by conflicting motivational drives that cause predated animals such as rodents to exert caution in their exploration, producing an

approach-avoidance conflict (see Montgomery, 1955). This conflict in motivational drives causes an anxiety state, with anxiety levels as the inverse of exploratory behaviours (Ennaceur et al., 2006).

The innate approach-avoidance conflict is widely utilised in animal research to study the extent to which an animal is willing to explore, and the anxiety that this produces. Researchers looking for treatments for human anxiety syndromes have taken advantage of conflict tasks to develop animal models of anxiety, back-validated them with current anxiety drugs, and used these animal models for drug screening of potential therapeutic compounds in behavioural conflict tasks.

More recently, pain researchers have begun using animal paradigms that study anxiety to begin modelling the affective components of pain. Pain is not only a sensory experience; the International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience", and researchers have struggled to develop affective measures of pain in animals (see Mogil, 2009). The co-morbidities that are associated with pain, such as anxiety, depression, and anhedonia (Meyer-Rosberg et al., 2001) make them potential behaviours to study in animals in pain states. Some laboratories are now engaged in modelling these behaviours, in parallel with pain behaviours (Callahan et al., 2008; Legg et al., 2009; Seminowicz et al., 2009).

### 3.1.3.1 Behavioural paradigms measuring anxiety-like behaviours

Many paradigms that exploit the approach/avoidance conflict are used to measure behaviour in animal models of anxiety (see Ramos, 2008). Anxiety, as discussed, can result from the conflict produced from being motivated to approach a novel environment (reward) and the innate avoidance behaviours that will protect naturally predated animals such as rodents. Therefore anxiety paradigms have components of rewarding exploration inherent in them, which can be measured in the paradigms described below. Indeed, this fact can confound research investigating anxiety behaviours, as an increase in approach/exploration can either be a measure of decreased anxiety, or increased novelty-seeking/impulsive behaviour (see Cryan and Holmes, 2005).

### 3.1.3.1.1 Elevated plus maze

The elevated plus maze (EPM) is one of the most popular test of anxiety. It consists of a plus-shaped maze, elevated off the floor, with two of the 'plus' arms being enclosed, and the other two open (see Figure 3-4). Rodents will naturally avoid the open arms and show general preference for closed arms, but will show exploratory behaviours by entering the exposed arms (Rodgers, 1997). Outcome measures in this test include the latency of entry into the open arms, number of entries into the open arms, and percentage of time spent there versus time in the closed arms. Anxiolytics (drugs that lower anxiety levels) will decrease the latency to enter the open arms, and increase the number of entries and amount of time an animal spends in the open arms. Rearing behaviour can also be scored as an index of exploratory behaviour.
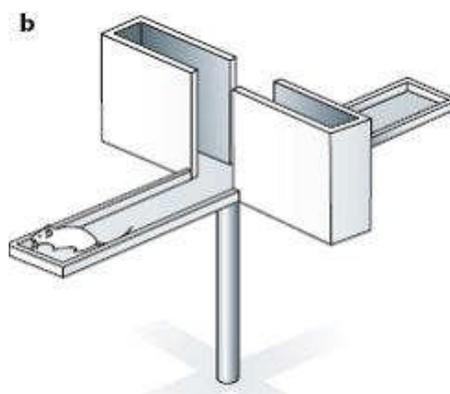


**Figure 3-4 – A schematic figure of the elevated plus maze for mice, showing the two open and two closed arms. Adapted from Cryan and Holmes (2005).**

### 3.1.3.1.2 Light/dark box test

The light/dark box, also called the black/white box, consists of two arenas connected to each other through a small opening. One arena is large, open, brightly lit and white; the other is smaller, covered, and black (see Figure 3-5). Rodents will tend to avoid the aversive white environment and outcome measures can include latency to entry, locomotive behaviour, number of entries into the light arena and number of rears. As with the EPM, anxiolytics will decrease latency to entry and increase number of entries into the light arena.
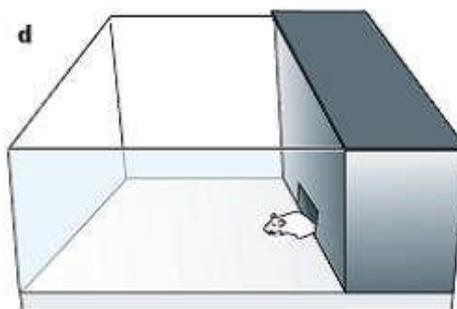
**Figure 3-5 – A schematic figure of the light/dark box test for mice. Adapted from Cryan and Holmes (2005).**

### 3.1.3.1.3 Open field test

First conceived in 1934, the open field test was designed to assess 'emotional reactivity' in rodents. Outcome measures that were regarded as indices of 'reactivity' were defecation and urination (Hall, 1934). Since then, the open field test has been widely used as a tool for assessing exploration, inquisitive behaviour, anxiety, memory and reaction to novelty (Ennaceur et al., 2006).

Open fields consist of a large, open arena. They can be square (see Figure 3-6), circular or rectangular; the arena can be bright or dark; the testing can be done in brightly illuminated conditions or in the dark; and length of testing time can vary between less than 5 minutes up to more than 30. This lack of standardisation can be problematic when comparing results between different laboratories. Measures of central exploration are often regarded as anxiety-related indices, where exploration into the centre will increase as anxiety decreases. Again, rearing behaviour can be scored as an index of exploratory behaviour.
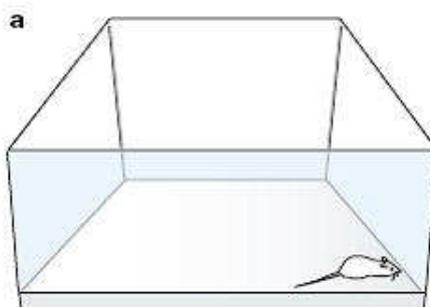


**Figure 3-6 – A schematic figure of the open field test. Adapted from Cryan and Holmes (2005).**

The main advantage of the open field test is its simplicity; it is not an operant task, so no special training is required. In addition, it will always yield results of some sort, depending on your outcome measures - although what those measures really mean can be subject to discussion (Stanford, 2007b; Stanford, 2007a; Ramos, 2008; Walsh and Cummins, 1976).

### 3.1.3.1.4 Novelty-induced hypophagia test

The inhibition of feeding produced by novelty, termed 'hyponeophagia', can be used to design studies that yield anxiety-related measures, and has been used in research to investigate the effects of chronic antidepressant treatment in both mice and rats (see Dulawa and Hen, 2005).

Hall (1934) first described an inverse relationship between feeding and defecation in animals exposed to a novel environment, and this fact has been exploited in the development of hyponeophagia paradigms, resulting in conflict tasks in which an animal has to decide between approaching and consuming a desirable food in a novel arena, or avoiding the novel environment for safety. Assessing hyponeophagia typically involves presenting rodents with food in a novel, and therefore anxiogenic, environment such as an open field or unfamiliar cage, and measuring the latency to feed and the amount consumed.

The food will either be familiar chow presented to food-deprived and therefore hungry animals (Shephard and Broadhurst, 1982a; 1982b),or will be a highly palatable food presented to satiated animals (Dulawa et al., 2004). The only training needed for this task is to familiarise the animals with the highly palatable food before testing.

Experiments using the NIH task have used an open field (Bilkei-Gorzo et al., 2002; Bodnoff et al., 1988; Britton and Britton, 1981) or a novel cage (Dulawa et al., 2004) as the novel environment. Open field NIH paradigms expand the behaviours that can be observed beyond measures of amount eaten and latency to eat, as other anxiety-like behaviours such as defecation and urination, and exploratory behaviours such as locomotion and rearing, can be scored and analysed.

The use of food as a reward makes these paradigms ethologically relevant, in that it can study animal behaviour in a situation which would be encountered in a natural habitat. As it is a non-operant task, little or no training is required.

## 3.1.3.2 Impulsivity as a reward-related behavioural measure

Impulsivity refers to the inability of an organism to suppress inappropriate responses. Definitions broadly include a lack of behavioural inhibition, including actions that are premature, mis-timed, or difficult to suppress or control (see Dalley et al., 2008). Impulsive behaviour is a type of reward behaviour - evidence from the addiction literature cites impulsivity as a risk factor in addiction and relapse (Perry and Carroll, 2008), and the neural substrates linked to impulsivity are closely linked to the mesolimbic dopaminergic system, with the fronto-striatal DA circuit suggested as a possible locus of dysfunction in pathological impulsivity (see Dalley et al., 2008). Clinically, impulsivity is also related to attention deficit disorders and schizophrenia (see Robbins, 2002).

Impulsivity has been divided into two major subtypes: impulsive choice (choice of a small, immediate reinforcer over a larger, delayed reinforcer) and inhibitory failure (inability to stop a particular behaviour). Delay discounting tasks are used as measures of impulsive choice, and the 5-choice serial reaction time (5CSRT) task is used to measure inhibitory failure. Both are operant tasks that require intensive training.

### 3.1.3.2.1 Delay discounting task

Performed in both animals and humans, in animal experimental paradigms, animals are trained to make a lever press/nose-poke to obtain a reward i.e. drug, food etc. One lever will always deliver a small, immediate reward, whereas another will always deliver a larger reward after a delay. The point at which each lever is chosen equally is called the indifference point. As the delay becomes longer, animals that display higher impulsivity will show a lower indifference point i.e. they will press the lever for the smaller, immediate reward more often than they will press to wait for the larger reward.

### 3.1.3.2.2 5-choice serial reaction time task (5CSRTT)

This complex paradigm measures inhibitory failure. The apparatus consists of a chamber with 5 horizontally-aligned apertures, above one of which a brief visual

stimulus, i.e. a light, will appear, and the animal can nose-poke to receive a reward. In the task, a large number of consecutive trials are presented and the subjects are required to wait during either a fixed or varied inter-trial interval (ITI). Nose-pokes that occur prematurely are thought to reflect higher levels of impulsivity (Robbins, 2002; Robinson et al., 2009).

Other tasks that measure inhibitory failure include the go/no-go task (subjects must respond, or withhold a response, according to the particular stimulus presented), and the stop signal reaction time (SSRT) task (where subjects must withhold a response already initiated).

### 3.1.3.3 Summary of anxiety and impulsivity tests

One advantage of the anxiety paradigms introduced above is that they do not require complex training schedules, unlike the tests for impulsivity. However, in the anxiety paradigms, outcome measures in one paradigm may not tally with results from another. For example, a meta-analysis of results from studies employing the EPM and open field find that there is no single anxiety related factor in rats (Ramos, 2008). Outcome measures can also be affected by other variables, i.e. time of day that testing is done, gender and individual differences (Broadhurst, 1958).

Impulsivity tasks are sensitive to subtle reward behaviours and demand high levels of cognitive attention, but they are complex and require long training periods, making them labour-intensive and time-consuming.

### 3.1.4  Summary of reward behaviour tests

All of the research presented above shows the wide variety of reward behaviour measurement. However, a large amount of studies have used direct drug administration to study drug addiction. Whilst the mechanisms of addiction in animals are relevant to the investigation of reward behaviours, it was decided that simpler general approach behaviours towards an ethologically valid reward are a more appropriate way to begin investigating the long-term cognitive behavioural effects of repeated early pain, which is the central aim of this thesis.

A hyponeophagia task was chosen as the paradigm for studying reward-related behaviours – a novelty-induced hypophagia (NIH) paradigm. It uses an ethologically relevant reward, food, and requires no further training, other than familiarising test animals with the food prior to testing. Furthermore, a variety of behaviours can be scored on an NIH task using an open field. This means that not only the response to reward can be studied, but also exploratory behaviours such as central zone entries and rearing, and classic 'emotionality' measures such as defecation and urination.

When designing the experiment, it was noted that previous experiments have used albino rat strains. Hooded Lister rats were chosen for these experiments, as they have been widely used in research investigating cognitive behaviour, for example fear-conditioned analgesia (Butler et al., 2008) and addiction research (Tessari et al., 2007).

A square open field arena has been used in previous tests (Britton and Britton, 1981; Bilkei-Gorzo et al., 2002). However, it was decided that the open field arena used in these experiments would be circular. The reason for this is that a square open field provides corners which an animal will naturally prefer to spend time in, as there are two walls which can shelter an animal. Making a circular open field removes corners, and so would increase the amount of exploration that an animal would be likely to engage in. To enhance the aversive nature of the environment further, it was decided that the arena would be bright white, and that testing would occur under full illumination (~1000 lux).

In line with much previous research showing that lab animals will work for sweet food rewards, and on the recommendation of staff in the Biological Services Unit at UCL, a sweet and palatable food treat was chosen that is easily quantifiable, cheap, and safe for animal consumption – Cheerios breakfast cereal (Nestlé). Cheerios are comprised of 79.2% carbohydrates (of which 40% is sugars), 3.6% fat, 5.8% fibre and 6.6% protein (www.cerealpartners.co.uk/brands/cheerios-honeynut.aspx) – a much higher sugar level than standard lab chow.

## 3.2  Hypotheses

The hypotheses for the experiments of this chapter state that:
- Repeated early injury will alter adult nociceptive sensory thresholds

- Repeated early injury will alter adult reward-related behaviour in the NIH task

- A single adult injury to a previously uninjured animal will alter reward-related behaviour in the NIH task

- A single adult re-injury to a previously injured animal will alter reward-related behaviour in the NIH task

- The outcome measures being investigated are a reflection of reward-related behaviours, exploration and emotionality.

## 3.3 Methods

To investigate the long-term effects of early-pain on later reward-related processing, animals were repeatedly injured as neonates, and the long-term sensory and cognitive effects investigated.

### 3.3.1 Experimental design

- All experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

- Litters of Hooded Lister rats were bred on-site from five breeding dams and two stud rats (Charles River Laboratories, Kent, UK). Hooded Lister animals were chosen as experimental subjects as they are widely used in cognitive tasks in the literature, have good eyesight in comparison to albino rat strains such as Sprague-Dawleys, and are highly inquisitive and likely to respond to environmental stimuli. Litters ranged in number of pups from 6 to 16.

- Animals were maintained on a 12 hour light/dark cycle and had access to food and water *ad libitum*.

- Males and females were used in all experiments.

Four main sets of experiments were carried out:

- The effects of repeated neonatal foot wound on nociceptive sensory processing in the adult were investigated (Figure 3-7)

- Next, the effect of repeated neonatal foot wound on reward-related processing in the adult, using a novelty-induced hypophagia paradigm, was investigated (Figure 3-8).

- The set of studies investigated how reward-related processing is affected by a single adult foot wound in animals either without (experiment 3) or with (experiment 4) a history of neonatal foot wound (Figure 3-9 and Figure 3-10 )

- The influence of Cheerios, the rewarding stimulus, was investigated by removing the Cheerios, or moving them to an alternative place within the arena, in animals with and without a history of foot wound after an adult re-injury. These were control sub-experiments in experiments 3 and 4.
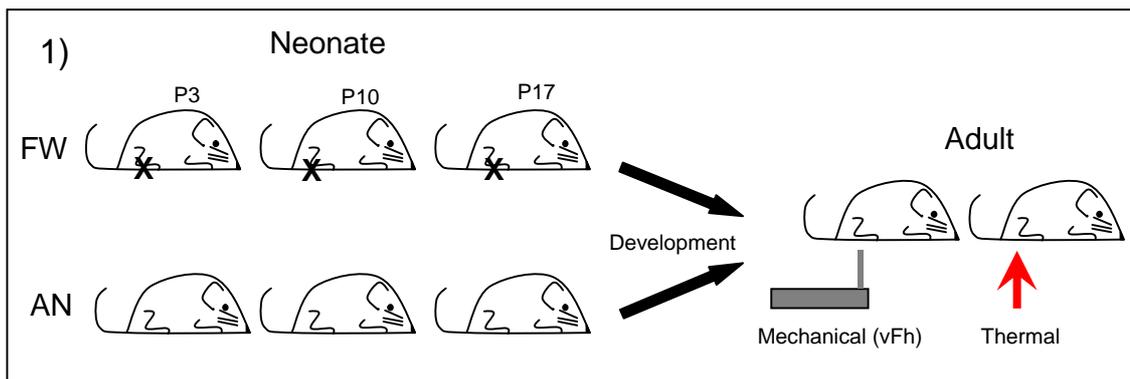


**Figure 3-7 – A schematic diagram to show experiment number 1 – effects of repeated neonatal foot wound on adult sensory nociceptive processing. FW = foot wound, AN = anaesthesia only.**
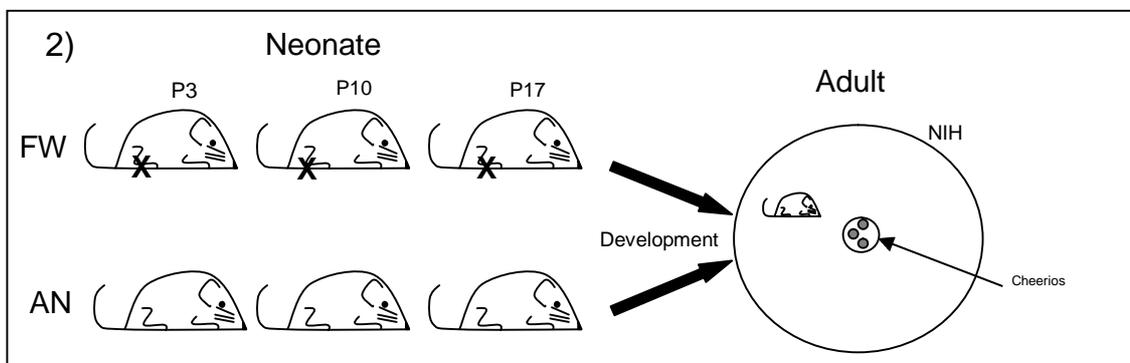


**Figure 3-8 – A schematic diagram to show experiment number 2 – the behavioural effects of repeated neonatal foot wound on adult reward-related processing in the novelty-induced hypophagia (NIH) task**
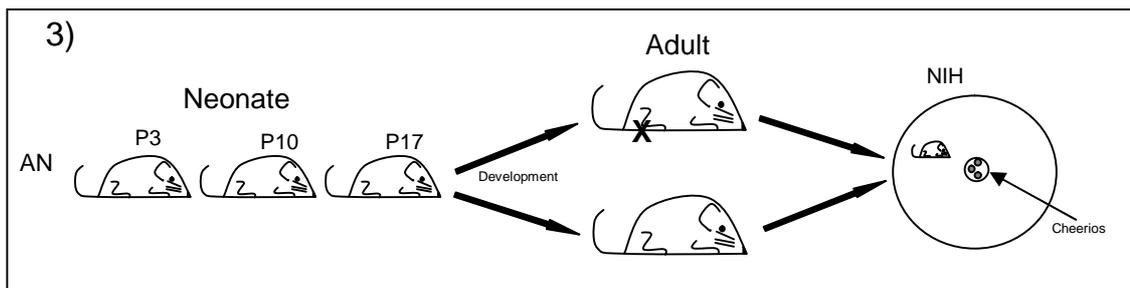
**Figure 3-9 – A schematic diagram to show experiment number 3 – the behavioural effects of a single adult foot wound in animals with no history of neonatal surgery.**
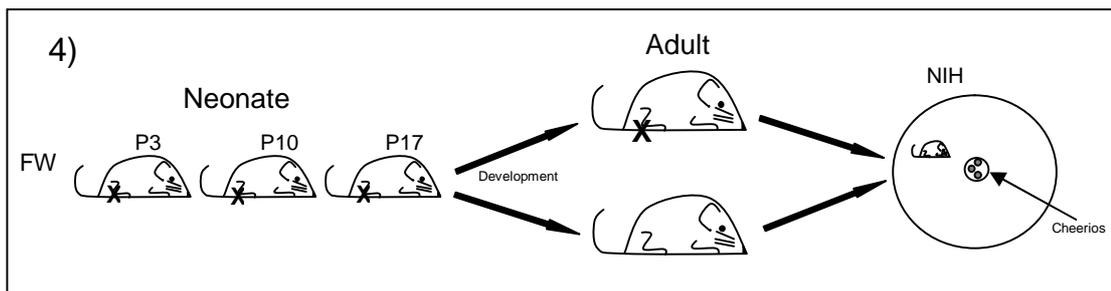


**Figure 3-10 – A schematic diagram to show experiment number 4 – the behavioural effects of a single foot wound in animals with a history of neonatal surgery**
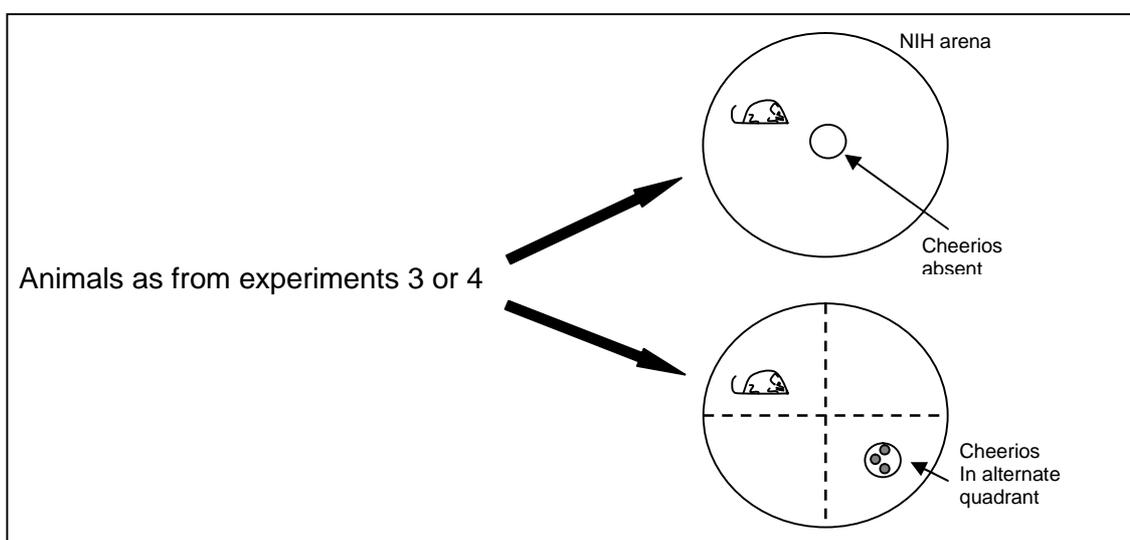


**Figure 3-11 – A schematic figure to show the sub-experiments of experiments 3 and 4 to investigate the influence of Cheerios, where they were either absent from the central zone (upper circle), or were moved to an alternate quadrant (lower circle).**

## 3.3.2 Foot wound surgery – neonatal

All animals (except controls) received plantar skin incision (referred to as foot wound) to the left (ipsilateral) hindpaw, at P3, P10 and P17. These ages were chosen for multiple reasons. Firstly, they span important periods of development of the animals (Walker et al., 2009b). Secondly, these ages cover a broad period of development that is likened to the gestational period in human neonates from 26 weeks up to adolescence (see Fitzgerald, 2005). These are ages at which is has been shown that injury affects later sensory processing in both humans (Walker et al., 2009a) and animals (Anand et al., 1999). Finally, these are also the time points at which the previous chapter has shown that dopaminergic signalling is active. This is important in the context of this

thesis in the hypothesis that early injury can interfere with reward-processing, due to dopaminergic signalling alterations.

The plantar skin incision model of post-operative pain (Brennan et al., 1996) was used in these experiments. It was chosen to mimic the clinical situation where premature and full-term neonates in the neonatal intensive care unit (NICU) receive multiple heel pricks per day. Its advantages are that it is well characterised (Brennan et al., 2005; Hamalainen et al., 2009) and easily reproducible at all postnatal ages.

- The entire litter (n=4-15) was separated from the dam. This ensured that all pups experienced the same amount of maternal deprivation and handling, both of which can confound nociceptive and cognitive processing in older animals (Walker et al., 2003a; Walker et al., 2008; de Medeiros et al., 2009; Champagne et al., 2003). Pups were placed on a heating pad for the duration of all surgeries and recovery periods.
- Half the litter were to receive foot wound, and half were control animals, to receive anaesthesia only. Pups were randomly assigned at P3 to each group.
- Anaesthesia was induced with 2-5% isoflurane in 2L/min oxygen, and maintained at 2% isoflurane for the 5-10 minutes of surgical duration. Anaesthesia-only control animals received the same duration of anaesthesia as surgically treated animals.
- The left hindpaw was cleaned pre-surgery with iodine solution using a cotton-bud. Control animals' paws were cleaned in the same manner.
- An incision was made in the paw using an 11 gauge scalpel blade, from the midpoint of the heel to the proximal border of the first footpad, at all age points (Figure 3-12).



**Figure 3-12 – A representative picture of the foot wound surgery in a P17 animal. The incision was made from the midpoint of the heel to the proximal border of the first footpad, and two stitches sutured the incision.**

- The underlying muscle and fascia were lifted and separated from surrounding tissue using bent-tip needle-toothed forceps.

- Tissue was sutured with two stitches using 5-0 Mersilk (Ethicon, Edinburgh, UK).

- The wound was disinfected with a cotton bud soaked in iodine to prevent infection. Control animals' paws were cleaned in the same manner

- Pups were returned to the holding and recovery container and recovery from anaesthesia monitored.

- Each pup was photographed after surgery/anaesthesia. As each animal has a specific and individual pattern of markings that is maintained throughout life (see example photos in Figure 3-13), the treatment groups that animals were assigned to were only revealed by comparisons between photographs (taken after each surgery) once all treatments, testing and analysis were finalised. This blinded the experimenter to each pup's treatment and removed the need to mark pups between surgeries, as well as blinding the experimenter to prior treatment of adult animals, meaning that experimenter bias was eliminated during analysis.

- After recovery of all pups, the entire litter was returned to the home cage. At P3 and P10, all animals were rolled in sawdust litter from the cage to prevent the dam rejecting the pups when returned.

- After the final surgery at P17, animals were weaned at P21-23 and housed 2-3 per cage, separated for gender, until behavioural testing was performed at adulthood (>P90). The animals were undisturbed during this period except for routine cleaning of cages.
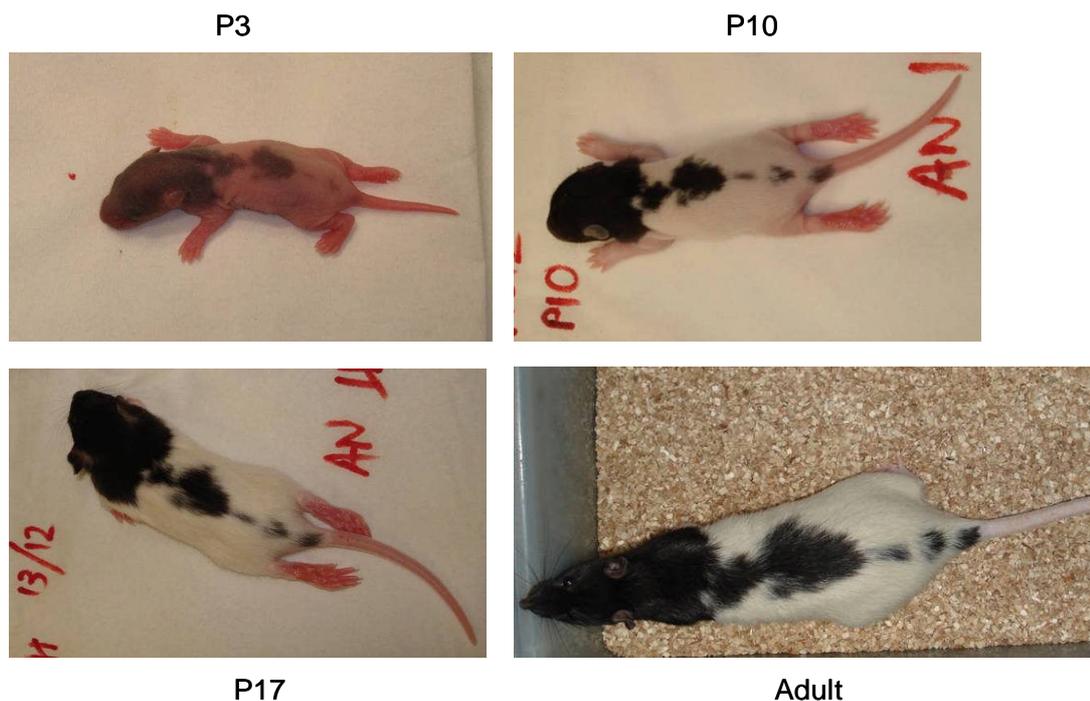
**Figure 3-13 – Example photographs to show that the markings on a P3 rat, at P10, P17 and in adulthood. The markings remain the same throughout the animal's life.**

### 3.3.3 Foot wound surgery - adult

- In experiments where animals were injured in adulthood, the following protocol was employed.

- Animals were removed from their home-cages and kept in a holding box with clean sawdust together with their cage-mates.

- Animals were removed one at a time, anaesthetised and plantar skin incision performed as above.

- For recovery, anaesthetised animals were placed in a separate box from pre-surgery cage-mates to prevent awake animals from interfering with recovering animals.

- Animals were returned to their home-cage after all cage-mates had recovered from surgery.

- Behavioural testing in the NIH paradigm was performed 2 days post-surgery.

### 3.3.4 Sensory testing in adulthood

Behavioural sensory testing was performed at 1 and 2 days prior to surgery to calculate baseline responses, and 24 hours, 48 hours and 5 days post-surgery.

### 3.3.4.1 Mechanical withdrawal thresholds

To assess the mechanical force that generated a nocifensive reflex withdrawal in experimental animals, von Frey hairs (vFh) were used. These are fibres of differing gauge that bend according to the force applied; the maximal force is generated at the point at which the hair bends. The increase in force between hairs is logarithmic, rather than linear. Table 3-1 illustrates the relationship between hair number and force (in grams) needed to cause the hair to bend:

| Hair | Force (g) |
|------|-----------|
| 10 | 1.95 |
| 11 | 3.2 |
| 12 | 5.9 |
| 13 | 10.3 |
| 14 | 16.3 |
| 15 | 24.8 |
| 16 | 41.7 |
| 17 | 65.3 |
| 18 | 102.4 |
| 19 | 156.8 |
| 20 | 220 |

**Table 3-1 – a table to show the relationship between von Frey hair number and stimulus intensity**

- Animals were placed into clear Plexiglas boxes elevated on a wire mesh. Before testing, animals were placed into the testing apparatus for one hour on the 3 days prior to testing, to habituate to the apparatus. On test days, animals were placed into the apparatus and left to habituate for 20 minutes prior to testing.
- Von Frey hairs were applied to the point of hair bending to the plantar surface of the left (ipsilateral) hindpaw, immediately proximal to the incision.
- 6 applications of hair were performed, with an inter-stimulus interval of at least 10 seconds.
- Testing began with hair number 10 and increased until threshold was reached.
- The withdrawal threshold was determined as the force at which the animal performed a reflex withdrawal to 3 out of the 6 stimuli.
- The right (contralateral) paw was then tested in the same manner after an interval of at least 5 minutes.

- After testing, animals were returned to their home cages and the apparatus thoroughly cleaned with 5% Trigene (MediChem International, Kent, UK) and 70% ethanol, then thoroughly dried, in order to remove any odours.

### 3.3.4.2 Thermal withdrawal thresholds

To assess the thermal sensitivity of the animals, a protocol adapted from the original described by Hargreaves et al (1988) is used.

- Animals were placed into clear Plexiglas boxes placed on a clear glass sheet. Before testing, animals were placed into the testing apparatus for one hour on the 3 days prior to testing, to habituate to the apparatus. On test days, animals were placed into the apparatus and left to habituate for 20 minutes prior to testing.

- The thermal withdrawal threshold testing apparatus (Plantar Test (Hargreaves' Apparatus) Ugo Basile, Italy) was placed under the glass sheet, enabling access to the plantar surface of the hindpaw without disturbance to the animal.

- A radiant beam of infra-red light was directed at the plantar surface of the hindpaw, immediately proximal to the incision, and latency to withdrawal reflex measured (in seconds). The latency was automatically recorded as the beam broke. To prevent tissue damage, the test was terminated after 20 seconds if no withdrawal had occurred.

- Three repeats were performed on each paw, with an inter-stimulus interval of at least 2 minutes, and the mean average withdrawal latency calculated.

- After testing, animals were returned to their home cages and the apparatus thoroughly cleaned with 5% Trigene and 70% ethanol to remove any dirt and odours, and then thoroughly dried.

### 3.3.5 Novelty-induced hyponeophagia (NIH) paradigm

- Hyponeophagia refers to the inhibition of feeding produced by exposure to novelty, and assessing it is usually done by presenting rodents with food in a novel and thus anxiogenic environment, i.e. an open field, and measuring the latency to eat and amount eaten. Animals are either food-deprived prior to testing, or are trained to consume highly palatable foods which are presented during testing (see Dulawa and Hen, 2005). In these experiments, animals were

not food-deprived at any stage, eliminating the potential confound on behaviour of hunger, and reducing ethical concerns.

- To investigate reward-related behaviours, a paradigm was developed based on hyponeophagia principles and involving aspects of classic conflict tasks, where the natural exploratory drive of an animal is in conflict with its fear of unfamiliar and aversive environments. In this paradigm, the animal had to overcome a novel, aversive environment to receive a palatable food reward that the animal was familiar with: Cheerios breakfast cereal (Nestlé).

- To habituate animals to the palatable foodstuff, all animals received a few Cheerios, placed onto the surface of their home-cage, for 3 days prior to testing. All animals were checked to confirm seeking and appetitive behaviour towards the treats in their home cage before testing in the NIH paradigm.

- All animals were tested between 10am and 12pm. Whilst this is in the sleep phase of nocturnal rodents' diurnal rhythms, all testing was done between these times to minimize confounds due to sleep/awake state.

- All testing was done in minimal noise conditions, as noise levels have been shown to affect results in open field paradigms (see Walsh and Cummins, 1976).

- On the day of testing, animals were removed singly from their home cage and placed into a holding box with clean sawdust.

- The holding box was taken into the testing room and the animal immediately placed into the testing arena. The experimenter then left the room immediately, as presence of an experimenter has been shown to alter open field behaviours (see Walsh and Cummins, 1976).

- The arena consisted of a circular, bright (>1000 lux) white open field 1.25m in diameter (see Figure 3-14), with a wall height of 50cm to prevent escape.

- A central zone was defined as an area with a 15cm radius around the central point of the arena (see Figure 3-14).

- A glass bowl was placed in the centre of the arena, containing 20 Cheerio food treats.

- Animals were placed into the arena equidistant from the food bowl and the edge of the arena and behaviour recorded using a webcam (Logitech Quickcam, Logitech ® Inc.) suspended above the arena for a period of 15 minutes.

- After the 15 minute test period, the experimenter returned to the room and animals were removed from the arena and placed back into the holding box, where a photograph was taken of their markings for blinding purposes. The holding box was returned to the room containing the home cages.

- Tested animals were retained in the holding box whilst the arena was cleaned in preparation for the next animal, and were kept separate from cage-mates awaiting testing, to prevent the tested animal communicating any stress to the as-yet untested animal, for example via ultra-sonic vocalisation (Litvin et al., 2007).

- Once both/all cage-mates had been tested, all animals were returned to their home-cages. The sawdust in the holding box was changed after each cage of animals had been tested.

- In between testing sessions, the arena was scrubbed and disinfected with 5% Trigene and 70% ethanol in preparation for the following animal. This was to remove any olfactory traces of the previous animal. Any remaining Cheerios were disposed of and the glass food bowl washed with hot water and disinfected with 70% ethanol.

- Two hours after testing, animals were sacrificed and the animals' brains were utilised for the experiments in the next chapter.
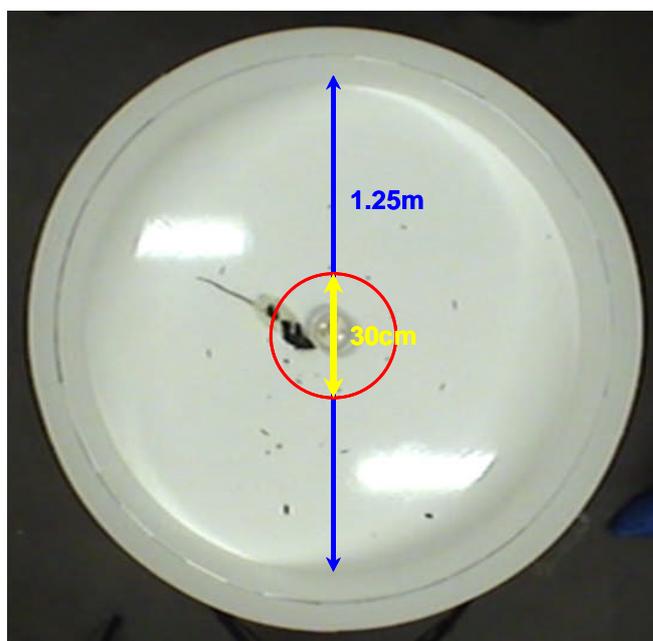


**Figure 3-14 – An aerial photograph taken from above the modified novelty-induced hypophagia (NIH) task. The arena is 1.25m in diameter, and the central zone was defined as a circle of 30cm diameter, placed directly in the centre of the arena.**

### 3.3.5.1 Quantification

Behavioural videos were manually scored by a blinded experimenter on a variety of outcome measures. These were separated for analysis purposes into anxiety-like behaviours, exploratory behaviours, and appetitive behaviours.

#### *3.3.5.1.1 Anxiety-like behaviours*

- Number of faecal boli
- Number of urine puddles (>5mm diameter)

#### *3.3.5.1.2 Exploratory behaviours*

- Number of rears
- Number of entries into the central zone (CZ)
- Percentage of time spent in the central zone
- These outcome measures were time-binned into 5 minute periods and also counted as total measures over the entire 15 minute recording period.

#### *3.3.5.1.3 Reward-seeking behaviour*

- Time taken to eat first Cheerio
- Number of Cheerios eaten (time-binned and total number)

### 3.3.6 Analysis

### 3.3.6.1 Sensory testing

- Percentage change from baseline after surgery was calculated. This was to control for individual differences in variable baseline thresholds.
- Analysis of sensory testing thresholds was performed using Prism Version 4 software (GraphPad, San Diego, USA). Data was compared by 2-way repeated measures ANOVA with day and treatment as variables and Bonferroni post-tests
- Graphs were prepared in Prism 4.

### 3.3.6.2 Hyponeophagia testing

- Analysis was performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, USA). Multiple comparisons between groups and outcome measures

were performed by multivariate analysis of variance (MANOVA), with treatment group as the Between-subjects factor and outcome measures as the Fixed factors. Bonferroni and Tukey's HSD post-hoc tests were performed to find which way any differences between groups and treatments lay.

- Analysis of time-binned data was performed by 2-way Repeated measures ANOVA with Tukey's Multiple Comparisons Test.

- Graphs were prepared in Prism 4.

- For both sensory and behavioural testing, results were considered statistically significant if $p < 0.05$.

## *3.4 Results*

The results presented in this chapter cover both sensory and cognitive modalities and investigate the long-term effects of a repeated early injury on adult reward-related behaviours. To begin, the results from the sensory testing in adulthood both pre- and post-injury are presented, followed by the results from behavioural testing in the novelty-induced hypophagia (NIH) paradigm.

### 3.4.1 Sensory testing

Both mechanical and thermal thresholds of injured and control animals were measured in adulthood (postnatal day (P)>90). This was to see what effect a repeated neonatal foot wound (see Methods) had on sensory processing in the adult animal at baseline, and after a single foot wound injury or re-injury in the adult animal. A reminder of the experimental design is shown in Figure 3-15:
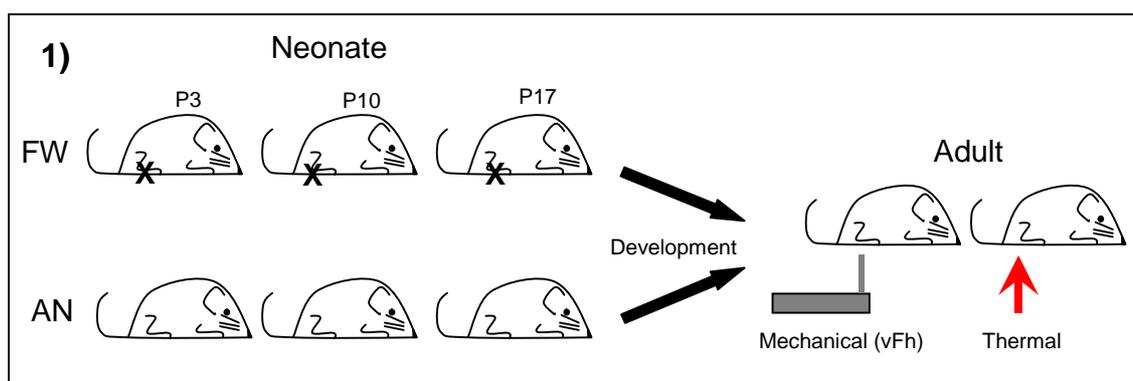


**Figure 3-15 – A schematic diagram representing the experimental design of the sensory testing experiments to measure mechanical and thermal withdrawal thresholds in neonatally injured or anaesthetised animals, both at baseline and after adult surgery. FW = foot wound, AN = anaesthesia only.**

Mechanical and thermal withdrawal thresholds were measured at 1, 2 and 5 days post-surgery in adulthood. Results after adult re-injury are presented below at the 2-day time point post-surgery. This is because this is the time-point of maximal change from baseline and so highlights treatment differences clearly. For full data for each time point after surgery, see Appendix 2.

The ipsilateral (wounded foot) and contralateral (non-wounded) hindpaw were tested (see Methods for experimental design and timeline). Genders were analysed separately at baseline and pooled after adult re-injury. The treatment groups are detailed in Table 3-2:

| Treatment group | Number per group |
|---|---|
| Male foot-wounded (FW) | 3 |
| Male anaesthesia control (AN) | 3 |
| Female foot-wounded (FW) | 3 |
| Female anaesthesia control (AN) | 3 |

**Table 3-2 – A table to show the treatment groups and number of animals per group for the sensory testing experiments**

### 3.4.1.1 Mechanical withdrawal thresholds

#### *3.4.1.1.1 At baseline, male mechanical withdrawal thresholds do not differ between injured and uninjured paws, or wounded and control animals*

There are no significant differences (2-way ANOVA) in mechanical withdrawal threshold between ipsilateral and contralateral foot, nor between treatment group (foot wounded and anaesthesia), in adult males at baseline, as shown in Graph 3-1:
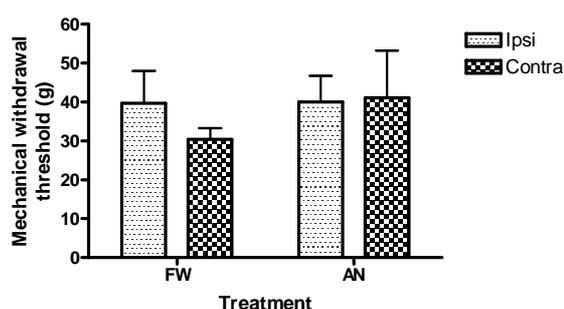


**Graph 3-1 - A bar graph to show the baseline mechanical withdrawal thresholds in male foot wounded (FW) or anaesthesia control (AN) animals in the ipsilateral and contralateral paws. Thresholds were measured in adulthood after repeated neonatal injury or anaesthesia.**

### *3.4.1.1.2 At baseline, female mechanical withdrawal thresholds are higher in neonatally injured animals.*

At baseline, the previously injured females (FW) show a significantly increased mechanical withdrawal threshold compared to the uninjured animals (AN), i.e. are less sensitive to punctate mechanical stimuli (Treatment: $F_{(1,8)}=$ 6.544, p<0.05; 2-way ANOVA with Bonferroni's post-tests).
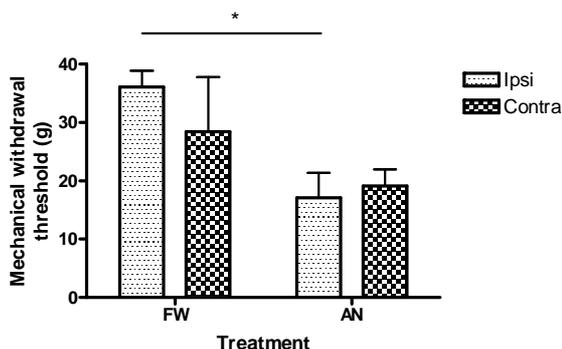


**Graph 3-2 – A bar graph to show the baseline mechanical withdrawal thresholds in female foot wounded (FW) or anaesthesia control (AN) animals in the ipsilateral and contralateral paws. Thresholds were measured in adulthood after repeated neonatal injury. *p<0.05; 2-way ANOVA with Bonferroni's post-tests.**

### *3.4.1.1.3 Mechanical withdrawal thresholds are lower in the ipsilateral paw 2 days post-injury*

After the adult animals are exposed to a single foot wound, mechanical withdrawal thresholds are significantly lower in the ipsilateral paw of neonatally injured (FW) animals 2 days after injury ($F_{(1,10)}=12.44$, p<0.05; 2-way ANOVA with Bonferroni's post-tests). This decrease in baseline is not significant in the neonatally anaesthetized (AN) control group.
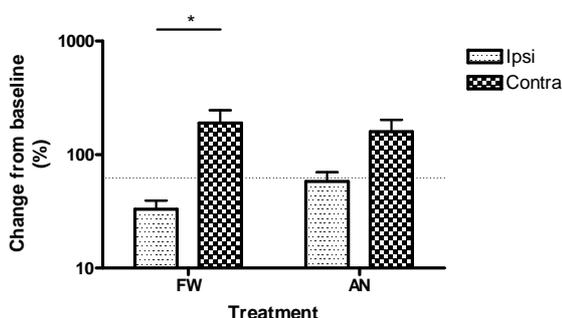


**Graph 3-3 - A bar graph to show the percentage change from baseline in mechanical withdrawal threshold 2 days post-injury in adulthood. The horizontal dashed line indicates baseline. Injured (ipsi) and uninjured (contra) paws are shown for previously injured (FW) and uninjured (AN) animals.  The Y axis is a logarithmic scale as the vFh increase in force (in grams) between each hair number is logarithmic. Percentage change from baseline was plotted to show changes in sensitivity more clearly.**

### 3.4.1.2 Thermal withdrawal thresholds

#### 3.4.1.2.1 Male baseline thermal withdrawal thresholds do not differ between paw or treatment

Graph 3-4 shows that there are no differences in thermal withdrawal threshold between ipsilateral and contralateral paws in either the injured or uninjured animals, and there are no differences between treatment group (FW and AN) either (2-way ANOVA).
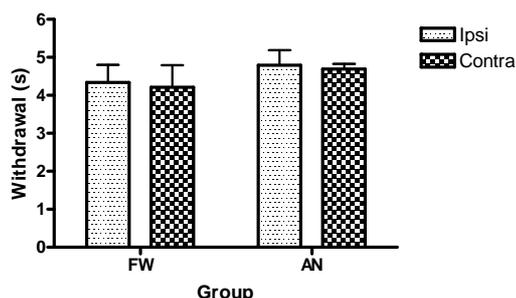


**Graph 3-4 - A bar graph to show the baseline thermal withdrawal thresholds for male injured and uninjured animals, in both ipsilateral and contralateral paws.**

#### 3.4.1.2.2 Female baseline thermal withdrawal thresholds are higher in the ipsilateral paw of the previously injured females

Graph 3-5 shows that the previously injured female rat shows a significantly higher basal thermal withdrawal threshold in the ipsilateral paw, compared to the contralateral paw and anaesthesia control animals (Paw $F_{(1,8)}=1.9$; Treatment $F_{(1.8)}=2.65$, $p<0.05$; 2-way ANOVA with Bonferroni's post-tests). This suggests that the female FW animal is hypoalgesic (i.e. less sensitive to thermal nociceptive stimuli) at baseline, in the injured paw only.
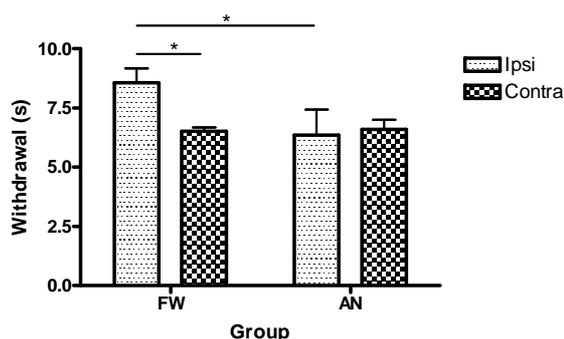


**Graph 3-5 - A bar graph to show the baseline thermal withdrawal thresholds for female injured and uninjured animals, in both ipsilateral and contralateral paws. \*p<0.05; 2-way ANOVA with Bonferroni's post-tests.**

#### 3.4.1.2.3 Thermal withdrawal thresholds are lower 2 days after single adult foot wound injury in neonatally injured animals

The ipsilateral paw of the previously injured animals shows a significant decrease in thermal withdrawal threshold 2 days post-injury (Paw: $F_{(1,10)}=28.59$, $p<0.001$; 2-way

ANOVA with Bonferroni's post-tests). This effect is not seen in the animals that were anaesthetised as neonates (AN).
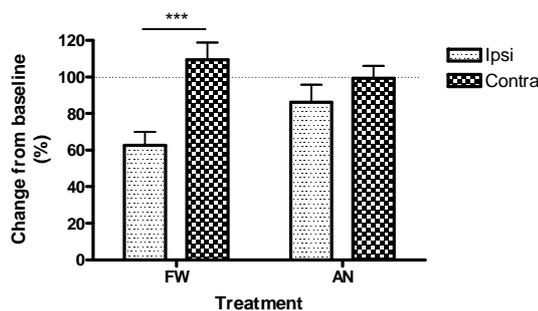


**Graph 3-6 – A bar chart to show the percentage change from baseline of thermal withdrawal thresholds 2 days after adult injury. The horizontal dashed line indicates baseline. (\*\*\*=p<0.001; 2-way ANOVA with Bonferroni's post-tests).**

### 3.4.1.3 Summary of sensory threshold testing

- The results presented above show that at baseline, male animals do not show any mechanical or thermal differences in withdrawal thresholds between ipsilateral and contralateral paws, regardless of whether they have a history of neonatal injury or not.

- Female animals that have been previously injured (FW) show a baseline hypoalgesia in the ipsilateral paw for both mechanical and thermal noxious stimuli.

- After a single adult injury, both mechanical and thermal withdrawal thresholds are lower in the ipsilateral paw 2 days post-surgery, in the neonatally injured animals only.

### 3.4.2 Repeated neonatal foot wound has little effect on adult reward-related behaviours

The results presented in this section will describe the effect of a repeated neonatal foot wound on adult baseline reward-related behaviours. A reminder of the experimental design is shown in Figure 3-16:
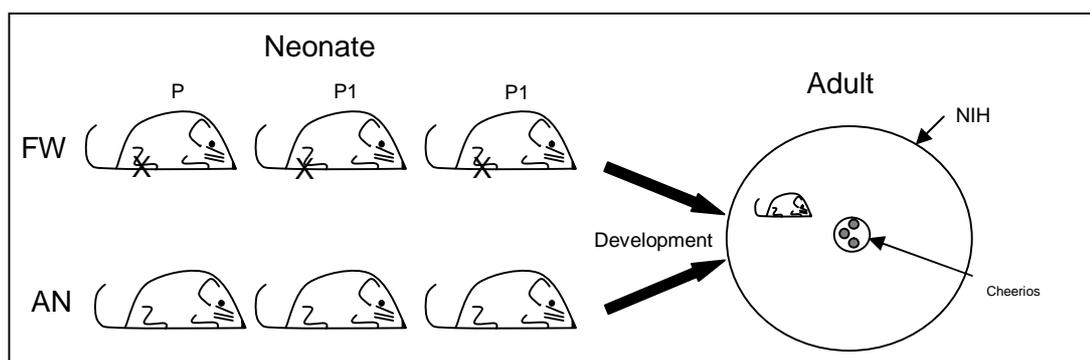
**Figure 3-16 – A schematic diagram showing the experimental design for the experiments investigating baseline adult reward behaviours in the NIH arena after repeated neonatal surgery.**

The behaviours were measured during a 15 minute testing period in the novelty-induced hypophagia (NIH) paradigm (see Methods). Outcome measures (i.e. number of rears) that can be separated into 5 minute time bins were separated, and are presented in time binned format in appendix 2 to show the variability of behaviours over the 15 minute testing period. The results from the 5-10 minute time bin only are shown in this section as many outcome measures show variable onset times; restricting the analysis to one time bin enables treatment effects to be shown more clearly.

Outcome measures have been classified into anxiety-related measures (faeces and urine), exploratory behaviours (rearing, central zone entries and time spent in central zone) and appetitive behaviours (time to first Cheerio and total number of Cheerios). Treatment groups and numbers of animals per group are described in Table 3-3 below:

| Group | Number of animals |
|---|---|
| Male foot wounded (M FW) | 19 |
| Male anaesthesia only (M AN) | 13 |
| Female foot wounded (F FW) | 19 |
| Female anaesthesia only (F AN) | 11 |

**Table 3-3 – a table to show the treatment groups and number of animals in each group.**

### 3.4.2.1 Anxiety-related measures are not affected by repeated neonatal surgery

#### 3.4.2.1.1 Faeces

Graph 3-7 illustrates the number of faecal boli produced by males and females over the 15 minute test period in the novelty-induced hypophagia test. There were no significant differences between treatment groups (FW versus AN)(2-way ANOVA). There was a significant effect of gender, showing that females produced fewer faecal boli ($F_{(1,58)}$=4.72, p<0.05; 2-way ANOVA).
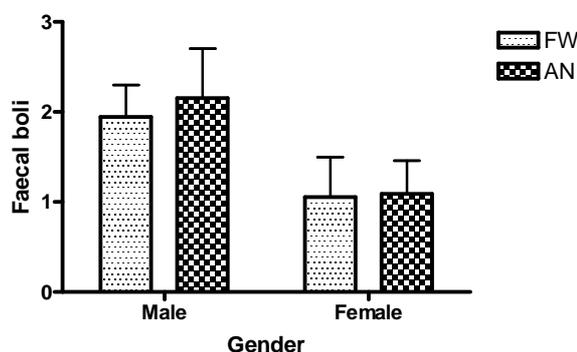


**Graph 3-7 – A bar graph to show the number of faecal boli produced by males and females when tested for 15 minutes in the novelty-induced hypophagia paradigm.**

#### 3.4.2.1.2 Urine

There were no differences in urination between gender or treatment group over the 15 minute test period in the NIH paradigm (2-way ANOVA).
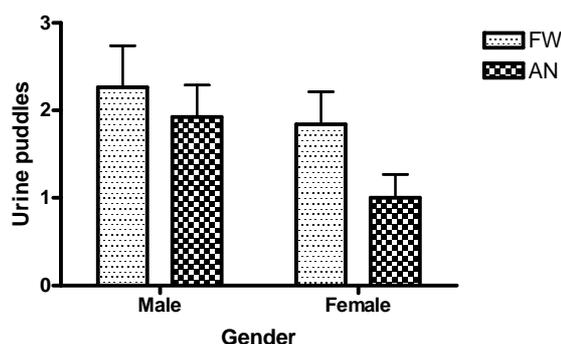


**Graph 3-8 – A bar graph to show the number of urine puddles (>5mm in diameter) produced by males and females when tested for 15 minutes in the novelty-induced hypophagia paradigm.**

### 3.4.2.2 Exploratory behaviours are not affected by repeated neonatal surgery

The data presented below is for the 5-10 minute time bin only. Data showing the results across the entire 15 minute testing period is presented in Appendix 2.

### 3.4.2.2.1 Number of rears

The number of male and female rears decreases over the testing period (see Appendix 2). Graph 3-9 shows the number of male and female rears in the NIH paradigm in the 5-10 minute time bin. There is no effect of treatment on the number of rears. There is a significant effect of gender on the number of rears, showing that females rear more than males (Gender $F_{(1,58)}$=12.05, p<0.001; 2-way ANOVA with Bonferroni post-tests).
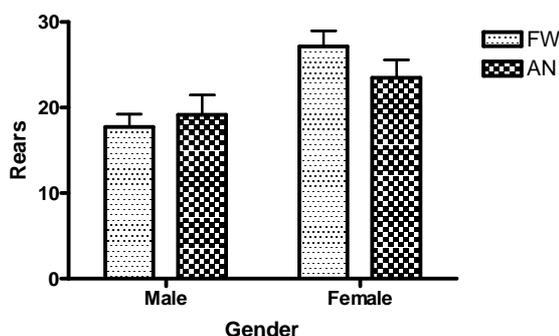


**Graph 3-9 – a bar graph to show the number of rears that both genders, foot wounded and anaesthesia-only, performed in the 5-10 minute time-bin.**

### 3.4.2.2.2 Central zone entries do not differ between treatment groups or genders

The number of entries that the anaesthesia group made into the central zone decreased over the 15 minute time period in both males and females (see Appendix 2). Graph 3-10 shows that there is no effect of treatment or gender on the number of central zone entries during the 5-10 minute time bin.
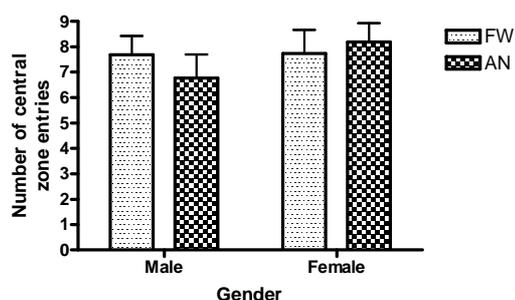


**Graph 3-10 – a bar graph to show the number of central zone entries that male and female foot wounded and anaesthesia-only animals performed during the 5-10 minute time bin.**

### 3.4.2.2.3 Time spent in central zone does not differ between treatment group or gender

The time that the animals spent in the central zone increased during the test period (see Appendix 2). There was no significant effect of treatment or gender in the 5-10 minute time bin (2-way ANOVA).
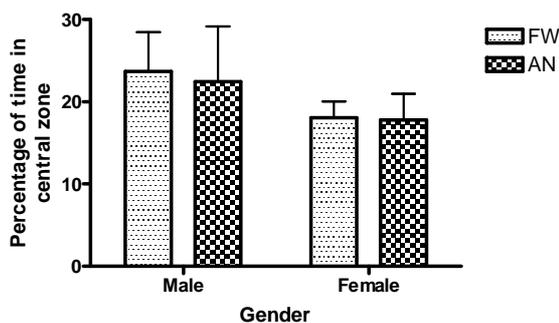
**Graph 3-11 – a bar graph to show the percentage of time spent in the central zone by both males and females in the 5-10 minute time bin.**

## 3.4.2.3 Appetitive behaviours in adulthood are altered by repeated neonatal foot wound

Animals that did not eat any Cheerios during the entire 15 minute testing period were excluded from this analysis. As this excluded a high number of animals, to see whether this had arisen due to chance or was an effect of treatment, Fisher's Exact Test was performed to compare the groups to an expected baseline of 100% eating behaviour. Table 3-4 shows that the M FW group, with a total of 7 animals out of 19 avoiding Cheerios, had significantly fewer animals that ate Cheerios than was expected ($p<0.008$; Fisher's Exact Test). Therefore at baseline, male injured animals are less likely to eat Cheerios in the NIH task.

| Treatment group | Number of non-eaters/total | P value from Fisher's Exact test |
|---|---|---|
| M FW | 7/19 | **0.008 **** |
| M AN | 3/13 | 0.22 |
| F FW | 3/19 | 0.12 |
| F AN | 1/11 | 0.5 |

**Table 3-4 – A table to show the number of non-eating animals in each treatment group and the P values of the Fisher's Exact test, comparing the number of non-eating animals to an expected eating baseline of 100%. **p<0.01.**

### 3.4.2.3.1 The time taken to eat the first Cheerio does not differ between treatment group or gender

Excluding the non-eaters, there are no significant differences between treatment group or gender in the amount of time taken to eat the first Cheerio (2-way ANOVA).
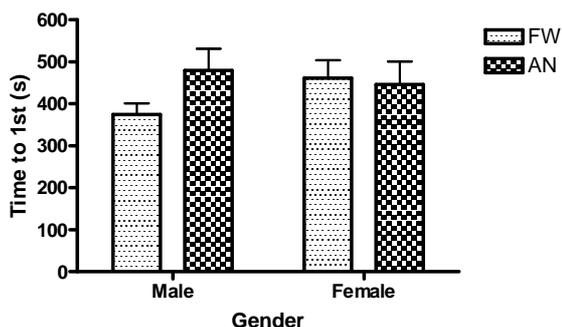
**Graph 3-12 – a bar graph to show the time taken to eat the first Cheerio in the male and female foot wounded and anaesthesia-only groups. Male FW n=12, M AN n=10, F FW n=16, F AN n=10.**

### 3.4.2.3.2 The number of Cheerios eaten

The number of Cheerios eaten in the 5-10 minute time bin did not differ between treatment groups. There was a significant effect of gender, showing that females ate fewer Cheerios during this time bin ($F_{(1,44)}$=6.7, p<0.05; 2-way ANOVA).
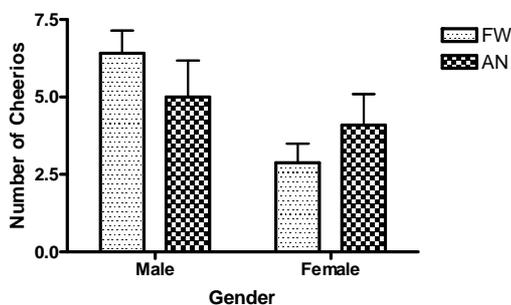


**Graph 3-13 – A bar graph to show the number of Cheerios eaten by males and females in the 5-10 minute time bin.**

## 3.4.2.4 Summary of the effect of a repeated neonatal injury on baseline adult reward-related behaviours.

- The results from the above section show only gender differences in reward-related behaviours at baseline in adulthood after an animal has had a repeated foot wound as a neonate.

- Anxiety-related behaviours, in this series of experiments defined as production of faeces and urine, show no differences between treatment groups, but show that females produce fewer faecal boli over the total testing period than males.

- Exploratory behaviours, defined as number of rears, entries into the central zone and time spent in the central zone, display no treatment group differences in the 5-10 minute time bin, but show that females rear more than males in the 5-10 minute time bin.

- Appetitive behaviours, in these experiments the time taken to eat the first Cheerio and the number of Cheerios eaten, show that a male with a history of foot wound is less likely to consume any Cheerios. Excluding non-eating

animals from the analysis shows that females eat fewer Cheerios in the 5-10 minute time bin.

### 3.4.3 The effect of a single acute foot wound in adulthood on reward related behaviours in animals with no prior history of injury

The results presented in this section will describe the effect that a single adult foot wound has on behaviour in the NIH of an animal with no prior history of surgery (anaesthesia-only animals from the above section). A reminder of the experimental design for this experiment is shown in Figure 3-17:
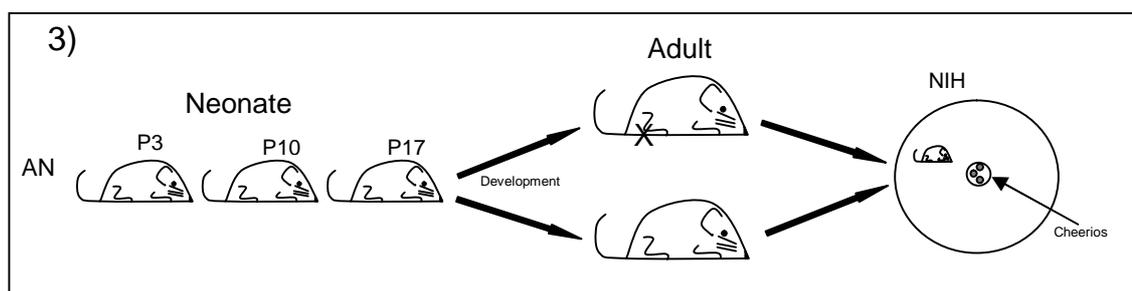


**Figure 3-17 – A schematic diagram to show the experimental design of the experiment to investigate the effect of a single adult foot wound on behaviour in the NIH arena of animals with no history of injury.**

As above, the behaviours were measured during a 15 minute testing period in the NIH paradigm; outcome measures have been classified into anxiety-related measures (faeces and urine), exploratory behaviours (rearing, central zone entries and time spent in central zone) and appetitive behaviours (time to first Cheerio and total number of Cheerios); and were time-binned accordingly. Testing was performed 2 days post-surgery. Data showing all time bins is presented in Appendix 2. The data presented below is from the 5-10 minute time bin only to show treatment effects more clearly. Treatment groups and numbers of animals per group are described in Table 3-5:

| Group | Number of animals |
|---|---|
| Male anaesthesia only (M AN) | 13 |
| Male anaesthesia plus adult wound(M AN+FW) | 14 |
| Female anaesthesia only (F AN) | 11 |
| Female anaesthesia plus adult wound (F AN+FW) | 13 |

**Table 3-5 – A table to show the treatment groups and number of animals in each group**

### 3.4.3.1 Anxiety-related measures are not affected by a single adult foot wound in animals with no history of injury

#### 3.4.3.1.1 Faecal bolus production is not affected by single adult foot wound

The results show that there are no significant differences in the production of faeces due to treatment in animals with no history of injury after a single adult foot wound. There is a significant effect of gender, showing that females produce fewer faecal boli ($F_{(1,47)}=5.64$, $p<0.05$; 2-way ANOVA). There is a trend that suggests that the number of faeces may increase in the adult injured animals, although this is not significant.
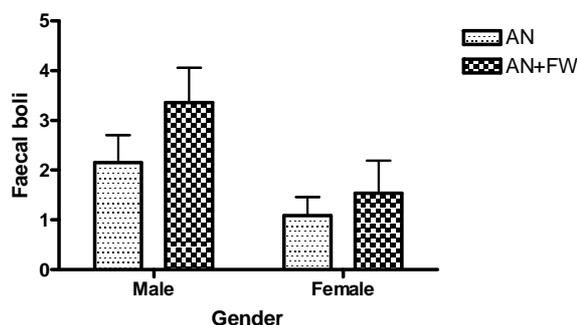


**Graph 3-14 – A bar graph to show the number of faecal boli produced in animals with no history of neonatal foot wound.**

#### 3.4.3.1.2 Urination is not affected by a single adult foot wound

There is no significant effect of treatment group on the number of urine puddles produced over the testing period of the NIH paradigm. There is a significant effect of gender, showing that females urinate less frequently ($F_{(1,47)}=12.68$, $p<0.001$; 2-way ANOVA).
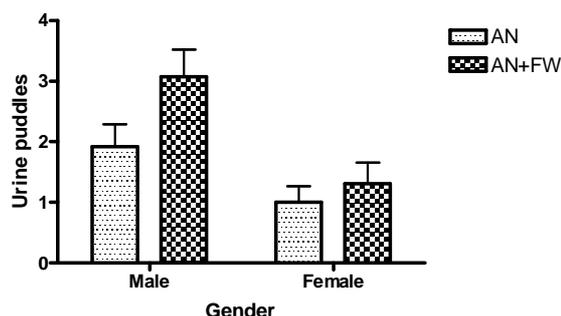


**Graph 3-15 – A bar graph to show the number of urine puddles (>5mm in diameter) produced during the NIH task in animals with no history of injury, after a single adult foot wound.**

### 3.4.3.2 Exploratory behaviours are affected by a single adult foot wound

#### 3.4.3.2.1 Rearing is decreased after a single adult foot wound in animals with no history of injury

Rears are decreased in both male and female adult-injured animals compared to controls (Treatment $F_{(1,47)}$=22.4, p<0.01; 2-way ANOVA with Bonferroni's post-tests). There was a significant effect of gender (Gender $F_{(1,47)}$=6.0, p<0.05; 2-way ANOVA). This shows that animals with no foot wound history, injured as adults, show less rearing exploratory behaviour.
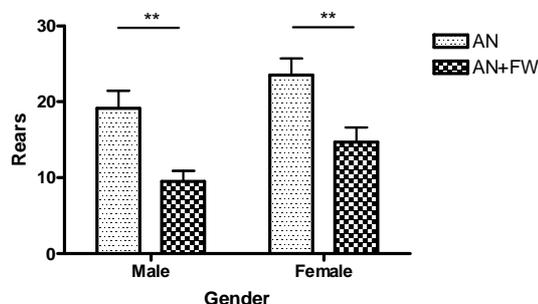


**Graph 3-16 – A bar graph to show rearing behaviour in the 5-10 minute time bin, in animals with a single adult foot wound and no history of prior injury. **p<0.05; 2-way ANOVA with Bonferroni's post-tests.**

### 3.4.3.2.2 Central zone entries are not affected by a single adult foot wound

There is no effect of treatment on the number of entries into the central zone during the 5-10 minute time bin in animals with no injury history. There is an effect of gender which shows that females enter the central zone more frequently than males ($F_{(1,47)}$=4.93, p<0.05; 2-way ANOVA).
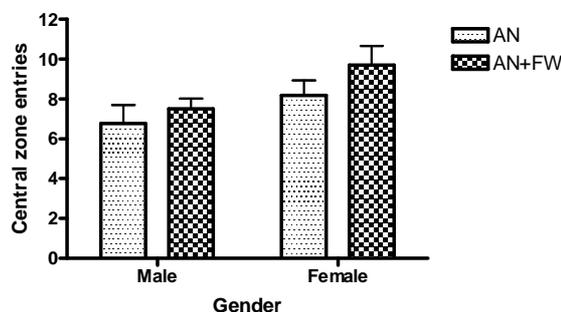


**Graph 3-17 – A bar graph to show central zone entries in the 5-10 minute time bin of the testing period, in animals with no injury history, after a single adult foot wound.**

### 3.4.3.2.3 Time spent in the central zone increases in animals injured as adults

Percentage of time spent in central zone is increased after a single adult foot wound in animals with no injury history. Graph 3-18 shows that the amount of time spent in the central zone is higher during the 5-10 minute time bin in both sexes of animals receiving a single adult foot wound, compared to those that do not (Treatment $F_{(1,47)}$=23.3, p<0.05 in males, p<0.001 in females; 2-way ANOVA with Bonferroni post-tests).
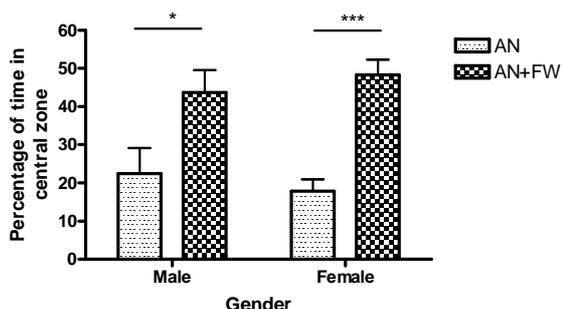
Graph 3-18 – A bar graph to show the amount of time spent in the central zone in the 5-10 minute time bin in animals with a single adult foot wound. *p<0.05; ***p<0.001; 2-way ANOVA with Bonferroni's post-tests.

## 3.4.3.3 Appetitive behaviours are not affected by a single adult foot wound in animals with no prior history of injury

As above, animals that did not eat any Cheerios during the entire 15 minute testing period were excluded from this analysis. Fisher's Exact test was performed to see whether the non-consumption of Cheerios was a result of treatment or not. A table to show the number of male and female non-eaters, and the Fisher's Exact p value, is shown below:

| Treatment group | Number of non-eaters/total | P value from Fisher's Exact test |
|---|---|---|
| M AN | 3/13 | 0.22 |
| M AN+FW | 2/14 | 0.48 |
| F AN | 1/11 | 0.5 |
| F AN+FW | 0/13 | n/a |

Table 3-6 – A table to show the number of non-eaters in each treatment group, and the P value from Fisher's Exact test comparing animals to an expected baseline of 100% consumption.

Table 3-6 shows that non-consumption by the animals was not due to an effect of treatment. Excluding the non-eating animals left the following numbers in each group:

| Treatment group | Number of animals (excluding non-eaters) |
|---|---|
| M AN | 10 |
| M AN+FW | 12 |
| F AN | 10 |
| F AN+FW | 13 |

Table 3-7 – A table to show the number of animals remaining in each treatment group once non-eaters had been excluded.

### 3.4.3.3.1 There is no difference between treatment groups in the time taken to eat the first Cheerio

There is no difference in the time taken to eat the first Cheerio between animals with a single adult injury and those without (2-way ANOVA).
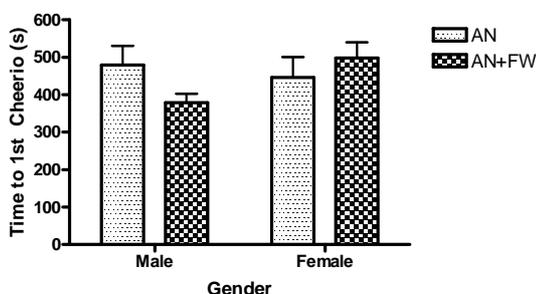


**Graph 3-19 – A bar graph to show the time taken for animals with no history of foot wound to eat the first Cheerio during the 15 minute testing period.**

### 3.4.3.3.2 Females eat fewer Cheerios than males in the 5-10 minute time bin

Eating behaviour does not begin until 5 minutes of testing, as shown in Graph 3-20. There were no significant treatment group differences, but a significant effect of gender, showing that females ate fewer Cheerios than males ($F_{(1,41)}$=6.26, $p<0.05$; 2-way ANOVA).
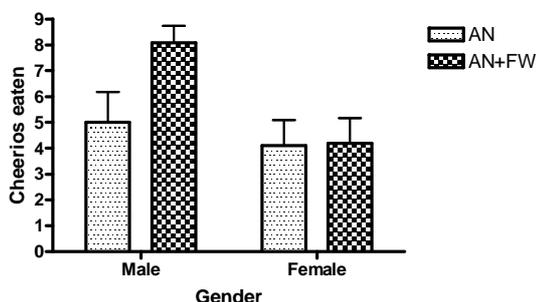


**Graph 3-20 - A bar graph to show the number of Cheerios eaten in the 5-10 minute time bin in animals with no prior history of foot wound, after a single adult foot wound.**

## 3.4.3.4 Removing the food reward alters exploratory behaviours in males and females with no history of injury

To investigate the effect that the presence of the food reward had in general on exploratory behaviours, the Cheerios were removed from the central bowl and separate litters of animals who received a single adult foot wound were tested. Table 3-8 shows the number of animals in each group.

| Treatment Group | Number of animals |
|---|---|
| M AN+FW Cheerios Present | 14 |
| M AN+FW Cheerios Absent | 5 |
| F AN+FW Cheerios Present | 13 |
| F AN+FW Cheerios Absent | 4 |

**Table 3-8 – A table to show the treatment group and numbers of animals per group in the presence/absence of Cheerios test.**

### 3.4.3.4.1 Anxiety measures are not significantly affected by the presence or absence of Cheerios

Neither male nor female anxiety measures, as indexed by number of faecal boli and urine puddles, are affected when Cheerios are removed from the NIH arena (2-way ANOVA).

### 3.4.3.4.2 The absence of Cheerios causes fewer entries into the central zone and decreases the amount of time spent there

Removing the Cheerios has the effect of decreasing the number of entries into the central zone (Graph 3-21) and decreasing the percentage of time spent there (Treatment $F_{(1,32)}=7.75$, $p<0.01$; 2-way ANOVA with Bonferroni's post-tests) as shown in Graph 3-22. These results show that the presence of Cheerios is a factor affecting exploratory behaviours in the NIH arena.
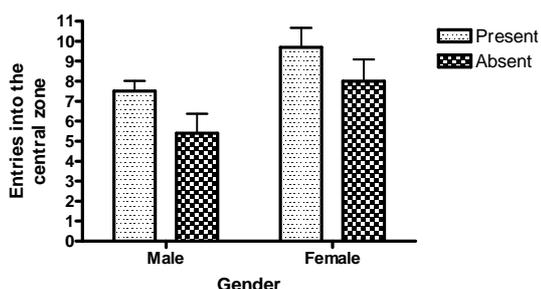


Graph 3-21 – A bar graph showing entries into the central zone during the 5-10 minute time bin in animals with no history of neonatal injury. There is an effect of gender, showing that females enter the central zone more than males ($F_{(1,32)}=5.35$, $p<0.05$; 2-way ANOVA).
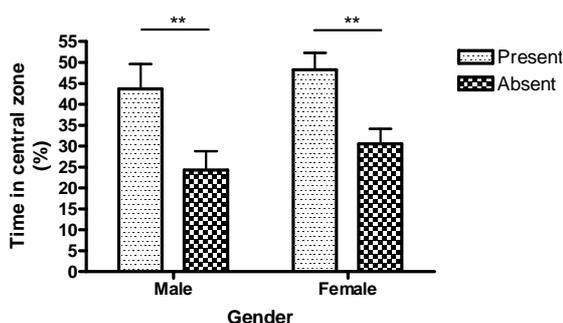


Graph 3-22 – A bar graph to show the effect of absence of Cheerios on percentage of time spent in the central zone in animals with no history of neonatal injury. **$p<0.01$; 2-way ANOVA with Bonferroni's post-tests.

## 3.4.3.5 Moving the food-bowl within the arena alters anxiety and approach behaviours in animals with no history of injury

The effect of moving the food-bowl from the centre to a less anxiogenic quadrant was investigated. Litters of animals with no history of neonatal surgery, who received a single adult foot wound, were tested with the food-bowl in an 'alternate quadrant' close to the edge of the arena (see Methods) and compared to the results from litters with the food-bowl in the centre. The central zone was defined as circling the food-bowl, regardless of its position. The groups and numbers of animals are shown in Table 3-9.

| Treatment group | Number of animals |
|---|---|
| M AN+FW Centre (Central) | 14 |
| M AN+FW Alternate Quadrant (AltQuad) | 3 |
| F AN+FW Centre (Central) | 13 |
| F AN+FW Alternate Quadrant (AltQuad) | 11 |

**Table 3-9 – A table to show the treatment groups and numbers per group when the food-bowl is moved from centre to edge (alternate quadrant).**

### 3.4.3.5.1 Anxiety measures are changed by moving the food-bowl to an alternate quadrant of the arena

The hypothesis that anxiety measures should be decreased when the food-bowl is moved away from the centre is fulfilled, as for both genders, there is significantly more urine production when the food-bowl is placed in the centre of the NIH arena (Quadrant $F_{(1,37)}$=5.22, p<0.05; Gender $F_{(1,37)}$=5.45, p<0.05; 2-way ANOVA with Bonferroni's post-tests). This effect is not significant when measuring defecation levels (2-way ANOVA).
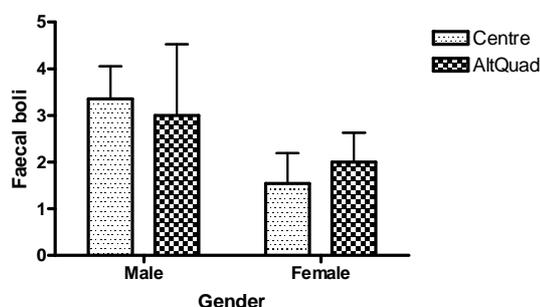


Graph 3-23 – Anxiety, as indexed by number of faecal boli, in animals with no injury history when the food-bowl is in the centre of the arena or an alternate quadrant (AltQuad).
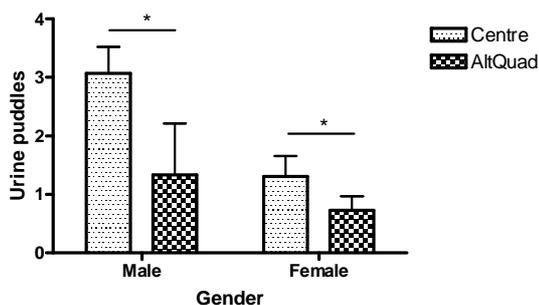
**Graph 3-24 – Anxiety, as indexed by urine puddles, in animals with no injury history, when the food-bowl is in the centre of the arena or an alternate quadrant. *p<0.05, 2-way ANOVA with Bonferroni's post-tests.**

### 3.4.3.5.2 Exploratory behaviours are minimally affected by the alternate placement of the food-bowl

Rearing behaviour during the 5-10 minute time bin is not affected by the placement of the food-bowl, and the percentage of time spent in the central zone is also the same wherever the bowl is placed (2-way ANOVA). However, entries into the central zone by females decrease when the Cheerios are placed into the alternate quadrant (Graph 3-25)(p<0.05; 2-way ANOVA with Bonferroni's post-tests). This is unexpected, as the number of entries was predicted to increase when the food-bowl was placed in an alternative, less anxiogenic, quadrant.
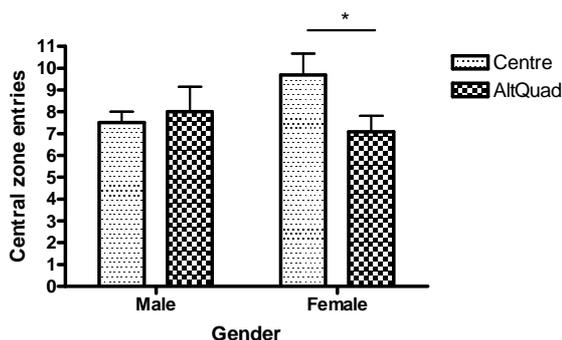


**Graph 3-25 – A bar graph to show the number of central zone entries during the 5-10 minute time bin in animals with no history of neonatal injury. *p<0.05; 2-way ANOVA with Bonferroni's post-tests.**

### 3.4.3.5.3 Appetitive behaviours are altered when Cheerios are placed in an alternate quadrant

It was expected that the time taken to eat the first Cheerio would decrease when the Cheerios were in the alternate quadrant. This is the case for females, as Cheerios are eaten sooner when the food-bowl was in the alternate quadrant (p<0.01; 2-way ANOVA with Bonferroni's post-tests)(Graph 3-26). There was no difference in the amount of Cheerios eaten during the 5-10 minute time bin when the Cheerios were placed in the alternate quadrant (2-way ANOVA).
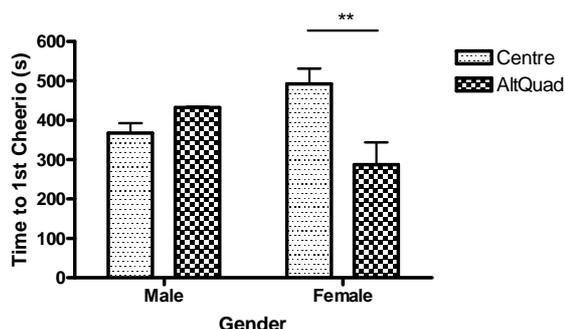
**Graph 3-26 – A bar graph to show the time taken to eat the first Cheerio in both males and females when the bowl is placed in an alternate quadrant. \*\*p<0.01; 2-way ANOVA with Bonferroni's post-tests).**

## 3.4.3.6 Summary of the effect of a single adult foot wound on previously uninjured animals

- The data in this section describe reward-related behaviours after a single adult injury in animals with no prior history of surgery. Time-binned data are presented from the 5-10 minute time bin. Data from all time-bins is presented in Appendix 2.

- Anxiety-related measures show no differences between animals that were injured or uninjured as adults.

- Exploratory behaviours, for both genders, show a similar pattern of results. Animals that receive a single adult injury (compared to uninjured controls) show decreased rearing behaviour, increased time spent in the central zone, but no differences in the number of entries into the central zone.

- Excluding animals that did not eat, the time taken to eat the first Cheerios did not differ between treatment groups. Females overall ate fewer Cheerios.

- Removing the Cheerios causes less time to be spent in the central zone. Placing the Cheerios in an alternate quadrant is less anxiogenic for both genders, and causes females to eat the Cheerios sooner. This confirms that Cheerios are rewarding, and that the centre of the arena is an anxiogenic environment.

### 3.4.4 The effect of re-injury to animals with a history of repeated neonatal foot wounds on reward-related behaviours

The results presented in this section will describe the effect that a single adult foot wound has on an animal with a prior history of surgery. Animals all had repeated neonatal foot wound surgery within the first 21 days of life. A reminder of the experimental design is shown in Figure 3-18:
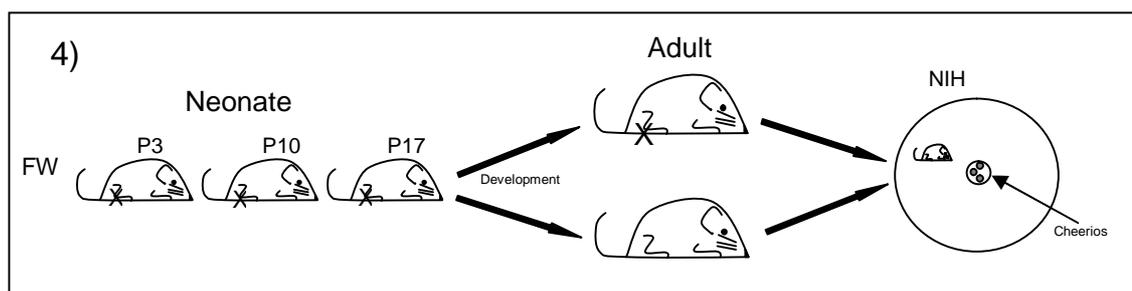
**Figure 3-18 – A schematic diagram to show the experimental design of the experiments investigating behaviours in the NIH arena after repeated neonatal injury and re-injury in adulthood.**

As above, the behaviours were measured during a 15 minute testing period in the NIH paradigm (see Methods); outcome measures have been classified into anxiety-related measures (faeces and urine), exploratory behaviours (rearing, central zone entries and time spent in central zone) and appetitive behaviours (time to first Cheerio and total number of Cheerios); these were time-binned accordingly. Time binned data, to show the effects of time on the outcome measures, are presented in Appendix 2. Data presented below is from the 5-10 minute time bin. Testing was performed 2 days post-surgery. Treatment groups and numbers of animals per group are described below:

| Treatment group | Number of animals |
|---|---|
| Male neonatal foot wound (M FW) | 19 |
| Male neonatal foot wound+adult foot wound (M FW+FW) | 14 |
| Female neonatal foot wound (F FW) | 19 |
| Female neonatal foot wound+adult foot wound (F FW+FW) | 12 |

**Table 3-10 –A table to show the treatment groups and number of animals per group in the experiment studying animals with a history of neonatal injury.**

### 3.4.4.1 Anxiety-related measures are affected by adult re-injury

Males with a history of foot wound defecate more after a single adult foot wound in adulthood, compared to animals that do not receive a foot wound in adulthood ($p<0.05$; 2-way ANOVA with Bonferroni's post-tests). There is a significant effect of gender, showing that females defecate less than males ($F_{(1,60)}=14.2$, $p<0.001$, 2-way ANOVA). There was no effect of re-injury on urine production (2-way ANOVA).
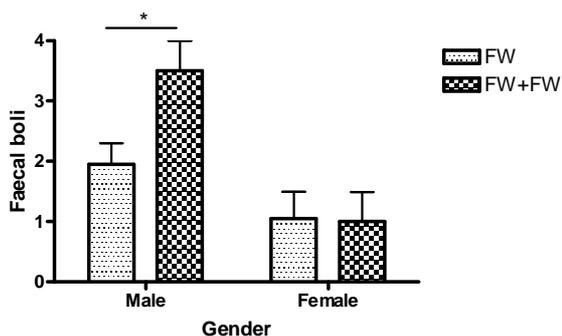
**Graph 3-27 - A bar graph to show the number of faecal boli produced by animals after a single adult foot wound in animals with a history of repeated neonatal foot wound injury. \*p<0.05; 2-way ANOVA with Bonferroni's post-tests.**

## 3.4.4.2 Exploratory behaviours are significantly altered by an adult re-injury

### 3.4.4.2.1 Rears are significantly decreased in animals with a history of neonatal injury

Animals that were re-injured as adults (FW+FW) reared significantly fewer times in the 5-10 minute time bin compared to control animals (FW)($F_{(1,60)}$=49.24, p<0.001; 2-way ANOVA with Bonferroni's post-tests). There was a significant effect of gender, showing that females rear more than males ($F_{(1,60)}$=21.75, p<0.001; 2-way ANOVA).
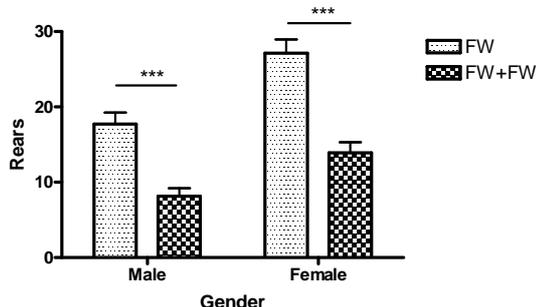


**Graph 3-28 – A bar graph to show the number of rears in the 5-10 minute time bin in animals with a history of neonatal repeated injury. \*\*\*p<0.001; 2-way ANOVA with Bonferroni's post-tests.**

### 3.4.4.2.2 Central zone entries are increased in females re-injured as adults

The number of entries into the central zone increased in females re-injured as adults (Treatment $F_{(1,60)}$=4.14, p<0.01; 2-way ANOVA with Bonferroni's post-tests).
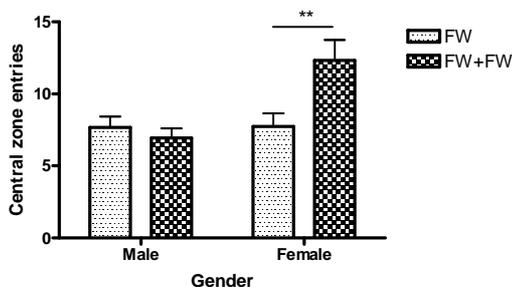


**Graph 3-29 – A bar graph to show the number of entries into the centre during the 5-10 minute time bin by animals with a history of repeated neonatal injury. \*\*p<0.01; 2-way ANOVA with Bonferroni's post-tests).**

### *3.4.4.2.3 Percentage of time in the central zone increases after adult re-injury.*

The percentage of time spent in the central zone is higher in all animals re-injured in adulthood (Treatment $F_{(1,60)}=22.26$, $p<0.01$; 2-way ANOVA with Bonferroni's post-tests).
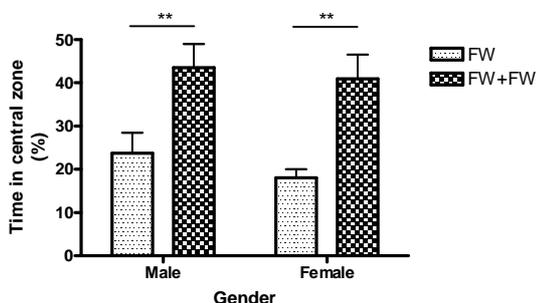


**Graph 3-30 – A bar graph to show the percentage of time spent in the central zone in the 5-10 minute time bin by animals with a history of neonatal injury. **p<0.01; 2-way ANOVA with Bonferroni's post-tests.**

## 3.4.4.3 Appetitive behaviours

As above, animals that did not eat any Cheerios during the entire 15 minute testing period were excluded from this analysis. Fisher's Exact test was performed to see whether the non-consumption of Cheerios was a result of treatment or not. A table to show the number of male and female non-eaters, and the Fisher's Exact p value, is shown below:

| Treatment group | Number of non-eaters/total | P value from Fisher's Exact test |
|---|---|---|
| M FW | 7/19 | **0.008** |
| M FW+FW | 2/14 | 0.48 |
| F FW | 3/16 | 0.12 |
| F FW+FW | 1/12 | 0.5 |

**Table 3-11 – A table to show the number of non-eaters in each treatment group, and the P value from Fisher's Exact test comparing animals to an expected baseline of 100% consumption. **p<0.01.**

Table 3-11 shows that non-consumption by the animals was due to an effect of treatment in the M FW group – repeated neonatal injury reduces the likelihood of consuming Cheerios during testing in adulthood. That this is not seen in the re-injured group (M FW+FW) suggests that a re-injury increases the likelihood of Cheerio consumption. Excluding the non-eating animals left the following numbers in each group:

| Treatment group | Number of animals (excluding non-eaters) |
|---|---|
| M FW | 12 |
| M FW+FW | 12 |
| F FW | 16 |
| F FW+FW | 11 |

**Table 3-12 – A table to show the number of animals in the treatment groups, excluding non-eaters.**

### 3.4.4.3.1 Time to eat the first Cheerio was not affected by an adult re-injury in animals with a history of neonatal injury.

The time taken to eat the first Cheerio was not different between animals that were re-injured as adults and those that were not (2-way ANOVA).
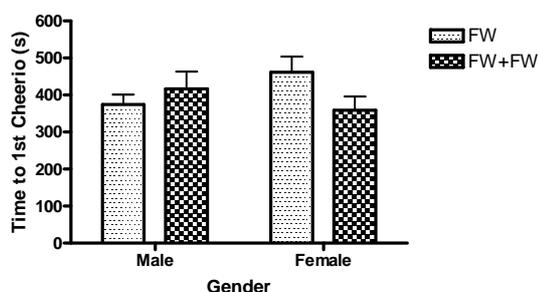


**Graph 3-31 –A graph showing the time in seconds before the first Cheerio was consumed, in animals with a history of neonatal foot wound.**

### 3.4.4.3.2 Cheerio consumption is affected by an adult re-injury

Cheerio consumption is increased in females re-injured as adults (Treatment $F_{(1,47)}=6.88$, p<0.01; 2-way ANOVA with Bonferroni's post-tests). There is a significant effect of gender, showing that females consume fewer Cheerios in the 5-10 minute time bin (Gender $F_{(1,47)}=11.5$, p<0.05; 2-way ANOVA).
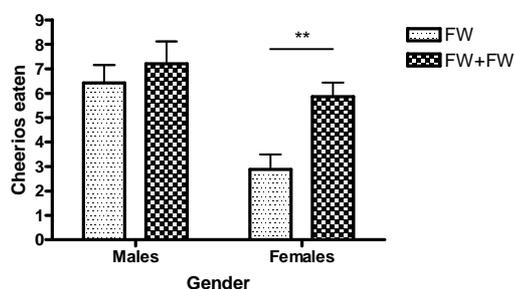


**Graph 3-32 - A bar graph to show the number of Cheerios eaten during the 5-10 minute time bin by animals with a history of foot wound. **p<0.01; 2-way ANOVA with Bonferroni's post-tests.**

## 3.4.4.4 Removing the food reward alters exploratory behaviours in males and females with a history of injury

As before, to investigate the effect that the presence of the food reward had in general on exploratory behaviours, the Cheerios were removed from the central bowl and separate litters of animals with a history of foot wound that were re-injured as adults were tested. Table 3-13 shows the number of animals in each group.

| Treatment Group | Number of animals |
|---|---|
| M FW+FW Cheerios Present | 14 |
| M FW+FW Cheerios Absent | 5 |
| F FW+FW Cheerios Present | 12 |
| F FW+FW Cheerios Absent | 4 |

**Table 3-13 – A table to show the treatment group and numbers of animals per group in the presence/absence of Cheerios test.**

### 3.4.4.4.1 Anxiety measures are not affected by the absence of Cheerios in previously injured animals

There is no effect of removing the Cheerios on the anxiety measures (faecal boli and urine production) of males or females with a history of neonatal repeated foot wound. There is a significant effect of gender, showing that females produce fewer faecal boli ($F_{(1,31)}$=13.21, p<0.001; 2-way ANOVA)(see Appendix 2).

### 3.4.4.4.2 Exploratory behaviours are affected by the presence or absence of Cheerios in animals with a history of injury

Animals that were re-injured as adults showed an increased number of rears when the Cheerios were absent (Treatment $F_{(1,31)}$=26.9; p<0.001; 2-way ANOVA with Bonferroni's post-tests)
Graph 3-33). There was a significant effect of gender, showing that males rear less than females ($F_{(1,31)}$=18.42, p<0.001; 2-way ANOVA).
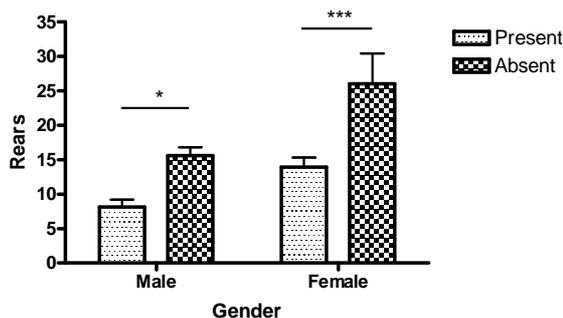
**Graph 3-33 - A graph to show the number of rears by both genders in the 5-10 minute time bin when Cheerios were present or absent in animals with a history of foot wound. *p<0.05; ***p<0.001; 2-way ANOVA with Bonferroni's post-tests.**

The number of entries into the central zone during the 5-10 minute time bin was not significantly affected by the presence or absence of Cheerios, although a trend suggests fewer entries when Cheerios were absent (Graph 3-34). There was a significant effect of gender, showing females enter the central zone more frequently (Gender $F_{(1,31)}$=15.22, p<0.001; 2-way ANOVA).
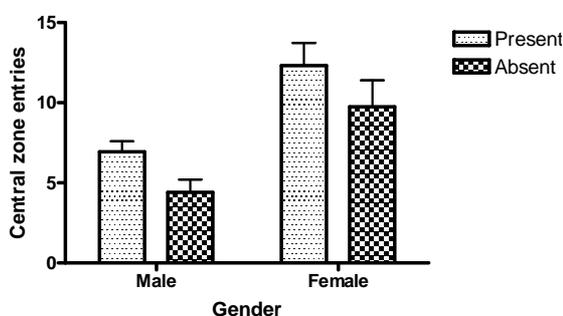


**Graph 3-34 – A bar graph to show the number of central zone entries during the 5-10 minute time bin when Cheerios are present/absent in animals of both gender with a history of neonatal repeated injury.**

The percentage of time spent in the central zone is lower in males with a history of FW injury when Cheerios are absent ($F_{(1,31)}$=6.78, p<0.05; 2-way ANOVA with Bonferroni's post-tests).
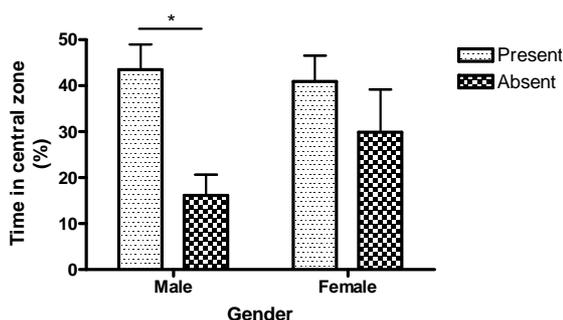


**Graph 3-35 – A graph to show the percentage of time spent in the central zone during the 5-10 minute time bin by both genders of animals with a history of repeated foot wound.. *p<0.05; 2-way ANOVA with Bonferroni's post-tests.**

## 3.4.4.5 Moving the food-bowl within the arena slightly alters reward-related behaviours in animals with a history of foot wound

The effect of moving the food-bowl from the centre to a less anxiogenic quadrant was investigated. Litters of animals with a history of foot wound that were re-injured as adults were tested with the food-bowl in an 'alternate quadrant' close to the edge of the

arena (see Methods), and compared to results from litters with the food-bowl in the centre. The central zone was defined as circling the food-bowl, regardless of its position. The groups and numbers of animals are shown in Table 3-14.

| Treatment group | Number of animals |
|---|---|
| M FW+FW Centre (Centre) | 14 |
| M FW+FW Alternate Quadrant (AltQuad) | 10 |
| F FW+FW Centre (Centre) | 12 |
| F FW+FW Alternate Quadrant (AltQuad) | 5 |

**Table 3-14 - A table to show the treatment groups and numbers per group when the food-bowl is moved from centre to edge (alternate quadrant).**

### 3.4.4.5.1 Anxiety measures are affected by the position of the food-bowl in animals with a history of injury

Unexpectedly, there is a significant effect on faecal bolus production when the bowl is moved away from the centre in animals with a history of repeated neonatal foot wound (Quadrant $F_{(1,37)}$=4.97, $p<0.05$; 2-way ANOVA). This is contrary to the hypothesis that moving the food-bowl to the edge of the NIH arena is less anxiogenic (anxiety-inducing). There is a significant effect of gender on both faeces and urine outcome measures, showing that males produce more faecal boli and urine over the 15 minute NIH testing time (Gender $F_{(1,37)}$=12.14 (faeces), $F_{(1,37)}$=7.52 (urine), $p<0.01$; 2-way ANOVA).
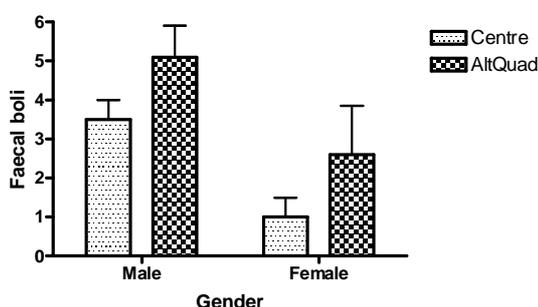


**Graph 3-36 – A graph to show the anxiety measures, as indexed by number of faecal boli produced, in animals with a history of neonatal injury when the Cheerios are central or placed in an alternate quadrant.**
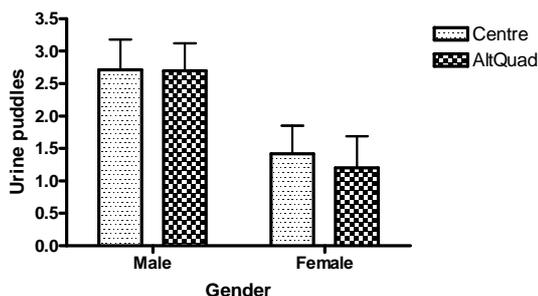
**Graph 3-37 - A graph to show anxiety, as indexed by urine production, in animals with a history of neonatal injury when the Cheerios are central or placed in an alternate quadrant.**

### 3.4.4.5.2 Exploratory behaviours of re-injured animals are mildly affected by placement of the food-bowl

The number of rears performed by females is significantly increased in females when the food-bowl is moved from the centre of the NIH arena (Quadrant $F_{(1,37)}=8.78$, $p<0.01$; 2-way ANOVA with Bonferroni's post-tests)(Graph 3-38).
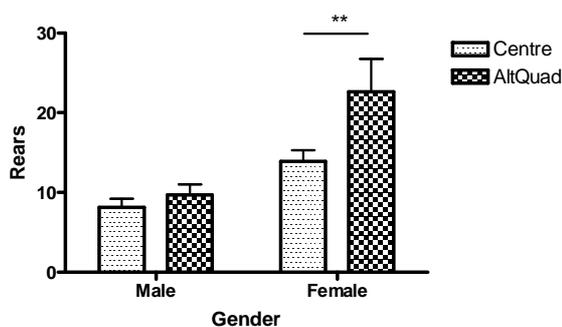


**Graph 3-38 – A graph to show the number of rears during the 5-10 minute time bin in animals with a history of foot wound that have been re-injured. \*\*$p<0.01$; 2-way ANOVA with Bonferroni's post-tests.**

There was no effect on the number of central zone entries or the amount of time spent around the food-bowl when Cheerios were placed in the alternate quadrant (2-way ANOVA), which was unexpected.

### 3.4.4.5.3 Appetitive behaviours by re-injured animals

As above, animals that did not eat any Cheerios during the entire 15 minute testing period were excluded from this analysis. Fisher's Exact test was performed to see whether the non-consumption of Cheerios was a result of treatment or not. A table to show the number of male and female non-eaters, and the Fisher's Exact p value, is shown below. Table 3-15 shows that non-consumption by the animals was not due to an effect of treatment or experimental design.

| Treatment group | Number of non-eaters/total | P value from Fisher's Exact test |
|---|---|---|
| M FW+FW Centre | 2/14 | 0.48 |
| M FW+FW AltQuad | 0/10 | n/a |
| F FW+FW Centre | 1/12 | 0.5 |
| F FW+FW AltQuad | 0/5 | n/a |

**Table 3-15 – A table to show the number of non-eaters in each treatment group, and the P value from Fisher's Exact test comparing animals to an expected baseline of 100% consumption.**

It was expected that the time taken to eat the first Cheerio would decrease when the Cheerios were in the alternate quadrant. This hypothesis is not fulfilled; there are no differences between central and alternate quadrant on the time taken to eat the first Cheerio for either gender (2-way ANOVA). The number of Cheerios eaten during the 5-10 minute time bin in animals with a history of foot wound was not affected by placement of the food-bowl.

## 3.4.4.6 Summary of the effect of a foot wound re-injury on animals with a history of neonatal foot wound

- The data in this section describe reward-related behaviours after an adult re-injury in animals with a history of neonatal foot wound surgery.

- Anxiety-related measures, as indexed by defecation, are increased in males that were re-injured as adults when they were tested in the NIH arena. Moving the food-bowl away from the centre of the arena increased defecation, which was unexpected, as it was hypothesised that moving the food-bowl away from the centre of the arena would decrease the anxiogenic quality of the NIH paradigm, and would decrease defecation accordingly.

- Exploratory behaviours, for both genders, show a similar pattern of results. In the 5-10 minute time bin, rearing behaviour decreases, and percentage of time spent in the central zone increases. Central zone entries increase in females only.

- Excluding animals that did not eat, the time taken to eat the first Cheerio did not differ between treatment groups for either gender, and neither did the number of Cheerios eaten during the 5-10 minute time bin.

- Removing the Cheerios increases rearing behaviour in both genders, and decreases the amount of time spent in the central zone in males only, showing that Cheerios as a reward are driving central zone entries in males.

- Placing the Cheerios in an alternate quadrant increases rearing behaviour in females only, but does not affect any other exploratory measure. This suggests that, in animals re-injured as adults, approaching the food-bowl is anxiogenic, regardless of its position.

## 3.4.5 Summary of all experiments

The results presented in this chapter have shown a variety of changes that are caused by repeated neonatal injury, single adult injury, or a re-injury in previously injured animals.

### 3.4.5.1 Sensory testing

- The effect of repeated neonatal surgery on sensory nociceptive thresholds at baseline and after an acute adult re-injury was first illustrated. Results showed that females that have been previously injured as neonates are hypoalgesic to mechanical and thermal stimuli at baseline. 2 days post-injury in adulthood, the ipsilateral paw is more sensitive to mechanical and thermal stimuli compared to contralateral paws, in neonatally injured animals in particular.

### 3.4.5.2 Anxiety-related measures

- When investigating animals with a history of neonatal injury at baseline, plus animals with no history of injury but a single adult foot wound, anxiety-related measures were not affected. However, upon testing animals re-injured as adults in the NIH paradigm, defecation increased, suggesting that a history of neonatal injury can increase anxiety levels after an adult re-injury.

### 3.4.5.3 Exploratory measures

- Exploratory measures at baseline in adulthood showed the same patterns over the total 15 minute testing period in animals either with or without a foot wound history – rearing and central zone entries decreased over the testing period and percentage of time spent in the central zone increased, presumably as animals became more familiar with their novel environment (see Appendix 2 for all data).

- Results became significantly different from controls when looking at the effects of adult injury – either a single injury in animals with no history of neonatal foot wound, or a re-injury in animals with a history of neonatal foot wound.

- Adult injury decreased rearing behaviour in all animals, regardless of background, suggesting an increase in anxiety-related exploratory behaviour. The percentage of time spent in the central zone was increased, suggesting an increased motivation to reach the food-bowl.

- Removing the Cheerios decreased the number of entries into the central zone and decreased the percentage of time spent there, suggesting that the Cheerios were the factor responsible for driving approach to the food-bowl. Moving the Cheerios to an alternate quadrant decreased anxiety measures, and decreased the latency to eat Cheerios in females with no history of surgery only. The lack of the same changes in animals neonatally injured and then re-injured as adults suggests that approach of the food-bowl is anxiogenic for these animals, regardless of its placement.

- In general, results were significant or more pronounced in females, particularly those with a history of foot wound.

### 3.4.5.4 Appetitive behaviours

- Males that had a history of foot wound were less likely to consume Cheerios at baseline in adulthood. However, upon injury in adulthood, appetitive behaviours were virtually the same across all groups, genders and conditions. This suggests that an adult injury increases the likelihood of eating Cheerios, regardless of the injury background of the animal.

## 3.5  Discussion

Briefly, the results from the experiments presented in this chapter have shown that repeated foot wound surgery during the first 3 weeks of an animal's life causes long-term changes in sensory nociceptive thresholds that persist into adulthood. Furthermore, when testing repeatedly injured animals in a cognitive behavioural paradigm, exploration and reward-related behaviours are subtly altered when animals are re-injured as adults.

As there were changes over time on both sensory and behavioural outcome measures, for the purposes of presenting differences due to treatment, specific time points after injury or time bins of the NIH task were chosen to analyse. For sensory testing, the time point chosen was 2 days post-surgery in adulthood. On the NIH task, the 5-10 minute time bin was chosen for data that had been split into time bins. Data presenting time courses for all sensory changes and behavioural outcome measures is presented in Appendix 2.

## 3.5.1  Sensory testing in adulthood

The results from the sensory testing experiments show that at baseline, pre-surgery in adulthood, males do not show significant sensory changes after repeated neonatal surgery, as measured by mechanical and thermal withdrawal thresholds. However, females show a baseline hypoalgesia (i.e. decreased sensitivity) to both mechanical and thermal stimuli.

After injury in adulthood, only the neonatally injured animals showed a significant drop in mechanical and thermal withdrawal thresholds in the ipsilateral paw at two days post-injury.

These results show that animals repeatedly injured as neonates display a baseline hypoalgesia to punctate mechanical and thermal noxious stimulation. However, upon re-injury in adulthood, the injured paw is made hypersensitive to mechanical and thermal stimulation. This effect is not seen in neonatally anaesthetised control animals.

### 3.5.1.1 Gender differences

That male sensory withdrawal thresholds at baseline were not significantly affected by neonatal injury is unexpected, as the literature consistently shows that male animals show altered sensory processing after neonatal injury (Anand et al., 1999; Bhutta et al., 2001; Lidow, 2002). The lack of significance in these results can be partially explained by the fact that the groups tested were very small, with only 3 animals per group. Sensory testing can be easily confounded by an animal's state at the time of testing (i.e. agitated, asleep), and coupled with individual differences in general reactivity to stimuli, it is perhaps not surprising that significant differences were not found with such small sample sizes. To clarify the effects of neonatal injury and its effect on adult baseline

behaviour and thresholds after adult injury, larger groups of animals would need to be tested.

Females, however, showed significant hypoalgesia to mechanical and thermal stimuli at baseline. This is in line with literature that has investigated the long-term effects of neonatal inflammation of the hindpaw. LaPrairie and Murphy (2007) injected carrageenan (an inflammatory agent) into the hindpaw on the first day of life and found that at postnatal day (P)40 and P60, both females and males showed an increased thermal withdrawal latency, and that the latency was 40% longer in females. This sexual dimorphism is suggested to be due to hormonal differences between gender during development, with female oestrogens possibly acting in a pro-nociceptive manner and male androgens acting in a protective manner (La Prairie and Murphy, 2007; see La Prairie and Murphy, 2010). This may help to explain the significant results seen in females rather than males.

## 3.5.1.2 A critical period for sensory changes

The injuries inflicted in these experiments were performed at P3, P10 and P17. According to various studies published, it is the injury at P3, during the 'critical period', that is crucial for causing long-term changes. Studies using both the plantar incision model and paw inflammation models have shown that long-lasting effects on sensory thresholds are produced when the injury occurs within the first week of life, suggesting a critical period of development during which noxious input will cause alterations in adult sensory thresholds (Wang et al., 2004; Ren et al., 2004; Walker et al., 2009b; La Prairie and Murphy, 2010).

During this sensitive time window, nociceptive processing is still immature. Whilst the anatomical connections in rats are present at birth, injury can influence the postnatal development of the sensory nociceptive system. Inflammation (induced by Complete Freund's adjuvant) causes an increase in primary afferent nerve input to the spinal cord (Ruda et al., 2000) and skin wounds cause long-lasting cutaneous hypersensitivity, increased sprouting of local sensory nerve terminals, and increased receptive field size in the spinal cord (Torsney and Fitzgerald, 2003).

### 3.5.1.3 Re-injury in adulthood

Previous research has shown that adult re-injury of a previously injured neonate exacerbates the effect of injury, compared to control animals uninjured as neonates. Coupled with an injury during the critical period for sensory development, this creates an adult animal with baseline hypoalgesia to noxious stimuli, but an enhanced nociceptive response to a new injury (see La Prairie and Murphy, 2010); interestingly, this is an effect also seen anecdotally in humans (Grunau et al., 1994; Hermann et al., 2006). The baseline hypoalgesia and exacerbated hyperalgesia after adult re-injury is replicated in these experiments. It has been suggested that this seemingly contrary state is mediated by a shift in the developing opioidergic tone in the brainstem of neonatally injured animals (La Prairie and Murphy, 2009).

The authors propose a working hypothesis for the effect whereby neonatal injury during the critical period (P0 to P8) causes increased afferent drive to central brain sites responsible for noxious processing, including the periaqueductal grey (PAG) and rostroventral medulla (RVM). Both these areas project directly to the spinal cord and constitute an important anti-nociceptive circuit, modulating pain processing in a top-down manner (Basbaum and Fields, 1984). The increased afferent drive caused by injury induces the continued release of endogenous opioid peptides from the PAG, and causes a long-term upregulation of these opioids. This upregulation is manifested as an increase in opioid peptides in the PAG (La Prairie and Murphy, 2009), and is seen in the adult rat, the upregulation of endogenous peptides lasting more than a week after inflammation (Williams et al., 1995).

Taken together with evidence that descending inhibitory control from the brainstem does not mature until the end of the second week of postnatal life (Boucher et al., 1998; see Fitzgerald, 2005), LaPrairie and Murphy suggest that the delayed opioidergic inhibitory control during the first two postnatal weeks may be the cause of the increased vulnerability of the immature rat nociceptive processing system to noxious insult during a critical period, a suggestion strengthened by the fact that naloxone, a mu opioid receptor antagonist, prevented expression of the baseline hypoalgesia seen in adulthood (La Prairie and Murphy, 2007).

## 3.5.2 Behaviour in the NIH paradigm after a single injury in adulthood

The results presented in this chapter explore the effect of a history of neonatal injury on adult reward-related behaviours. However, to understand the effects upon behaviour of a single injury in adulthood, testing in the NIH arena was performed on animals with no history of neonatal injury who received a single foot wound in adulthood and outcome measures were compared to animals who had received no injury at all.

The results showed that rearing behaviour, which is an index of exploratory behaviour (see Walsh and Cummins, 1976), was decreased in animals after a single adult foot wound. In addition to this, the amount of time these animals spent in the central zone was increased. This is an anxiogenic (anxiety-inducing) position in which to be, as the animal is exposed in the centre of the arena; however anxiety levels, as measured by defecation and urination, were not affected by a single acute injury.

Therefore it is possible to speculate that animals with no history of injury but acutely injured as adults show a reduced exploratory drive, suggesting that the rewarding effects of exposure to novelty in the NIH arena are attenuated (Hummel et al., 2008). However, these animals may be more motivated to seek a food reward, reflected by the increased amount of time spent in the central zone of the NIH arena. This is supported by data showing that removing the Cheerios from the food-bowl causes a decrease in time spent in the central zone by these animals.

Studies from pain researchers have shown that chronic pain causes alterations in behaviour on complex cognitive behavioural paradigms (see Mogil, 2009). This experiment, investigating the effect of a single adult foot wound, is one of few showing that acute pain in adulthood can also cause behavioural alterations in the same complex paradigms (van der Kam et al., 2008; Hummel et al., 2008; LaBuda and Fuchs, 2000).

## 3.5.3 Behaviour in the NIH paradigm in animals injured in infancy

There was little effect on any outcome measure when animals that had been repeatedly injured as neonates were tested at baseline on the NIH task in adulthood, with outcome

measures mainly reflecting gender effects i.e. that female animals rear more than males and defecate less.

However, when neonatally-injured animals were re-injured in adulthood and tested in the NIH paradigm, clear behavioural differences were highlighted. Defecation was increased, as were entries into the central zone, percentage of time spent there, and number of Cheerios eaten. A concomitant decrease in rearing behaviour by both genders was seen. Taken together, these results suggest that exploratory drive (as seen by rearing behaviour) is decreased in re-injured animals, but motivational drive to reach a palatable food reward is increased. The increase in defecation levels suggests that this motivation to reach the Cheerios is performed despite an increase in anxiety levels (Broadhurst, 1958).

These results suggests that early neonatal injury may not affect adult reward-related behaviours *per se*, but that subtle shifts in reward processing may occur when the animal is again placed in a pain state in adulthood. This is in line with the literature from pain researchers showing that re-injury of a previously injured animal in adulthood causes enhanced hyperalgesic responses (Bhutta et al., 2001; La Prairie et al., 2008).

### 3.5.4 Control experiments show Cheerios are rewarding

These experiments rely on the rewarding aspect of the chosen food. Therefore important control experiments were done in the NIH arena to check that animals were responding to the presence of food treats, and that placement of the food reward was anxiogenic whilst in the centre of the arena.

To begin, testing animals injured as neonates on their baseline behaviour towards food reward in the NIH arena showed an intriguing result: there were a high number of animals that did not eat any Cheerios during the testing period. This was unexpected as home cage behaviour had shown that all animals would universally eat the Cheerios very shortly after their presentation. A test to see whether the lack of consumption was due to coincidence showed that the number of non-eaters was significantly higher than would be expected. Therefore the consumption of a food treat was less likely in animals with a history of surgery, suggesting a possible baseline anhedonic state in these animals.

To check that the animals were responding to the presence of Cheerios, and that central placement of the food-bowl was anxiogenic, the Cheerios were removed from the food-bowl, or the bowl placed into an alternate, less anxiogenic quadrant of the arena. Significant changes in behaviour were seen only in animals injured in adulthood, particularly in those animals with a history of repeated neonatal foot wound. Taken with the data showing a baseline decrease in Cheerio consumption by neonatally injured males, this suggests that an adult injury increases the likelihood of Cheerio consumption.

The appetitive aspect of the Cheerios was proven as animals spent less time in the central zone when the Cheerios were removed, regardless of their injury background, and the data suggests that the number of entries into the centre were also decreased by removal of the Cheerios. In fact, in animals with no history of injury, the absence of a food reward shows a trend for increased defecation, possibly indicating that the presence of Cheerios may help to decrease anxiety levels in animals trained to consume these palatable treats.

When moved to an alternate quadrant, it was expected that anxiety levels would decrease as the approach towards the Cheerios became less aversive. This was the case in animals with no foot wound history, as urination frequency was higher in both genders when the food-bowl was centred in the arena. It was also expected that the number of central zone entries and percentage of time spent there would increase, and the latency for animals to consume their first treat would decrease. This, however, was not seen, possibly due to the large variation in these outcome measures.

Overall, these results suggest that Cheerios are the main factor causing approach of the food-bowl, and that approaching and spending time at the food-bowl is an anxiogenic experience, particularly in animals with a history of neonatal injury.

### 3.5.5  Technical considerations

Animal behaviour is difficult to interpret due to the large variability within and between subjects and the risk of anthropomorphism as experimenters extrapolate an animal's state or trait at any given time from its behaviour. Accordingly, there are many potential

variables that need to be taken into account when designing any animal experiment, and inherent limitations in the testing methods used to measure behaviours.

## 3.5.5.1 Sensory testing limitations

The experiments in this chapter studying sensory withdrawal thresholds were performed on a very small number of animals due to breeding difficulties and time constraints. In addition, each animal will have its own method of response to a noxious stimulus, and there are always differences in the behavioural state of each animal at the time of testing. For example, regardless of the level of habituation to the testing apparatus, some animals will always fall asleep after 20 minutes in the test chamber, whereas other animals will remain wide awake. Taken together with the magnification of variance produced by the logarithmic increase in force as von Frey hair number increases, it is perhaps less surprising that the results from the mechanical withdrawal threshold experiments in this chapter were not statistically significant.

## 3.5.5.2 Experimental design limitations

### 3.5.5.2.1 The novelty-induced hypophagia (NIH) paradigm

The principles of hyponeophagia, or the inhibition of feeding upon exposure to novelty, have been useful for studying the effects of anti-depressant drugs, and the NIH paradigm has been posited as the most valid application of hyponeophagia principles (see Dulawa and Hen, 2005). There are numerous types of validity that define whether a test is appropriate, including face, construct, predictive, content, criterion, internal and external validities, amongst others. The first three on this list - face, construct and predictive - are widely used to critically appraise behavioural tests, and are briefly discussed below.

#### 3.5.5.2.1.1 Face validity

Face validity is a measure of how well a test *appears* to measure the outcomes of interest. The NIH task is high in face validity as it uses an ethologically relevant reward, food, in an environment akin to something an animal would experience in its natural habitat, and utilises inherent behaviours such as exploration as measures of interest. Conversely, tasks such as intra-cranial self-stimulation, whilst identifying specific brain

regions for reward, do not take place in ethologically relevant environments or rely on naturally occurring behaviours for measures of success.

### 3.5.5.2.1.2 Construct validity

Construct validity refers to the accuracy with which a test assesses the variable it is intended to assess. That the food reward was shown to affect appetitive and exploratory behaviours shows that the NIH task did indeed have a rewarding component, and the significant effects of treatment further underline the construct validity of the NIH, as it measured differences in outcome variables that were due to treatment.

### 3.5.5.2.1.3 Predictive validity

Predictive validity is defined as the ability of a model to make accurate predictions about the human phenomenon of interest. Hyponeophagia tasks demonstrate strong predictive validity for the anxiolytic effects of drug treatments (Thiébot et al., 1984; Dulawa et al., 2004), but its predictive validity for application to pain research will require further work i.e. validation with analgesics, anxiolytic drugs, and other pain medications. Taken together, the face and construct validity of the NIH paradigm are high, and confirming the predictive validity of it warrants further investigation.

Ambulation, an outcome measure that is reliably predictive of anxiety in open field tasks (see Walsh and Cummins, 1976), was not measured in this experimental setting. However, other parties have argued that locomotion is not a good index of emotionality (Stanford, 2007a; Rodgers, 1997). Instead, in these experiments, defecation and urination, also reliable outcome measures, were recorded, and yielded significant effects of gender and treatment, in line with previous research (Broadhurst, 1958).

Animal behaviour is notoriously difficult to interpret. Animals do not have the capacity to report back on their pain states, motivations, or internal mental state, so conclusions are extrapolated based on often crude behavioural measures, and the danger of anthropomorphism is clear. The best way for an experimenter to overcome these problems is to be aware of the limitations of animal experiments, and to acknowledge the potential variables that can confound outcome measures. Below is a brief list of variables that were taken into account when designing the NIH paradigm:

### 3.5.5.2.1.4   Effects of maternal care and deprivation

Maternal deprivation is a powerful factor that can affect brain development in neonatal rat pups. Maternal style of the nursing dam i.e. licking and grooming behaviour can interfere with brain development, affecting the stress-regulating hypothalamic-pituitary-adrenal (HPA) axis, and epigenetic regulation of glucocorticoid receptors in the hippocampus (Weaver et al., 2004). It can also affect pain behaviours in adulthood (Champagne et al., 2003; Champagne and Meaney, 2006; Champagne and Meaney, 2007; Clinton et al., 2007). To decrease the impact of this between-litter variability, all litters were to be bred from the same dams. To control for the effects of maternal deprivation, all litters of animals experienced the same duration of deprivation and handling during surgery, and a combination of 5 breeding dams and 2 stud males were used for breeding, to produce a variation in maternal style.

### 3.5.5.2.1.5   Individual differences

A potentially major confound in all animal experimentation is the presence of individual differences, which refers to the natural variations in response to environmental stimuli (see Pawlak et al., 2008). High levels of individual differences cause large variation in behavioural data. Indeed, the presence of these differences can give rise to selective breeding whereby sub-strains of animals are created i.e. the high responding/low responding strains of rat (see Blanchard et al., 2009).

### 3.5.5.2.1.6   Noise levels

High noise levels are acknowledged as a stressor in behavioural tests (see Walsh and Cummins, 1976). To prevent this confounding the results, all testing was done in low noise levels. However, noise levels in the corridor upon which the behavioural testing was done were occasionally raised i.e. by the entry telephone ringing; this clearly altered behaviour in the NIH arena, as seen by freezing behaviour from the video recordings, but was an unavoidable confound.

### 3.5.5.2.1.7   Odour

Both ambient odours and urine/faecal traces left in the NIH arena could have potentially affected behaviours i.e. conferring the anxiety status of the previously tested animal. To control for this, the transfer box used to move animals from their home cage room to the testing room was regularly cleaned and fresh sawdust used for every cage of animals. The NIH arena and food-bowl was thoroughly scrubbed, cleansed and dried between the

testing of each animal, using disinfectant and high ethanol percentage solutions to remove all odour traces.

#### 3.5.5.2.1.8 Environmental conditions during development

Environmental enrichment during development affects brain development and adult behaviour (Sale et al., 2009). To control for this variable, environmental enrichment in the form of objects placed in the home cage that animals could interact with were provided for all animals throughout their lifetimes.

#### 3.5.5.2.1.9 Variations in hunger levels

Access to food and water was allowed for all animals *ad libitum*, in an attempt to reduce any confounding factors of hunger. In theory, this should mean that approach to the food in the NIH arena was due to its palatability, rather than by a motivational hunger drive.

#### 3.5.5.2.1.10 Social interaction

Social interactions i.e. via ultrasonic vocalisation between animals has been shown to affect pain behaviours and outcome measures on open field tasks (see Chesler et al., 2002; Walsh and Cummins, 1976). To limit the effects of these, animals were kept separate after testing until all cage-mates had been testing in the NIH arena.

#### 3.5.5.2.1.11 Gender differences

There are clear gender differences in a huge variety of animal behaviours, including pain behaviours and behaviours in reward-related and exploratory paradigms (see Walsh and Cummins, 1976; Bevins and Besheer, 2005; La Prairie and Murphy, 2007). For this reason, genders were not pooled and were analysed separately.

## 3.5.5.3 Summary of technical considerations

With any experiment utilising laboratory animals and measuring behaviour, many considerations regarding experimental design and potentially confounding factors have to be taken into account. As many of these as possible were acknowledged and controlled for in these experiments; this makes the significance of results arising from treatment conditions more robust, and conclusions drawn firmer.

## *3.6 Conclusions*

The results presented in this chapter show that repeated plantar skin incision during the neonatal period in rats causes sensory alterations that occur well into adulthood, and that this model of injury affects adult reward-related behaviours on the novelty-induced hypophagia task. To date, the literature has intensively studied the effects of this injury model on pain behaviours (Brennan et al., 2005; Banik et al., 2006; Walker et al., 2009b). The experiments presented above are the first to investigate the long-term cognitive behavioural effects of the plantar skin incision model during infancy, and are in line with the aims of pain researchers to produce pain models and outcome measures that are more relevant to the human pain experience.

To find brain areas and processing systems that are responsible for alterations in behaviour, it is important to help determine the neurobiological mechanisms of early pain on later reward-related processing. The next set of experiments in this thesis attempts to find a neurobiological correlate of the altered behaviour, investigating the orexinergic system of the lateral hypothalamus.

# 4  Chapter 4 – The orexin system as a neurobiological correlate for reward behaviours

As shown in the previous chapter, a repeated injury during early neonatal life can alter exploratory and approach behaviours towards a food reward upon re-injury in adulthood. The aim of this chapter was to investigate whether the orexinergic system of the lateral hypothalamus is a potential neurobiological correlate of this altered behaviour, in an attempt to find a biomarker of the shifted reward-related behaviours.

## *4.1  Introduction - The orexin system*

The orexin system has been the subject of much research over the past decade, and has a suggested role in a variety of systems. For this thesis, it is its proposed role in reward processing that is most intriguing.

### 4.1.1  Orexin peptides and receptors

Orexins A and B (also known as hypocretins 1 and 2) are two small peptides, 33 and 28 amino acids in length respectively. Both are produced from the same precursor peptide, prepro-orexin, by proteolytic cleavage (de Lecea et al., 1998). They were originally identified as ligands that bound to an orphan G-protein coupled receptor (GPCR), now termed orexin receptor-1 ($OX_1R$), with $OX_2R$ then identified as a receptor for orexin B (Sakurai et al., 1998). Orexin A binds to both receptors with an equal affinity, whereas orexin B shows higher affinity for $OX_2R$. The $OX_1R$ couples solely to the $G_q$ subclass of GPCRs, whereas the $OX_2R$ couples to both the $G_q$ and $G_{i/o}$ subclasses. Activation of both causes increased levels of intracellular calcium, resulting in the activation of the $G_q$ pathway to stimulate phospholipase C (PLC). Orexins A and B are unique among hypothalamic peptides, in that they can cause release of both GABA and glutamate, and can therefore influence both fast inhibitory and excitatory neurotransmission (van den Pol et al., 1998). Interestingly, orexins A and B, and their receptors, show a high degree of homology that is conserved across mammalian species, suggesting that the roles performed by orexins may be similar across species (Tsujino and Sakurai, 2009). Figure 4-1 shows a schematic diagram of the orexin peptides and their receptors:
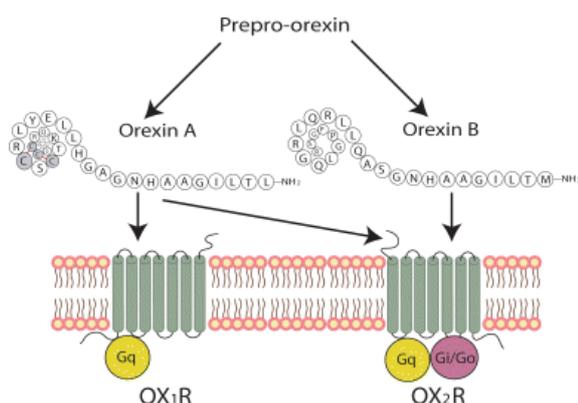
**Figure 4-1 – A schematic representation of the orexin system. Orexins A and B are both produced by cleavage of prepro-orexin, and have slightly different affinities to the $OX_1R$ and $OX_2R$ G-protein coupled receptors. Both GPCRs are coupled to the $G_q$ pathway, and $OX_2R$ is also coupled to the $G_{i/o}$ pathway. Taken from Holland and Goadsby (2007).**

## 4.1.2 Distribution and projections of orexinergic cells, peptides and receptors

Orexins are produced exclusively by just a few thousand cells located in the perifornical and lateral parts of the hypothalamus (PFA and LH) (see Boutrel and de Lecea, 2008). Cells project widely throughout the entire neuraxis, sending particularly dense connections to the paraventricular thalamic nucleus (PVN), arcuate nucleus of the hypothalamus, and locus coeruleus (LC) (Peyron et al., 1998; Nambu et al., 1999). In addition, orexinergic pathways from the hypothalamus project to the ventral tegmental area (VTA) and nucleus accumbens (NAcc), and from there to prefrontal cortices (see Figure 4-2) (Fadel and Deutch, 2002). These projections are particularly important for the orexins' role in reward processing (see Aston-Jones et al., 2010).

The orexinergic cells of the hypothalamus receive inputs from the parabrachial nucleus, medial and lateral preoptic areas, basal forebrain, central amygdala, and bed nucleus of the stria terminalis (BNST), but are also reciprocally connected with many of the LH's output areas i.e. the VTA and dorsal/medial parts of the hypothalamus, as well as laminae I, II and X of the spinal cord (van den Pol, 1999).
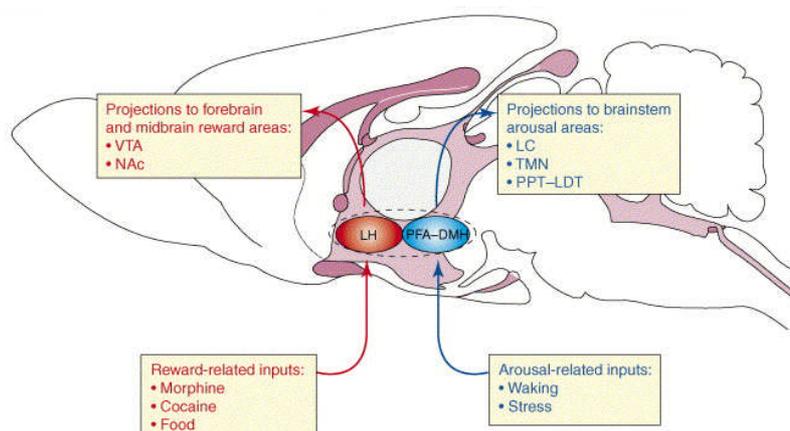
**Figure 4-2 – A schematic diagram to show the orexinergic projections from the lateral (LH) and perifornical-dorsomedial (PFA-DMH) hypothalamus, and their discrete roles. LH cells are activated by reward-predicting stimuli and project to mesolimbic dopaminergic neurons i.e. the ventral tegmental area (VTA) and nucleus accumbens (NAc). PFA-DMH neurons are activated by arousal-related stimuli, and project to the locus coeruleus (LC), pedunculopontine nucleus and lateral dorsal tegmental nucleus (PPT-LDT), and tuberomammillary nucleus (TMN), which are involved in regulation of circadian rhythms and stress. From Harris and Aston-Jones (2006).**

Orexin receptors (both 1 and 2) are found throughout the brain and spinal cord, with higher expression in areas involved in regulation of feeding, sleep, autonomic control and memory (Trivedi et al., 1998; Tsujino and Sakurai, 2009).

Outside of the central nervous system, orexin peptides and their receptors are also found in the enteric nervous system, the neuroendocrine system, pancreas, stomach, heart, kidneys, testes, and adrenal glands, suggesting a broader role of orexins in brain-gut axis control, cardiovascular function, and reproduction (Korczynski et al., 2006). Clearly, orexin plays an important part in a wide array of physiological functions.

Orexin peptide and receptor mRNA is present in the rat at an early stage during development. Orexin B mRNA is present from embryonic day (E) 19, and orexin peptide mRNA is found in the LH cells of newborn rat pups. In addition, application of orexins to LH tissue in postnatal day (P) 1 to 14 animals shows that both orexins are profoundly excitatory (van den Pol et al., 2001). This shows that orexins are present and excitatory during the first few weeks of postnatal life, and therefore may be involved in tuning the developmental control of arousal, feeding and reward processing. Research has shown that the first few weeks of postnatal life are a critical period of development

in an organism's life (see Fitzgerald, 2005), therefore external interference during early postnatal development, i.e. by repeated injury, has the potential to cause imbalances in orexin signalling that could persist into adulthood.

### 4.1.3 Dual roles for orexins in arousal and reward

It is generally accepted that the orexins play major roles in both reward and arousal (see de Lecea et al., 2006; Boutrel and de Lecea, 2008). Much evidence suggests that these functions are regulated by separate sub-divisions of the hypothalamus - the perifornical-dorsomedial areas (PFA-DMH) and the lateral hypothalamus (LH) (Fadel and Deutch, 2002; Harris and Aston-Jones, 2006; Aston-Jones et al., 2010).

### 4.1.3.1 Arousal and the role of orexins

Chemelli et al (1999) first showed that orexins have a crucial role in regulating the sleep-wake cycle by showing that mutation of the prepro-orexin gene produced mice with a phenotype remarkably similar to that of narcolepsy in humans. Narcolepsy is a chronic neurological disorder, characterised by sudden, intrusive episodes of sleep that interrupt normal waking. The authors suspected that, although the hypothalamus was classically implicated in regulation of homeostatic mechanisms (Bernardis and Bellinger, 1993), the neuroanatomical distribution of orexins suggested a possible role for them in the sleep-wake cycle (Peyron et al., 1998; Date et al., 1999). Indeed, the authors found that orexin knockout (KO) mice displayed sudden episodes of REM sleep (characterised by EEG/EMG recordings) during their awake phase. Mutations of the $OX_2R$ gene that caused narcolepsy in dogs (Lin et al., 1999), and the discovery that human narcoleptics have few orexin cells and negligible levels of orexin in cerebrospinal fluid (Siegel, 2004), cemented the orexins' roles in maintenance of the waking state.

Narcoleptic patients are widely treated with highly addictive amphetamine-related drugs, yet rarely become addicted (Harris and Aston-Jones, 2006). In addition, orexin KO mice do not develop morphine dependence to the same extent as wild-type animals (Georgescu et al., 2003), and display dopaminergic hypo-function to psychostimulants (Mori et al., 2010). These findings suggest that orexin has a role in addiction and reward processing – a role that has been investigated, confirmed and widely studied in the last decade.

## 4.1.3.2 Orexins and reward processing

The lateral hypothalamus had been known as a crucial area for reward processing for decades before the orexins were discovered. Experiments in the 50s and 60s had shown that self-stimulation of the lateral hypothalamus is rewarding for an animal (Olds and Milner, 1954; Olds, 1958; Olds, 1962). Self-stimulation of the LH is more robust than that of stimulation in other areas (see Gallistel et al., 1981), and self-administration of opioids directly into the LH had also been shown (Cazala et al., 1987). Identification of orexins within these cells hinted that they may have a role in reward.

### *4.1.3.2.1 Cue-drug association learning*

The role for orexins in reward processing was firmly established by two papers using a conditioned place preference (CPP) paradigm to investigate reward function, suggesting that orexins are crucial for the learning involved in associating a cue (i.e. a CPP chamber) with a drug reward.

Georgescu and colleagues (2003) showed that orexin neurons of the lateral, but not more medial hypothalamus, contain mu opioid receptors (MORs) and respond to chronic morphine. Using CRE-LacZ transgenic mice, increased LacZ gene expression was seen in orexin cells after chronic morphine. In addition, orexin KO mice developed attenuated morphine-induced CPP. The authors suggested a role for the orexin system in molecular adaptations to morphine.

Harris et al (2005) further cemented the orexins' role in reward processing by showing that activation of the LH orexin cells, but not PFA-DMH orexin cells, was strongly linked to preferences for cues associated with both drug (morphine and cocaine) and food (i.e. natural) rewards. Using a CPP paradigm, the authors showed that the activation of orexin cells, as shown by co-expression of Fos (a protein product of *c-fos*, an immediate early gene), was strongly correlated to the strength of preference animals displayed towards a drug or food-paired chamber.

Later studies showed again that morphine-induced CPP stimulates LH orexin neurons during conditioning, but only when the morphine is administered in a novel drug-paired environment – home-cage administration or a novel environment alone will not induce

CPP (Harris et al., 2007b). Antagonism of orexin signaling (using SB 334867 (SB), an orexin A antagonist) in the VTA, a site receiving orexin projections, prevented learning of morphine-induced CPP, but not expression of previously learnt associations (Narita et al., 2006). In addition, disconnection of the LH to the VTA, by unilateral VTA lesion and concurrent injection of SB into the opposite VTA region, showed that CPP could be blocked (Harris et al., 2007b). Taken together, this evidence showed the importance of the connection of the LH to the VTA, and suggested a role for orexins in learning cue-drug associations.

### 4.1.3.2.2 Abstinence

Abstinence is a reward-related phenomenon in which orexins may play a role. Chronic drug administration followed by forced abstinence causes dramatic increases in preference for the previously administered drug, but a decrease in preference for alternative drugs or natural rewards i.e. food, and causes long-term changes in reward sensitivity (Markou and Koob, 1991; Epping-Jordan et al., 1998; Aston-Jones and Harris, 2004). Regions that show increased Fos expression (i.e. increased cell activation) during abstinence include the basolateral amygdala, shell of the nucleus accumbens (NAcc) and LH – activation of which has been localized to the orexinergic cells (see Aston-Jones et al., 2009). Other studies have confirmed an increase in orexin activation and orexin mRNA levels during abstinence and withdrawal (Georgescu et al., 2003; Zhou et al., 2008), suggesting that orexinergic signalling may be driving drug-seeking behaviours in animals in a withdrawn state.

### 4.1.3.2.3 Reinstatement

A further role for orexins comes from investigating the phenomenon of reinstatement, where an animal shows drug-seeking behaviours to previously extinguished cues. Extinction is performed by repeatedly pairing an addicted animal to a previously drug-paired CPP chamber, until the animal no longer displays drug-seeking behaviours. Reinstatement is posited to model relapse in human addicts (Yahyavi-Firouz-Abadi and See, 2009).

Harris et al (2005) showed that artificially activating the orexin neurons (using rat pancreatic peptide (rPP) – a Y4 agonist that activates Y4 receptors on orexin cells) reinstated an extinguished drug or food-seeking behaviour, an effect that was

completely blocked by prior administration of the orexin antagonist SB. Furthermore, injection of orexin A directly into the VTA reinstated previously extinguished morphine-seeking behaviours (Harris et al., 2007b). Recent studies have shown that orexin administration causes reinstatement of nicotine-seeking behaviour (Plaza-Zabala et al., 2010), and blockade of orexin signalling attenuates cue-induced reinstatement of extinguished cocaine and ethanol-seeking (Smith et al., 2009; Lawrence et al., 2006), showing that the orexins are involved in modulation of the response to a range of addictive drugs.

### 4.1.3.3 Orexins strengthen the synaptic plasticity of dopamine cells in the VTA and therefore affect the mesolimbic dopamine system

Glutamatergic transmission in the VTA (the origin of the mesolimbic dopaminergic system) activates dopamine (DA) cells, and DA cell activation and plasticity of neuronal output is implicated in drug reward and addiction (Hyman et al., 2006). Drugs of abuse increase DA cell firing rates, leading to potentiation of synapses that lasts over a long period (termed long-term potentiation – LTP). LTP is characterised by insertion of glutamatergic NMDA (N-methyl-D-aspartic acid) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate) receptors into the post-synaptic cell membrane. Electrophysiological studies investigating the firing properties of VTA DA cells receiving orexin inputs have shown that orexin interacts with glutamatergic signaling in DA cells, suggesting a potential influence on drug abuse behaviour.

Korotkova et al (2003) first showed that orexins excite DA cells in the VTA. Later, Borgland et al (2006) co-administered the orexin antagonist SB along with cocaine, and showed that the glutamate-dependent LTP usually seen after cocaine administration was blocked. In addition, application of orexin A to midbrain slice preparations caused LTP at DAergic synapses due to the insertion of postsynaptic NMDA receptors, thereby facilitating LTP. Orexin B also potentiates NMDA receptor function postsynaptically, as well as increasing glutamatergic transmission to VTA neurons via a PKC-dependent mechanism (Borgland et al., 2008). Finally, orexin cells selectively promote motivation for positive reinforcers (such as high fat food) via the strengthening of VTA synaptic connectivity, but do not produce any synaptic strengthening after aversive stimuli such as footshock (Borgland et al., 2009).

The PFC, in particular the medial subdivision (mPFC), is an important region for regulating goal-directed behaviours and impulse control, is linked to reward and addictive behaviours (Kalivas and McFarland, 2003) and is strongly reciprocally connected to the VTA. Intra-VTA infusion of orexin A caused increased extracellular DA levels in the prefrontal cortex (PFC), indicating that orexin signalling in the VTA, particularly the caudal subdivision, causes activation of the mesolimbic dopaminergic system (Vittoz and Berridge, 2005; Vittoz et al., 2008).

As orexin neurons are stimulated by reward-associated cues, it is possible that orexins and glutamate, released by the LH and mPFC respectively, may be acting upon DA neurons in the VTA simultaneously, placing orexins in an important neuromodulatory position to augment mPFC inputs and strengthen learning of cue-drug associations (see Aston-Jones et al., 2010). Taken together, these data suggest that orexin neurons of the LH are necessary for the drug-induced neuroplasticity at glutamatergic synapses in the mesolimbic dopaminergic system.

### 4.1.3.4 Overlapping arousal and reward functions

Stress responses are dependent on the activation of arousal mechanisms, and studies of orexin cells in medial hypothalamic areas have shown a role for orexins in stress-associated reward behaviours i.e. stress-induced drug reinstatement (see Boutrel and de Lecea, 2008). There is much reciprocal connectivity between the orexin cells spread across the PFA-DMH and the LH, which is not surprising as the motivation to maintain internal homeostatic states and seek reward depends heavily on the arousal state of the animal.

When an organism is subject to a stressor, the hypothalamo-pituitary-adrenal (HPA) axis is activated. Synthesis of corticotrophin-releasing factor (CRF) is induced in the paraventricular nucleus (PVN) of the hypothalamus, and CRF in turn stimulates pituitary corticotroph cells to produce adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal glands to produce adrenaline and cortisol, causing behavioural arousal i.e. increased heart rate and blood pressure, and increased activity levels (see Figure 4-3).
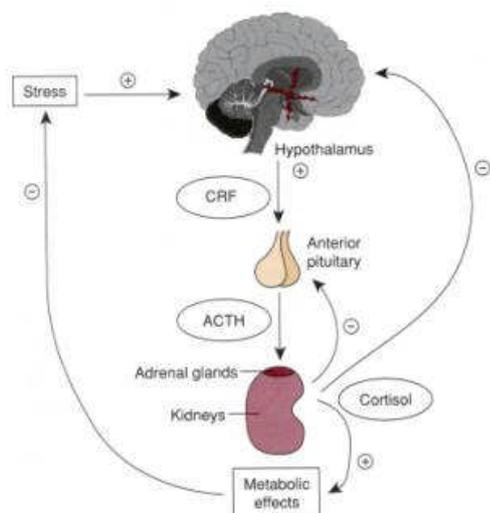
**Figure 4-3 – A schematic diagram of the HPA axis in humans. Hypothalamic cells produce CRF in response to a stressor, causing pituitary cells to produce and release ACTH. This, in turn, stimulates the adrenal glands, which secrete cortisol. Cortisol provides negative feedback to the hypothalamus and pituitary to decrease CRF production and release, and terminate the stressful response. Adapted from http://www.montana.edu/wwwai/imsd/alcohol/Vanessa/vwhpa.htm.**

CRF-containing terminals form synapses onto PFA-DMH orexin cells, and can directly depolarize these cells – an effect lost in CRF-receptor KO mice (Winsky-Sommerer et al., 2004). CRF blockade also prevents the orexin-induced reinstatement of cocaine-seeking behaviour (Boutrel et al., 2005). Furthermore, CRF release from cells in the PVN in response to nicotine administration, which is normally anxiogenic, was prevented by orexin antagonism within the PVN (Plaza-Zabala et al., 2010).

Together, these results show that components of the HPA axis and orexin system are closely linked, and may help to explain the increase in orexin mRNA seen after drug withdrawal, as the animal is subjected to the stressful and aversive state of abstinence.

## 4.1.3.5 Orexins and feeding behaviour

The name 'orexin' arises from the term 'orexigenic', meaning to stimulate appetite, and was given to the orexins due to their ability to induce feeding behaviour when injected intracerebroventricularly (i.c.v.) or to specific hypothalamic sites in rats (Sakurai et al., 1998; Dube et al., 1999). In addition, it was found that levels of the mRNA encoding prepro-orexin increase during fasting, pointing to an important role in regulation of appetite (Sakurai et al., 1998). Later studies found that orexin cell activation also drives reward-seeking of a high fat food in satiated rats (Harris et al., 2005; Borgland et al., 2009) and mice (Sharf et al., 2010), providing a strong suggestion that orexin cell activation may affect the seeking and/or consumption of the food treats used as a reward in the experiments described in this thesis.

The role of orexins in feeding behaviour is mediated by the connectivity of the LH to the NAcc (Stratford and Kelley, 1999). Orexin A injection into the NAcc shell induced feeding behaviour (Thorpe and Kotz, 2005), and stimulation of the NAcc shell caused high-fat feeding behaviour which depended on orexin signalling in the VTA (Zheng et al., 2007).

Further evidence linking orexin and motivation for feeding of high fat food comes from the study of "reward-based feeding behaviour", or food consumption beyond homeostatic needs. Activation of orexin cells was shown in animals that were expecting their daily meal, and upon exposure to a cue related to a conditioned palatable food (chocolate) in sated rats (Choi et al., 2010). In addition, Choi et al found that administration of i.c.v. orexin A increased food intake, and caused an increase in the progressive ratio break-point for food pellets i.e. the point at which rats discontinued lever pressing to obtain a food reward. These effects were reversed after orexin antagonism.

The role of orexins in feeding behaviour, particularly the motivation to obtain palatable foods when the animal is sated, places orexin as a strong candidate to reflect the behaviours monitored in the novelty-induced hypophagia (NIH) paradigm, which contains a sweet, sugary foot treat at its centre.

## 4.1.3.6 Orexins play a role in nociception

The orexin cells of the lateral hypothalamus are reciprocally connected to areas involved in nociceptive processing, including the periaqueductal grey (PAG), parabrachial nucleus (PBN) and laminae I, II and X of the spinal cord (Peyron et al., 1998; van den Pol, 1999). Lateral hypothalamic stimulation inhibits spinal cord neuronal responses to peripheral noxious stimulation (Carstens et al., 1983; Carstens, 1986), and orexins A and B have analgesic properties in animal models of postoperative pain (Cheng et al., 2003), neuropathic pain (Yamamoto et al., 2003a; Suyama et al., 2004), diabetic neuropathy (Kajiyama et al., 2005), and inflammatory pain models (Yamamoto et al., 2003b), when administered both intrathecally (i.t.) and i.c.v. (Bingham et al., 2001; Mobarakeh et al., 2005). Orexins have also been linked to cluster headaches and migraine (see Holland and Goadsby, 2007).

Bingham et al (2001) suggest the existence of an orexinergic descending inhibitory system, whereby orexins modulate spinal cord processing to reduce ascending excitation. The authors suggest that this functions in a manner similar to, but independent from, endogenous opioids, as naloxone had no effect on orexin A's analgesic actions. Orexins have been shown to excite both excitatory relay neurons in the spinal cord and inhibitory GABAergic neurons in the brainstem (Siegel, 2004; Liu et al., 2002), so to extend this theory, it is possible to speculate that orexins directly activate ascending projection cells in the spinal cord, and simultaneously activate inhibitory GABAergic neurons, which dampen the ascending nociceptive response. When coupled with descending inhibition from the brainstem which utilises endogenous opioids (Fields, 2007), this could greatly enhance inhibitory modulation. Support for this theory comes from prepro-orexin KO mice, which show no differences in mechanical or thermal nociceptive withdrawal thresholds at baseline, but demonstrate greater hyperalgesia after peripheral inflammation (when descending inhibitory mechanisms are presumably activated) compared to wild-type animals (Watanabe et al., 2005).

The evidence suggests that orexins may play an important modulatory role in spinal pain transmission and contribute to descending inhibitory drives, further highlighting the potential of orexins as neurobiological correlates of behaviour in animals subjected to repeated neonatal and adult painful stimuli.

In summary, the role of orexin cells in reward processing, stress, arousal, and nociception places them in a strong position as candidates for reflecting the behavioural changes seen after adult injury to neonatally-injured animals.

## 4.2 Hypotheses

In this chapter, I test the following hypotheses:

- That the behavioural changes seen in animals after a repeated early injury in neonatal life will be correlated with activation of the lateral hypothalamic orexin cells, identifying orexin as a neurobiological correlate of reward-related behaviours in injured animals.

- That, as with the behavioural changes seen, the results will be more pronounced in neonatally-injured animals that are re-injured in adulthood.

- That orexin cell activation will be seen in relation to presentation of a food reward, Cheerios breakfast cereal, in the NIH arena.

## *4.3 Methods*

The experiments described in this chapter were to investigate the neurobiological correlate of behaviour in the novelty-induced hypophagia (NIH) paradigm in animals with a history of foot wound injury as neonates, after re-injury in adulthood. The orexin system of the lateral hypothalamus was investigated as a potential system for reflecting these effects. Activation of the orexin system was shown by the co-expression of c-Fos in orexin-positive cells. C-Fos is a protein product of *c-fos*, an immediate early gene. It is restricted to the nucleus, and was used in these experiments as a marker of cell activation, with maximal expression expected at 90-120 minutes post-stimulus (see Kovács, 2008). It has been widely used as a marker of cell activation in experiments investigating the roles of orexins (Georgescu et al., 2003; Harris et al., 2005; Harris et al., 2007b; Choi et al., 2010).

### 4.3.1 Experimental design

The tissue for the experiments in this chapter originated from the animals tested in the novelty-induced hypophagia paradigm, as described in the previous chapter. Animals were repeatedly injured (plantar skin incision) or anaesthetised as neonates (on postnatal days (P)3, P10 and P17), and adult behaviour upon testing in the NIH arena was studied. As described previously in Chapter 3, the NIH arena is a behavioural paradigm for testing anxiety, exploratory and reward-related behaviours. Animals were tested in the arena and then returned to their home cages before tissue collection. See Chapter 3 Methods for explanation of foot wound surgery and the NIH arena. The designs for the experiments in this chapter are explained below:

## 4.3.1.1 Experiment 1: Investigating activation of the orexin system after single adult foot wound in animals with no history of injury

To see the effect that a single adult foot wound has on orexin cell activation in animals with no history of injury, tissue from animals that were repeatedly anaesthetised as neonates was compared when the animals were given a single foot wound or re-anaesthetised as adults, and tested in the NIH arena. Figure 4-4 illustrates the design of this experiment:
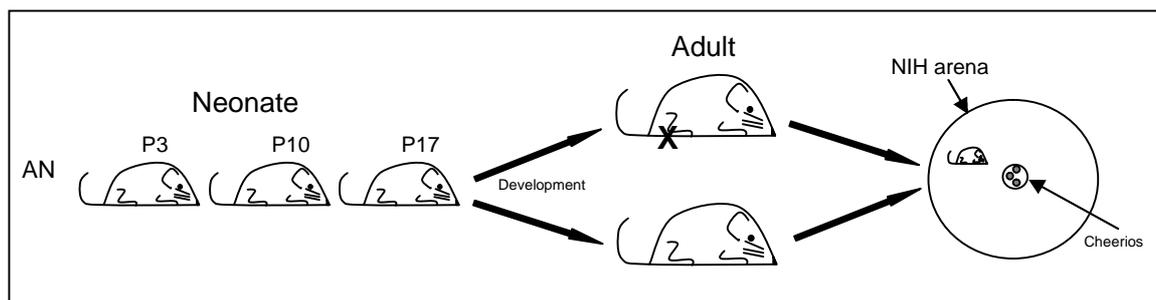


**Figure 4-4 – A schematic diagram to illustrate the design of Experiment 1. Animals repeatedly anaesthetised as neonates were injured or re-anaesthetised as adults, and orexin activation after testing in the NIH arena investigated.**

## 4.3.1.2 Experiment 2: Investigating baseline activation of the orexin system in animals with a history of repeated neonatal injury

To see what effect testing in the NIH arena had at baseline on the orexin system, tissue from neonatally foot-wounded animals or anaesthesia-only controls was compared. Figure 4-5 is a schematic diagram to illustrate the design of this experiment:
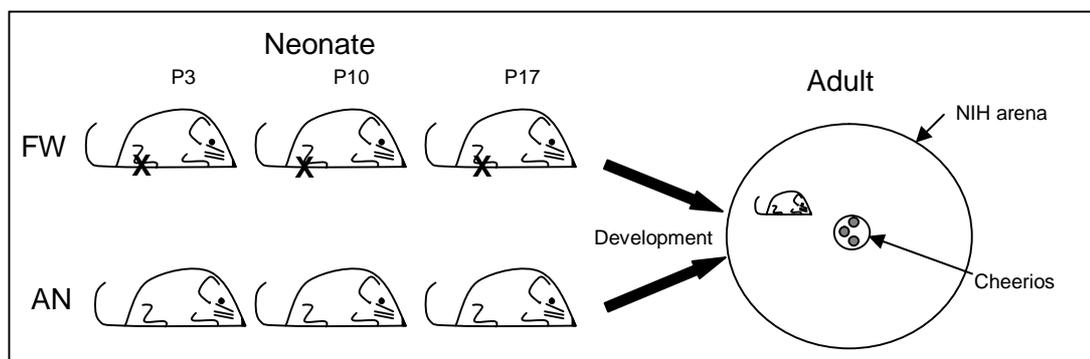


**Figure 4-5 – A schematic diagram to show the design for Experiment 2. The activation of the orexinergic system was compared after testing in the NIH arena between animals that received repeated neonatal foot wound or anaesthesia, but received no adult treatment.**

## 4.3.1.3 Experiment 3: Investigating activation of the orexin system after adult foot wound in animals repeatedly injured as neonates

To see what effect a history of injury had on adult orexin activation after testing in the NIH arena, tissue from animals that were repeatedly wounded as neonates was compared when animals were re-injured or anaesthetised as adults. Figure 4-6 illustrates the design of this experiment:
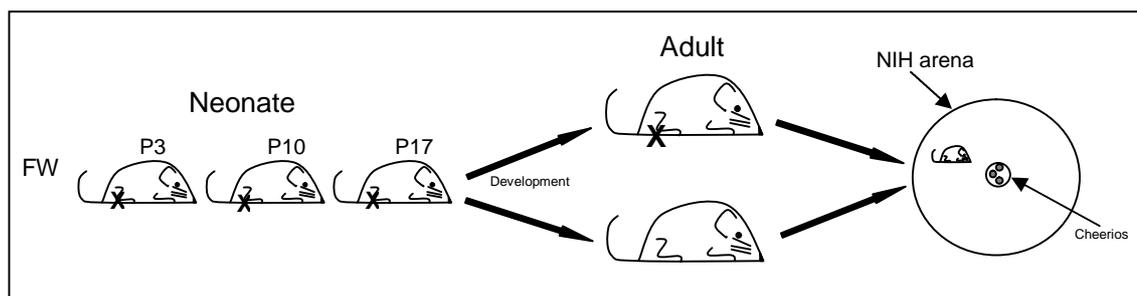


**Figure 4-6 – A schematic diagram to illustrate the design of Experiment 3. Animals repeatedly injured as neonates were re-injured or anaesthetised as adults, and orexin activation after testing in the NIH arena investigated.**

## 4.3.1.4 Experiment 4: Investigating the effect of Cheerio consumption on the lateral hypothalamic orexin system.

To investigate whether consumption of Cheerios or the presence of a food reward activates orexin cells, two additional comparisons of orexin cell activation were performed.

### *4.3.1.4.1 Experiment 4a*

Firstly, tissue from animals that had not consumed any Cheerios during the 15 minute NIH testing period was compared to tissue from animals that did consume Cheerios.

Neonatally foot-wounded males, uninjured in adulthood, were used for this comparison. Out of 19 animals in this group, 7 did not consume any Cheerios. A Fisher's Exact test showed that this was a significantly higher proportion of non-consuming animals than was expected (p=0.008). Figure 4-7 is a schematic to show the design of this experiment:
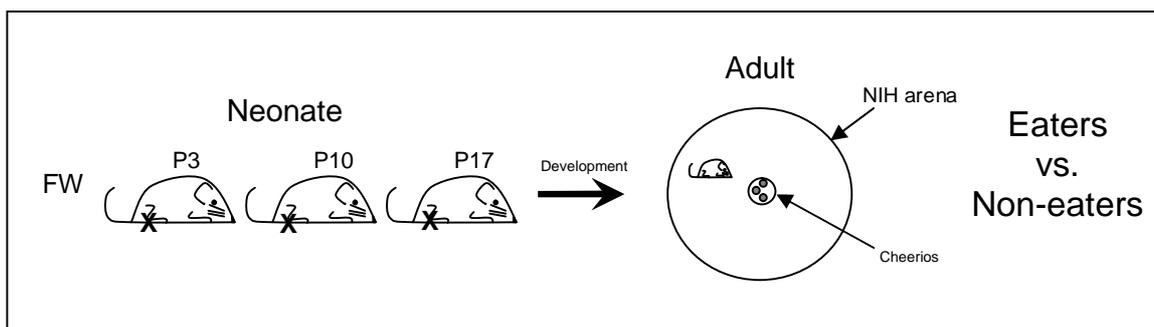
**Figure 4-7 – A schematic diagram to illustrate the design for Experiment 4a. Repeatedly neonatally-injured males were tested in the NIH paradigm in adulthood, and the activation of the orexin system was compared between animals that consumed Cheerios during the NIH test, and those that did not.**

### 4.3.1.4.2 Experiment 4b

Secondly, the activation of orexin cells was compared in tissue from animals that had access to Cheerios in the NIH arena to tissue from animals that had no access to Cheerios in the NIH arena. Figure 4-8 is a schematic to show the design for this experiment:
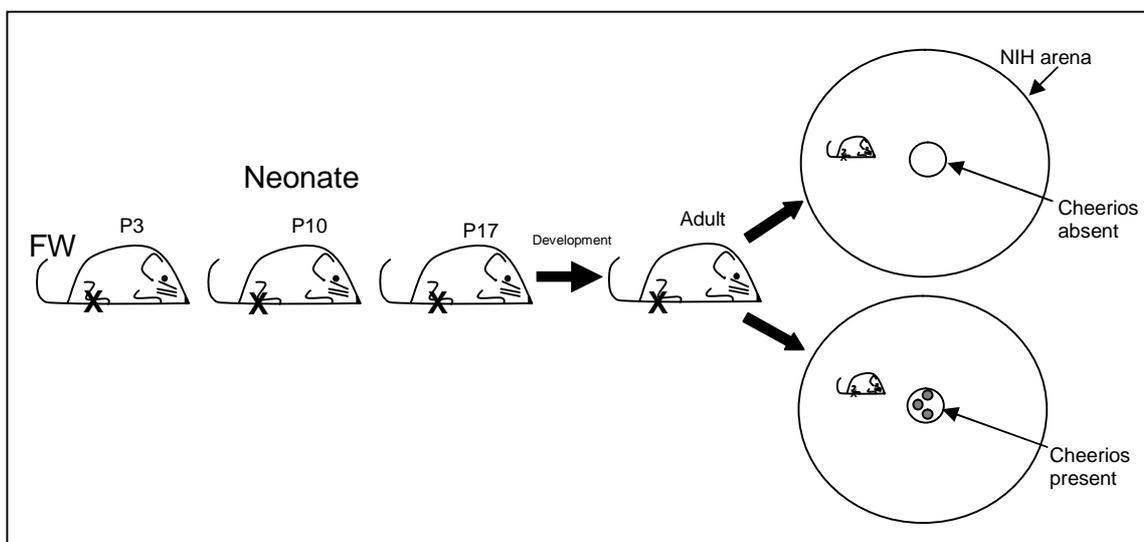


**Figure 4-8 – A schematic diagram to illustrate the experimental design for Experiment 4b. Animals that were repeatedly injured as neonates were re-injured as adults, and tested in the NIH arena either in the presence or absence of Cheerios in the central food bowl. Activation of the orexin system was compared.**

For all the above experiments, tissue was harvested two hours after testing in the NIH arena. This is because it is the maximal time point of c-Fos expression and is a time

point widely used in other experiments investigating orexin cell activation (Georgescu et al., 2003; Harris et al., 2005; Harris et al., 2007b; Choi et al., 2010).

Animals were sacrificed by intraperitoneal (i.p.) injection of an overdose of Euthatal (Merial Animal Health Limited, Harlow, UK), and transcardially perfused with 4% paraformaldehyde (PFA – see Appendix 1: Solutions). Brains were dissected out, post-fixed in 4% PFA for 4 hours, then transferred to a 30% sucrose and azide solution (see Appendix 1) and stored at 4°C.

### 4.3.2  Definition of the lateral hypothalamus

The co-ordinates of the lateral hypothalamus (LH), as defined by the stereotaxic atlas of Paxinos and Watson (2004), are as follows:

<div align="center">

From Bregma:

Anteroposterior: -3mm to -5mm

Dorsoventral: -7mm to -9mm

Mediolateral: +1.5mm to 3mm

</div>

This is a large region, so previous studies have further defined the area as lateral to the fornix (Harris et al., 2005; Aston-Jones et al., 2009). Figure 4-9 illustrates the definition and localisation of the lateral hypothalamus, as used for these experiments:
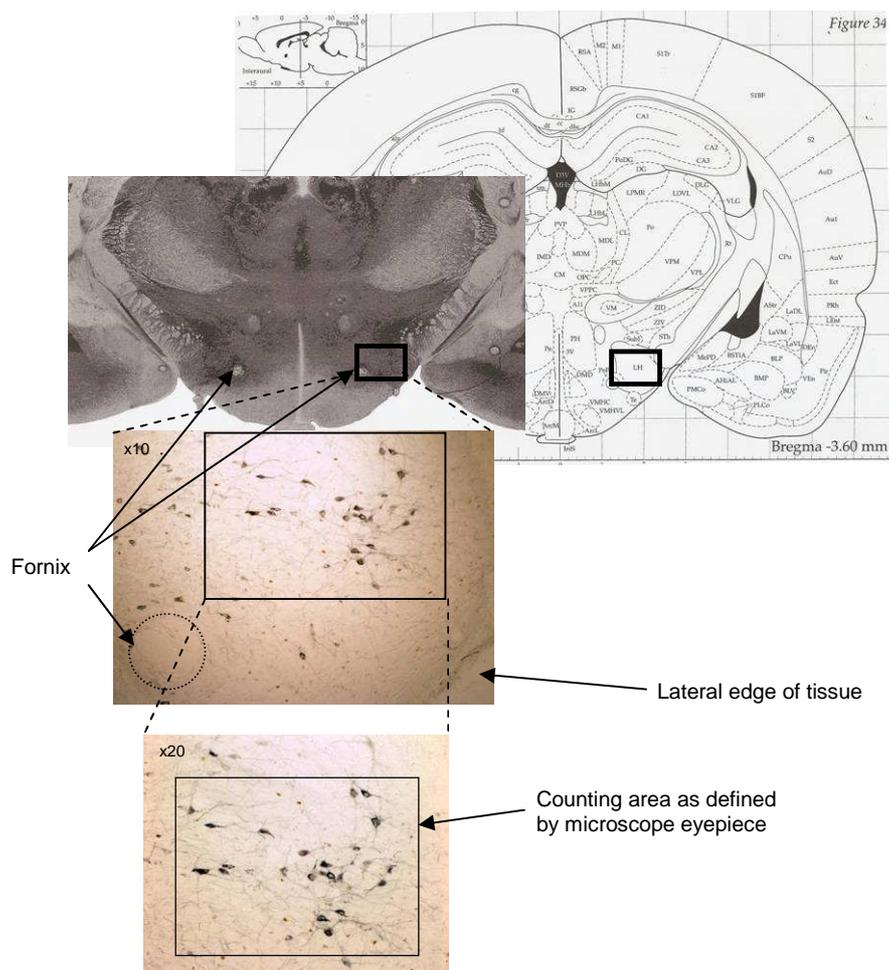
**Figure 4-9 – A figure to show the localization and definition of the lateral hypothalamus for cell counting purposes. The top two panels show the LH, outlined by a black rectangle, as defined by the stereotaxic atlas of Paxinos and Watson (2004). The lower two panels show x10 and x20 magnifications of the LH. Black cytoplasmic staining shows orexin-positive cells. The dotted circle indicates the fornix; cell counting was performed laterally to this landmark. The black rectangle in the x20 magnification (bottom panel) represents the counting area as defined by the microscope eyepiece.**

### 4.3.3 Immunohistochemistry

The following protocol was used for immunostaining:

- 40µm sections of the lateral hypothalamus were cut on the freezing microtome and placed into wells containing 5% sucrose in azide (see Appendix 1: Solutions).

- Sections were transferred to Röhren tubes (Sarstedt, Leicester, UK), 6 sections per tube, each at a distance of 240µm from the last, and blocked in 5% horse serum plus hydrogen peroxide for 1 hour (see Appendix 1).

- C-Fos primary antibody (Calbiochem, Nottingham, UK) was placed onto the sections at a 1:10,000 dilution (1µl per 10ml) in TTBS (see Appendix 1). The tubes were incubated overnight at room temperature.

- Sections were washed with 0.1M PB (see Appendix 1), 3 times, for 10 minutes per wash.

- The c-Fos secondary antibody was placed onto the sections. This was biotinylated anti-rabbit, raised in goat (Vector Laboratories Inc., CA, USA), at a 1:500 dilution (2µl per ml) in TTBS. Sections were incubated for 2 hours at room temperature.

- Sections were washed as above.

- The avidin-biotin protocol was used to amplify the signal. Using the Vectastain ABC kit (Vector Laboratories Inc., CA, USA), 1µl of 'A' and 1µl of 'B' per ml TTBS were stirred for 30 minutes.

- Avidin-biotin solution was placed onto the sections for 1 hour at room temperature.

- Sections were washed, as above.

- To stain the c-Fos, brown DAB (3,3'-diaminobenzidine) staining was performed. The DAB solution was prepared according to the kit instructions (Vector Laboratories Inc., CA, USA). Sections were incubated for ~6 minutes in DAB solution or until staining of nuclei was apparent, transferred to deionised water to end the reaction, then washed with 0.01MPB and 0.1M PB, for two minutes each.

- The orexin-A primary antibody (Santa Cruz Biotechnology Inc., CA, USA) was placed onto the tissue at a concentration of 1:1000 (1µl per ml) in TTBS and incubated overnight at room temperature.

- Sections were washed.

- The orexin secondary antibody was placed onto the sections. This was biotinylated anti-goat, raised in horse (Vector Laboratories Inc., CA, USA) at a dilution of 1:500 (2µl per ml) in TTBS for 2 hours at room temperature.

- Sections were washed.

- Avidin-biotin solution was made up as above and mixed for 30 minutes.

- The AB solution was placed onto the tissue for 1 hour at room temperature.

- Sections were washed.

- To stain the orexin, blue DAB (3,3'-diaminobenzidine) staining was performed. The DAB solution was prepared according to the kit instructions (Vector Laboratories Inc., CA, USA). Sections were incubated for up to 2 minutes in DAB solution or until cytoplasmic staining was apparent, transferred to PBS (see Appendix 1), then washed in deionised water and 0.1MPB for two minutes each.

- Sections were then placed into 0.01M PB for mounting onto gelatinised slides.

- Slides were dried in the dark overnight at room temperature, then dehydrated and coverslipped with Histoclear (Flowgen Bioscience, Nottingham, UK).

### 4.3.3.1 Immunohistochemistry controls

Control tubes of sections were prepared to check for the effects of non-specific binding of the antibodies (where the antibody binds to the tissue at random instead of to the protein of interest). The control tubes underwent the same washing and staining as the experimental tubes at all stages of the protocol, except the sections were not exposed to the antibody at the relevant stage of the protocol, and were placed into 0.1M PB instead.

Tubes were prepared under one of the four following conditions:

1. The c-Fos primary antibody was absent

2. The c-Fos secondary antibody was absent

3. The orexin primary antibody was absent

4. The orexin secondary antibody was absent

Control images are not shown, as staining was so faint that tissue could not be seen.


### 4.3.4 Visualisation and counting of sections

- All counting was done by a blinded experimenter. Unblinding was not performed until all slides had been counted.

- Slides were visualised using a light microscope (Nikon E800). C-Fos staining was seen as brown nuclear staining. Orexin staining was seen as a cytoplasmic dark blue/black stain. Only sections that contained orexin cells were counted.

- The lateral hypothalamus was identified according to the above criteria. Sections were counted bilaterally.

- Cell counting took place at x20 magnification. The viewfinder within the microscope eyepiece defined the area to be counted (see Figure 4-9).

- Orexin-positive cells, c-Fos positive nuclei, and the number of co-expressing cells were counted manually.

- 5 sections per animal were counted, with 5-8 animals per group. Numbers per group are specified in the Results section.

### 4.3.5 Analysis of cell counts

- From raw cell counts, the percentage of orexin-positive cells that were co-expressing c-Fos was calculated.

- Averages for each animal were calculated, and then group averages calculated.

- 2-way ANOVAs were performed with GraphPad Prism version 4 software to investigate group and gender differences. Bonferroni's post-hoc tests were performed to find the direction of any differences.

- Pearson's correlation coefficient was calculated for data that was being correlated with behavioural outcome measures.

- ANOVA and calculation of Pearson's correlation coefficient are parametric methods of analyses, therefore all data was checked to conform to a normal distribution (D'Agostino and Pearson omnibus normality test) prior to analysis. All data passed normality tests.

## *4.4 Results*

The results from the experiments in this chapter show that the orexin system is sensitive to a rewarding stimulus, and that an injury in adulthood, particularly if the animal has a history of neonatal injury, alters the activation of the orexinergic system.

Immunostaining of the lateral hypothalamus was performed after animals had been tested in the novelty-induced hypophagia (NIH) arena. C-Fos (an immediate early gene) was used as a marker of cell activation. All cell counts were performed blind. Orexin cells were cytoplasmically stained, and c-Fos staining was confined to nuclei (see Figure 4-10). Percentage of orexin cells co-expressing orexin and c-Fos are therefore said to be 'activated' orexin cells.
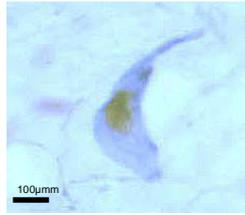
**Figure 4-10 – A representative image of an orexin-positive cell co-expressing c-Fos in the nucleus.**

Representative images of lateral hypothalamic sections from male animals are presented on the next page:

- 'AN' refers to an animal repeatedly anaesthetised as a neonate.
- 'AN+FW' refers to an animal repeatedly anaesthetised as a neonate and receiving a single adult foot wound
- 'FW' refers to an animal that received a repeated neonatal foot wound.
- 'FW+FW' refers to an animal that received a repeated neonatal foot wound and has been re-injured as an adult.
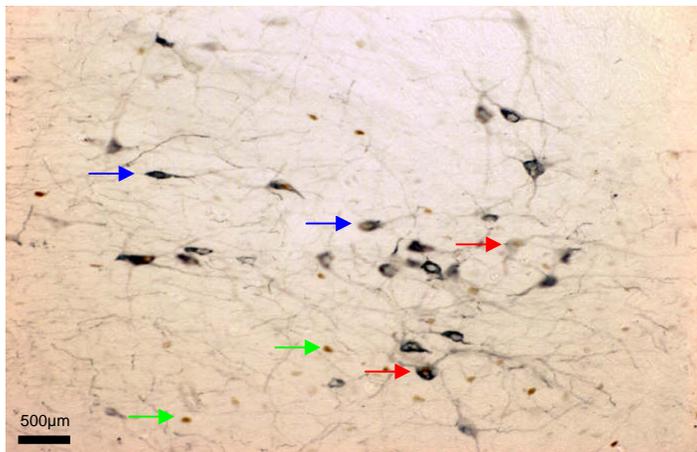
**Figure 4-11 – A representative image of the lateral hypothalamus of a control 'AN' animal. Examples of c-Fos-expressing nuclei are indicated by green arrows, orexin cells by blue. Co-expressing cells are indicated by red arrows.**
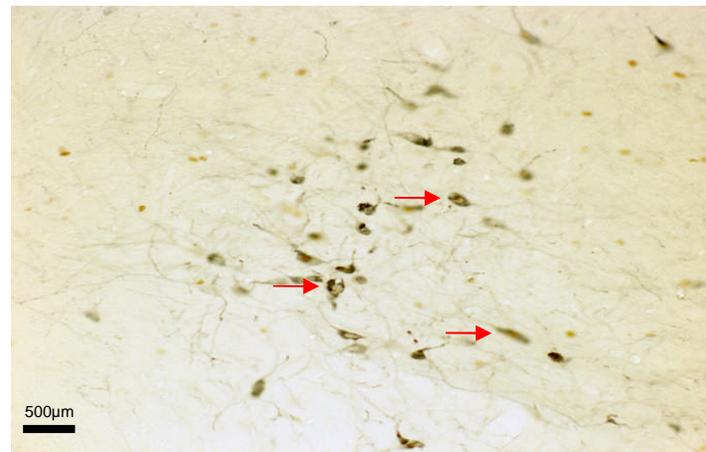


**Figure 4-13 - A representative image of the lateral hypothalamus of a 'FW' animal. Co-expressing cells are indicated by red arrows.**
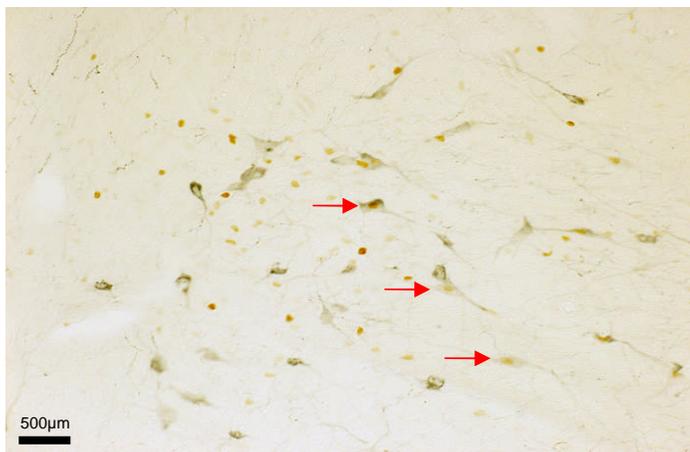


**Figure 4-12 - A representative image of the lateral hypothalamus of an 'AN + FW' animal. Co-expressing cells are indicated by red arrows.**
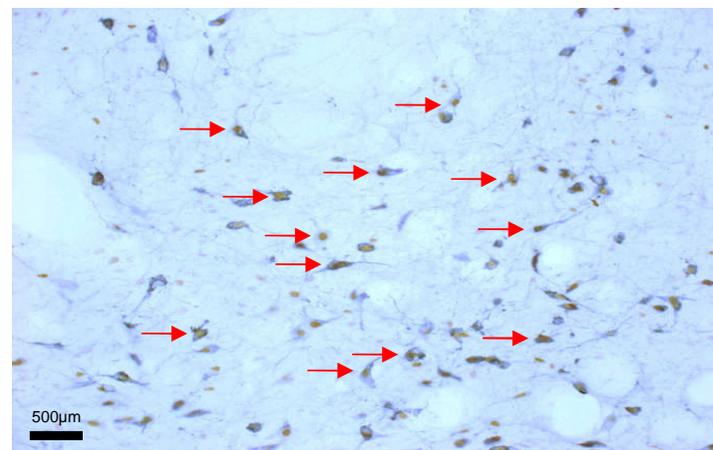


**Figure 4-14 - A representative image of the lateral hypothalamus of a 'FW+FW' animal. Co-expressing cells are indicated by red arrows.**

## 4.4.1 Experiment 1: Orexin cell activation is not affected by a single adult foot wound in animals with no history of injury, and does not correlate with reward-related behaviours

As shown in Chapter 3, a single adult foot wound causes changes in behaviour - rearing is decreased and the percentage of time spent in the central zone increases. The activation of the orexin system was investigated in these animals to see if the orexin system reflects the behavioural changes seen.

Percentage of orexin cells co-expressing c-Fos was calculated in animals that had no history of foot wound and were either injured or re-anaesthetised as adults, after the animals were tested in the NIH paradigm. 'AN' refers to animals that were repeatedly anaesthetised as neonates. 'AN+FW' refers to neonatally anaesthetised animals that received a single adult foot wound. Animals that did not eat any Cheerios were excluded from the analysis.

Table 4-1 shows the raw data generated from this experiment. There was a significant effect of treatment on the number of orexin cells ($F_{(1,23)}=4.89$, $p<0.05$; 2-way ANOVA), which showed that adult-injured animals (both genders) had more orexin cells per section than animals with no adult injury. However, there was no significant effect of gender or treatment on the percentage of orexin cells co-expressing c-Fos, as illustrated in Graph 4-1. This data shows that the activation of orexin cells induced by testing in the NIH arena is not changed by a single adult foot wound in animals with no history of neonatal injury.

| Treatment group | Number | Orexin-positive | c-Fos-positive | Number of co-expressing cells | % orexin cells co-expressing c-Fos |
|---|---|---|---|---|---|
| M AN | 6 | 42.4±3.7 | 31.6±6.9 | 6.5±1.4 | 16.4±4.5 |
| M AN+FW | 7 | 54.6±5.2 | 28.1±3.2 | 9.8±1.3 | 18.4±2.8 |
| F AN | 7 | 40.1±3.2 | 44.2±7.0 | 9.4±2.4 | 22.2±4.0 |
| F AN+FW | 7 | 54.1±9.1 | 41.2±7.5 | 10.9±1.9 | 16.7±2.8 |

**Table 4-1 – A table to show the treatment groups, number of animals per group, number of orexin- and c-Fos-positive cells, and number and percentage of orexin-cells co-expressing c-Fos in animals with no history of injury, injured (AN+FW) or not (AN) as adults.**
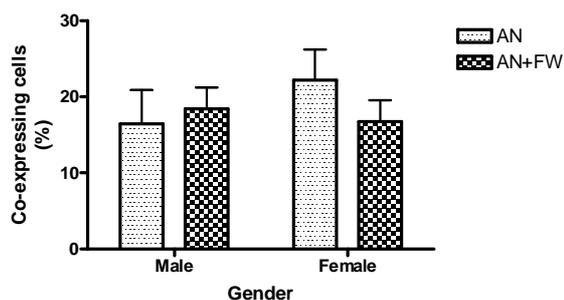
Graph 4-1 – A graph to show the percentage of orexin cells co-expressing c-Fos in animals with no history of injury (AN), compared to those with no history of injury given a single adult foot wound (AN+FW).

To investigate if the activation of orexin cells is correlated to the outcome measures that were significantly affected by a single adult injury, namely rears and the percentage of time spent in the central zone (both in the 5-10 minute time bin), Pearson's correlation coefficient (R) was calculated for both rears and central zone percentage, in males and females with no history of injury, either with or without a single adult foot wound.

There were no significant correlations between activation of orexin cells and the two chosen behavioural outcome measures in either gender or after any treatment, suggesting that the orexin system does not reflect behavioural changes in animals with no history of repeated neonatal injury. These data are shown in Table 4-2.

| Treatment group | Rears | | Time in central zone (%) | |
|---|---|---|---|---|
| | Pearson's R | P value | Pearson's R | P value |
| M AN | 0.55 | 0.25 (n.s.) | -0.48 | 0.33 (n.s) |
| M AN+FW | -0.16 | 0.73 (n.s.) | 0.32 | 0.49 (n.s.) |
| F AN | 0.25 | 0.59 (n.s) | 0.61 | 0.15 (n.s.) |
| F AN+FW | 0.22 | 0.64 (n.s.) | -0.16 | 0.74 (n.s.) |

Table 4-2 – A table to show Pearson's correlation coefficient for the number of rears/percentage of time spent in the central zone, both in the 5-10 minute time bin, correlated to the percentage of orexin cells co-expressing c-Fos, in animals with no history of injury.

## 4.4.2 Experiment 2: The presence of a 'pain history' does not alter baseline orexin cell activation, or correlate with reward-related behaviours

Next, to investigate the effect a history of injury has on baseline activation of the orexin system, the percentage of orexin cells co-expressing c-Fos was calculated in animals that had either a history of foot wound (FW) or control animals that did not (AN), after

the animals were tested in the NIH paradigm. 'FW' refers to animals that had a repeated neonatal foot wound at postnatal day (P)3, P10 and P17. 'AN' refers to animals that were only anaesthetised on the same days as neonates. Animals that did not eat any Cheerios were excluded from the analysis.

Comparing the genders and treatment groups showed that the number of orexin cells per animal did not differ (2-way ANOVA). The number of c-Fos-positive nuclei was higher in both groups of females, possibly suggesting an effect of generalised increased arousal in these animals (Gender $F_{(1,24)}$=5.47, p<0.05; 2-way ANOVA). However, the percentage of orexin cells co-expressing c-Fos was not different between gender or treatment, suggesting that neither gender nor treatment affect the baseline activation of the orexin system after testing in the NIH paradigm (see Graph 4-2). Table 4-3 illustrates the mean cell counts and calculated percentages of co-expressing cells (± SEM).

| Treatment group | Number | Orexin-positive | c-Fos-positive | Number of co-expressing cells | % orexin cells co-expressing c-Fos |
|---|---|---|---|---|---|
| M FW | 7 | 43.2±4.2 | 28.2±7.3 | 7.0±1.5 | 15.5±2.6 |
| M AN | 6 | 42.4±3.8 | 31.6±6.9 | 6.5±1.4 | 16.4±4.5 |
| F FW | 8 | 46.3±2.6 | 47.5±5.9 | 10.2±1.5 | 22.3±3.6 |
| F AN | 7 | 40.1±3.2 | 44.2±7.0 | 9.4±2.4 | 22.2±4.0 |

**Table 4-3 - A table to show the treatment groups, number of animals, orexin- and c-Fos-positive cell counts and number and percentage of orexin cells co-expressing c-Fos (± standard error) for animals repeatedly injured (FW) or anaesthetised (AN) as neonates.**
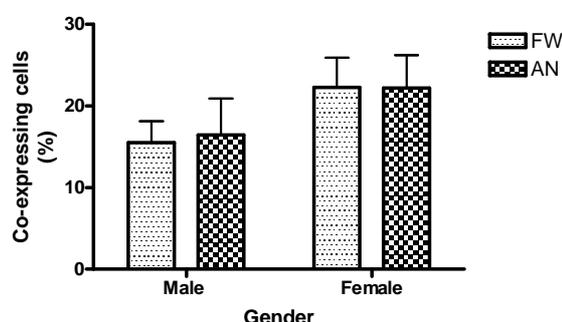


**Graph 4-2 – A bar graph to show the percentage of orexin cells co-expressing c-Fos in males and females injured or anaesthetised as neonates and tested in adulthood in the NIH arena.**

To investigate whether the activation of the orexin cells is correlated to reward-related behaviours, Pearson's correlation coefficient was calculated in the above groups of animals. The outcome measures used were the number of rears and percentage of time

spent in the central zone (both during the 5-10 minute time bin); this was because these are the most consistently robust outcome measures as seen in Chapter 3.

The results showed that there are no correlations between activation of orexin cells and reward-related behaviours (rears and percentage of time spent in the central zone) in animals of either gender, with or without a history of repeated neonatal foot wound, when tested at baseline in the NIH arena in adulthood. These data are shown in Table 4-4.

| Treatment group | Rears | | Time in central zone (%) | |
|---|---|---|---|---|
| | Pearson's R | P value | Pearson's R | P value |
| M FW | 0.69 | 0.08 (n.s.) | -0.46 | 0.30 (n.s.) |
| M AN | 0.55 | 0.25 (n.s.) | -0.48 | 0.33 (n.s.) |
| F FW | 0.02 | 0.96 (n.s.) | -0.58 | 0.13 (n.s.) |
| F AN | 0.25 | 0.59 (n.s) | 0.61 | 0.15 (n.s.) |

**Table 4-4 – A table to show Pearson's correlation coefficient for the number of rears/percentage of time spent in the central zone, both in the 5-10 minute time bin, correlated to the percentage of orexin cells co-expressing c-Fos, in animals with no history of injury.**

## 4.4.3 Experiment 3: Re-injury of an animal with a history of injury increases orexin cell activation, and correlates with rearing behaviour

To investigate whether a re-injury in adulthood to animals with a history of neonatal injury affected orexin cell activation, the percentage of orexin cells co-expressing c-Fos was calculated in re-injured animals after the adult animals were tested in the NIH paradigm. 'FW' refers to animals that were repeatedly injured as neonates but received no adult injury. 'FW+FW' refers to neonatally injured animals that also received a single adult foot wound. Animals that did not eat any Cheerios were excluded from the analysis. Table 4-5 shows the cell count data from this experiment:

| Treatment group | Number | Orexin-positive | c-Fos-positive | Number of co-expressing cells | % orexin cells co-expressing c-Fos |
|---|---|---|---|---|---|
| M FW | 7 | 43.2±4.2 | 28.2±7.3 | 7.0±1.5 | 15.5±2.6 |
| M FW+FW | 7 | 48.2±4.9 | 46.8±14.4 | 13.7±3.1 | 27.2±4.1 |
| F FW | 8 | 46.3±2.6 | 47.5±5.9 | 10.2±1.5 | 22.3±3.6 |
| F FW+FW | 7 | 52.9±4.2 | 40.8±5.9 | 10.1±3.5 | 20.2±3.1 |

**Table 4-5 – A table to show the treatment groups, number of animals, orexin- and c-Fos-positive cell counts and number and percentage of orexin cells co-expressing c-Fos (± standard error) for animals repeatedly injured as neonates, and re-injured (FW+FW) or not (FW) as adults.**

There was no difference in orexin-positive and c-Fos positive cell numbers between genders or treatments (2-way ANOVA).

There was, however, a significant difference in percentage of orexin cells co-expressing c-Fos between treatment groups in the male animals (p=0.016, 1-tailed unpaired t-test). This result shows that when an animal with a 'pain history' is re-injured as an adult, the orexinergic system is more active after testing in the NIH arena, rising from ~15% of orexin cells activated at baseline to ~27% in re-injured animals (Graph 4-3).
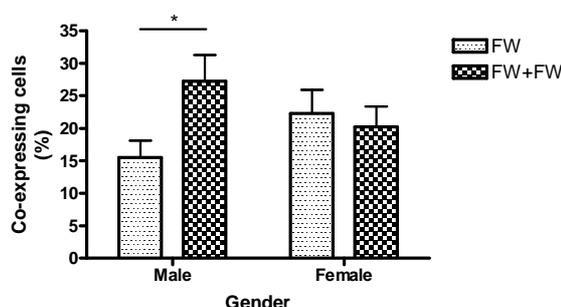


**Graph 4-3 – A graph to show the percentage of orexin cells co-expressing c-Fos in animals with a history of neonatal injury (FW), compared to those with a history of injury given a single adult re-injury (FW+FW).**

To investigate whether the orexin cell activation correlated to the reward-related behaviours investigated in Chapter 3, Pearson's correlation coefficient was calculated. The behavioural outcome measures investigated were rears and percentage of time spent in the central zone (both during the 5-10 minute time bin), as these were the outcome measures significantly affected by an adult re-injury in animals with a previous history of foot wound. The data are presented in Table 4-6:

| Treatment group | Rears | | Time in central zone (%) | |
|---|---|---|---|---|
| | Pearson's R | P value | Pearson's R | P value |
| M FW | 0.69 | 0.08 (n.s.) | -0.46 | 0.30 (n.s.) |
| M FW+FW | **-0.83** | **0.04 (*)** | 0.02 | 0.96 (n.s.) |
| F FW | 0.02 | 0.96 (n.s.) | -0.58 | 0.13 (n.s.) |
| F FW+FW | 0.42 | 0.35 (n.s.) | 0.51 | 0.25 (n.s.) |

**Table 4-6 – A table to show Pearson's correlation coefficient for the number of rears/percentage of time spent in the central zone, both in the 5-10 minute time bin, correlated with the percentage of orexin cells co-expressing c-Fos, in animals with a 'pain history' either with or without a re-injury in adulthood. Significant results are shown in bold. *p<0.05.**

The results show that orexin cell activation negatively correlates (r= -0.83) with the number of rears performed in the NIH arena i.e. as the co-expression of c-Fos in orexin cells increases, the number of rears decreases (represented in Graph 4-4). This result proves that activation of the orexin system is sensitive to reward-related behaviours in animals with a 'pain history', re-injured in adulthood.
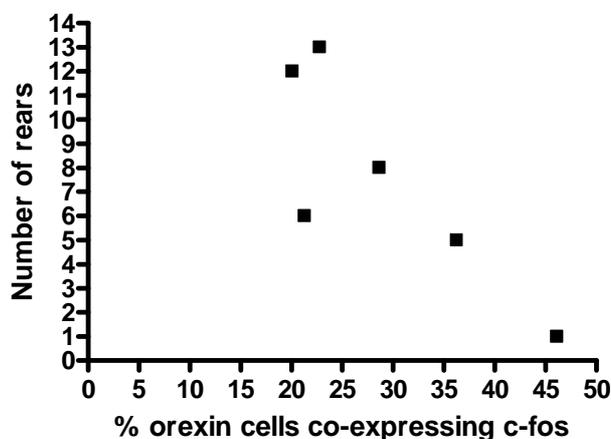


**Graph 4-4 –A scatterplot to show the correlation between the percentage of orexin cells co-expressing c-Fos and the number of rears performed by the male FW+FW group during the 5-10 minute testing time bin in the NIH arena.**

## 4.4.4 Investigating the effect of Cheerio consumption on the lateral hypothalamic orexin system

To investigate whether consumption of Cheerios or the presence of food reward activates orexin cells, two additional comparisons of orexin cell activation were performed.

## 4.4.4.1 Experiment 4a: Comparing orexin cell activation in animals that consumed Cheerios during the NIH test period to those that did not

It was expected that all animals would consume Cheerios during the NIH test period, as anecdotal evidence showed that all animals rapidly consumed Cheerios when presented with them in their home cages. However, male animals that had been repeatedly injured as neonates produced a high number of non-eating animals when tested in the NIH arena in adulthood. Analysis showed that there were significantly more non-eating animals than were expected (see Chapter 3, p127). To investigate what effect Cheerio consumption had on orexin cell activation, the percentage of orexin cells co-expressing c-Fos was investigated in animals that consumed, or did not consume, Cheerios. Table 4-7 shows the number of eating and non-eating animals that were used for this analysis:

| Eaters | Non-eaters |
|--------|------------|
| 7 | 5 |

**Table 4-7 – A table to show the number of eating and non-eating animals analysed in the male neonatally-injured group.**

There was no difference in the number of orexin cells between eating or non-eating animals, but there was significantly more c-Fos activation in the non-eating animals ($F_{(1,24)}$=7.56, p<0.05; 2-way ANOVA). This could be a reflection of increased arousal during the test period. The data are shown in Table 4-8:

| Cell type | Eater | Non-eater |
|-----------|-------|-----------|
| Orexin-positive | 43.2±4.2 | 42.2±5.7 |
| C-Fos-positive | 28.2±7.3 | 41.8±10.1 |
| Co-expressing (number) | 7.0±1.5 | 10.0±2.5 |
| Co-expressing (%) | 15.5±2.61 | 22.4±3.4 |

**Table 4-8 – A table to show the mean (± standard error) number of orexin and c-Fos-positive cells, and the percentage of cells co-expressing both, in eating and non-eating males.**

When comparing the percentage of orexin cells co-expressing c-Fos between eating and non-eating animals, there was no difference between groups (p=0.13, Unpaired t-test), suggesting that the consumption of Cheerios does not affect orexin cell activation.
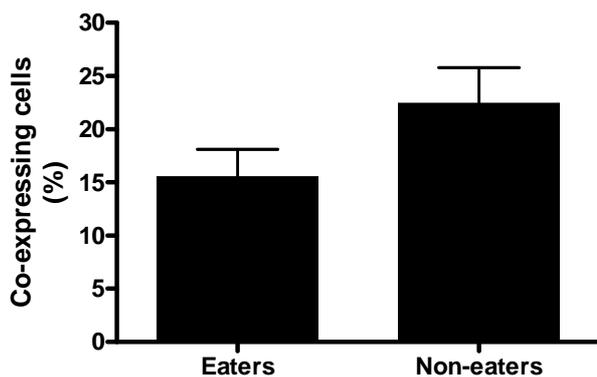
**Graph 4-5 – A bar graph to show the percentage of orexin cells co-expressing c-Fos in animals that did or did not consume Cheerios during the NIH test period.**

## 4.4.4.2 Experiment 4b: Orexin cell activation is significantly lower in animals that had no access to food reward during the NIH test period

To investigate whether the presence of Cheerios affected activation of the orexin system, orexin cell activation was compared between animals that had Cheerios present in the food bowl of the NIH arena ('Present'), and those that were presented with an empty food bowl in the centre of the NIH arena ('Absent').

As the re-injured male animals with a 'pain history' were the only group that showed a significant effect of treatment on orexin cell activation, these animals were compared between the 'present' and 'absent' conditions. In addition, the data was compared to a 'baseline' group – animals with a history of foot wound but no adult injury. Table 4-9 shows this data:

| Treatment group | Number | Orexin-positive | c-Fos-positive | Number co-expressing orexin and c-Fos | % orexin cells co-expressing c-Fos |
|---|---|---|---|---|---|
| M FW Present | 7 | 48.2±4.5 | 46.8±7.5 | 13.7±3.1 | 27.2±3.1 |
| M FW Absent | 5 | 57.4±5.4 | 32.6±8.8 | 7.9±1.2 | 14.8±3.7 |
| Baseline | 7 | 43.2±4.2 | 28.2±7.3 | 7.0±1.5 | 15.5±2.6 |

**Table 4-9 – A table to show the treatment groups, number of animals, cell counts and number and percentage of orexin cells co-expressing c-Fos (± standard error) for animals that had access to Cheerios in the NIH arena and those that did not have access to Cheerios, as well as a 'baseline' group.**

Comparing the percentage of orexin cell activation between the 'present', 'absent' and 'baseline' conditions showed that orexin cell activation is higher in re-injured male

animals when Cheerios are present in the NIH arena, and compared to baseline activation (p<0.05; One-way ANOVA with Bonferroni's post-tests). When Cheerios are absent, the activation of the orexin system is the same as that at baseline:
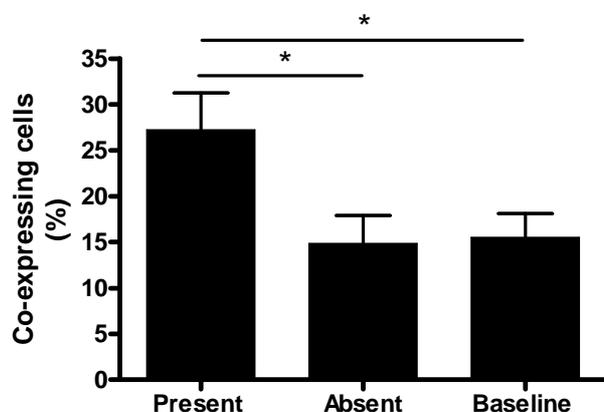


**Graph 4-6 – A bar graph to show the percentage of orexin cells co-expressing c-Fos in neonatally injured animals re-injured in adulthood, when the Cheerios were present or absent during testing in the NIH arena. 'Baseline' data refers to animals with a 'pain history' but no adult re-injury, tested in the presence of Cheerios. *p<0.05; one-way ANOVA with Bonferroni's post-tests.**

This data shows that activation of the orexin system is sensitive to food reward, but only in male animals with a history of neonatal pain that are then re-injured in adulthood.

To see if the presence or absence of Cheerios correlated to changes in reward-related behaviours, Pearson's correlation coefficient was calculated for the number of rears and the percentage of time spent in the central zone (shown in Table 4-10). Behaviourally, the number of rears increased when the Cheerios were absent, and the percentage of time spent in the central zone decreased.

| Treatment group | Rears | | Time in central zone (%) | |
|---|---|---|---|---|
| | Pearson's R | P value | Pearson's R | P value |
| Present | **-0.83** | **0.04 (*)** | 0.02 | 0.96 (n.s.) |
| Absent | **0.9** | **0.04 (*)** | -0.60 | 0.29 (n.s.) |
| Baseline | 0.69 | 0.08 (n.s.) | -0.46 | 0.30 (n.s.) |

**Table 4-10 – A table to show Pearson's correlation coefficient for the number of rears and percentage of time spent in the central zone (both in the 5-10 minute time bin), correlated with the percentage of orexin cells co-expressing c-Fos at baseline and in animals with a 'pain history' re-injured in adulthood, when the Cheerios are present or absent. Significant results are shown in bold. *p<0.05.**

The results show that when Cheerios are present, the number of rears is negatively correlated (r= -0.83) to the activation of the orexin cells (see Graph 4-4). However, when the Cheerios are absent, the number of rears is positively correlated (r=0.9) with

the activation of orexin cells (see Graph 4-7). There is no correlation to either behavioural outcome measure in the Baseline group.
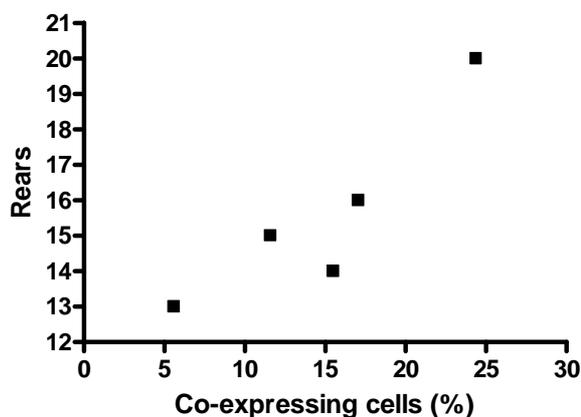


**Graph 4-7 – A scatterplot to show the correlation between the percentage of orexin cells co-expressing c-Fos and the number of rears performed by the male FW+FW group during the 5-10 minute testing time bin in the NIH arena when Cheerios are absent.**

Therefore, when Cheerios are absent, orexin cell activation is lower than when Cheerios are present; and the number of rears increases as orexin cell activation increases. However, when Cheerios are present, orexin cell activation is higher, and the higher the activation, the fewer the number of rears.

### 4.4.5 Summary

- The activation of the orexin system of animals with no history of neonatal injury is not affected by a single injury in adulthood. Furthermore, the activation of orexin cells does not correlate to the behavioural changes seen in these animals after a single adult injury, suggesting that the orexin system is not a sensitive biomarker of reward-related behaviours in animals without a history of injury.

- Animals that had a history of repeated neonatal foot wound did not show any differences in orexin cell activation when compared to anaesthesia-only controls. Furthermore, the activation of orexin cells did not correlate to reward-related behaviours in these animals, suggesting that the orexin system is not a sensitive biomarker of reward-related behaviours in animals with a history of injury, tested at baseline.

- Males with a history of repeated neonatal foot wound show an increase in the percentage of orexin cells activated when re-injured in adulthood. In addition, this activation of orexin cells correlates with the number of rears performed, showing that activation of the orexin system reflects reward-related behaviours in males with a 'pain history' when they are re-injured as adults.

- Additional studies to investigate the effect of Cheerio consumption on orexin cell activation showed that whether animals eat or not is irrelevant to orexin activation. However, when comparing animals that had access to Cheerios in the NIH arena versus those that did not, orexin activation is lower when the Cheerios are absent. This suggests that the orexin system is sensitive to the presence of a reward in animals with a 'pain history', and could be used as a neurobiological marker of reward in animals that are tested in the NIH arena.

- In addition, when Cheerios are absent, and levels of orexin activation are low, there is a positive correlation between cell activation and rearing behaviour.

- Taken together, these results show that the orexin system is sensitive to the presence or absence of a food reward in animals with a history of repeated neonatal injury. In addition, the magnitude of activation of the system correlates with the behavioural outcome measures studied in Chapter 3.

## *4.5  Discussion*

The results presented in this chapter show that the orexin system can reflect aspects of reward, but only in male rats with a history of neonatal pain when re-injured in adulthood. In these animals, orexin cell activation is sensitive to the presence or absence of food reward and correlates to behavioural outcome measures.  This suggests that the orexin system could be a potential biomarker of reward-related behaviours in this specific group of animals.

## 4.5.1 A single injury in adulthood does not influence orexin signalling

The results showed that when an animal with no prior history of injury receives a single foot wound in adulthood, activation of the orexin system after behavioural testing in the novelty-induced hypophagia (NIH) paradigm is not different to that of control animals, who received no adult injury. This suggests that orexin cells are not activated after NIH arena testing in animals with an acute injury.

It was thought possible that an acute injury may have increased orexin activation, as the literature has shown that orexins have analgesic effects on animal pain models of inflammation and neuropathy and may be acting to dampen ascending nociceptive input

(Bingham et al., 2001; Yamamoto et al., 2003a; Kajiyama et al., 2005). This analgesic effect has also been shown in the plantar skin incision model of post-operative pain – the same pain model used in these experiments (Cheng et al., 2003). However, any potential analgesic effect, as seen by an increase in orexin signalling, was not seen in these experiments.

This is most likely because the studies showing the orexins' analgesic effect used very different testing protocols to those used in these experiments. For example, Bingham et al (2001) studied orexin A's effect 1-5 minutes after infusion, and Cheng et al (2003) intrathecally infused orexin beginning 20 minutes after surgery, and continued up to 180 minutes post-surgery. In the experiments presented in this chapter, orexin activation was investigated 48 hours after surgery, meaning that any immediate inflammatory and incision effects on orexin signalling were not measured. Perhaps if testing in the NIH arena had been performed at a time closer to injury i.e. 20 minute post-surgery, changes in orexin activation may have been evident. This, however, was not the aim of these experiments, which was to see how early injury affects adult reward-related behaviours, and whether orexins are linked to these changes.

In addition to the testing time point differences, the NIH arena is not a test of acute nociception and is not designed as such. Unlike the papers investigating orexin analgesia, which included utilisation of the Hargreaves hotplate apparatus to test thermal hyperalgesia, von Frey hairs to study mechanical hypersensitivity, and abdominal acid injection to test visceral pain responses (Bingham et al., 2001; Yamamoto et al., 2003b; Suyama et al., 2004), the NIH arena was designed to measure exploratory behaviours and approach to a reward. This would further explain why a single acute adult injury did not affect orexin cell activation after NIH testing, 2 days after surgery.

## 4.5.2 Animals with a history of neonatal injury do not show altered orexin activation after reward testing in adulthood

To investigate the effect of a 'pain history' on reward-related behaviours, animals that were repeatedly injured as neonates (or repeatedly anaesthetised control animals) were tested in the NIH arena in adulthood at baseline i.e. with no further injury/anaesthesia.

The previous chapter has shown that there were no behavioural differences between these groups of animals, and the results from this chapter show that there was no difference in orexin cell activation either.

Baseline activation of orexin cells (i.e. percentage of orexin cells co-expressing c-Fos) was around 15-22% in all groups, both male and female. This is in line with the literature on orexin cell activation, which also shows control/baseline LH orexin cell activation at around 10-20% (Harris et al., 2005; Harris et al., 2007b; Aston-Jones et al., 2009).

### 4.5.3 Orexin cell activation does not reflect whether an animal consumes a food reward or not

Increases of c-Fos expression in orexin cells have been shown when animals are exposed to a cue previously linked to a high-fat food reward (Harris et al., 2005), and upon anticipation and consumption of a palatable foodstuff i.e. chocolate (Choi et al., 2010). Orexin antagonism has shown decreases in the self-administration of high-fat food (Nair et al., 2008) and prevention of the feeding induced by nucleus accumbens stimulation (Zheng et al., 2007). Recent work has suggested that orexin is responsible for signalling positive reinforcers and rewarding stimuli, in particular high-fat foods as well as drugs of abuse, but not aversive stimuli (Borgland et al., 2009). For these reasons, one hypothesis of these experiments was that consumption of Cheerios, acting as a food reward, would be related to orexin cell activation. However, when comparing activation in animals that voluntarily consumed Cheerios during the NIH test to those that did not, no differences were seen.

One explanation for this may be that the group of animals within which this consummatory behaviour was investigated were males with a history of neonatal injury, with no further adult injury. Whilst this group displayed a significantly higher number of non-eating animals than was expected (see page 127, Chapter 3), the animals that ate Cheerios showed no differences in any behavioural outcome measure (compared to controls) when tested in the NIH arena in adulthood. This result suggests that a neonatal foot wound, which decreases the likelihood of eating Cheerios in the NIH, is not linked

to orexin cell activation, and does not support the hypothesis that consumption of a food reward is linked to orexin cell activation in animals repeatedly injured as neonates.

### 4.5.4 Animals with a history of neonatal injury show increased orexin activation after re-injury in adulthood

When males with a history of neonatal foot wound were re-injured in adulthood and tested in the NIH arena, the percentage of orexin cells co-expressing c-Fos was significantly increased (27%) compared to males with a similar history but no adult re-injury (15%). This result, unlike the previous result, supports hypotheses 1 and 2 of this chapter - that early neonatal injury affects orexin cell activation upon reward testing in adulthood, but similar to the behavioural results, it is upon re-injury in adulthood that differences become apparent.

The increase in percentage of orexin cells co-expressing c-Fos seen between males with a 'pain history', and those with the same history plus an adult re-injury, is around 10%. This is not as large as increases elsewhere in the literature that investigate orexin cell activation in food-cued conditioned place preference (CPP) tasks, which record percentage increases of 20-40%, similar to that induced by morphine and cocaine-conditioned protocols (Harris et al., 2005). Choi et al (2010) showed overall activation of orexin cells within the LH as reaching up to 80%, which is much higher than the highest activation of orexin cells seen in these experiments, at 27%.

However, the studies above utilise conditioning tasks to investigate reward behaviours, where the animal has learnt to associate a particular reward with a specific environment or operative response i.e. lever press. The NIH arena does not require any pre-training, and does not rely on conditioned learning or cues. The only training performed before NIH arena testing was familiarisation of animals with Cheerios presented in their home cages. Therefore it could be argued that animals had learnt to associate the smell of Cheerios with the motivation to consume them, and that this odour was acting as a conditioned cue. However, the lack of training to pair the odour 'cue' to any specific novel environment would preclude this type of classical conditioning, and indeed Harris et al (2007b) showed that it was the pairing of the food/drug to a novel environment which specifically activates orexin cells and led to suggestions that orexins are critically

involved in learning drug-cue associations. Therefore the data presented in this chapter are the first to date to show that orexin cell activation is possible after testing in a non-conditioned behavioural paradigm.

In addition to increased orexin cell activation seen in males re-injured in adulthood, the percentage of orexin cell activation was correlated to a behavioural outcome measure - specifically, the number of rears performed during the 5-10 minute time bin. Rears were negatively correlated to percentage of orexin cells co-expressing c-Fos. This fulfils the hypothesis that orexin cell activation is linked to behavioural outcome measures from NIH testing, and is in line with published literature showing correlations of orexin cell activation to preference scores in CPP testing paradigms (Harris et al., 2005; Harris et al., 2007b; Smith et al., 2010). It is unusual however that, as orexin cell activation increases, rearing decreases, as it would be expected that increased activation would be associated with increased rearing, due to the rewarding aspect of exploration of a novel environment (see Bevins and Besheer, 2005). However, Li et al (2010a) found that microinjections of orexin into the paraventricular nucleus (PVN) (which receives orexin innervation from the hypothalamus) reduced rearing behaviour in the open field. The PVN produces corticotrophin-releasing factor (CRF) under conditions of stress and arousal, so it is possible that the increased orexin cell activation seen when animals were tested in the NIH arena is linked to activation of arousal mechanisms (see de Lecea et al., 2006; Boutrel and de Lecea, 2008).

The increase in orexin cell activation after adult re-injury and correlation to rearing behaviour was not seen in females, which could perhaps be due to hormonal confounds. The fact that behavioural outcome measures were altered in females (see Chapter 3), but there were no significant differences in orexin cell activation, suggests that the orexin system may be vulnerable to hormonal interference during the oestrus cycle. This is the case with stress regulation via the HPA axis (see Kudielka and Kirschbaum, 2005) and with nociceptive processing (La Prairie and Murphy, 2007). As the oestrus cycle was not monitored in these experiments, this could explain variance within results from the female animals, and a lack of significant differences in orexin cell activation between treatment groups.

### 4.5.5 Animals with a history of neonatal injury display an orexin system sensitive to the presence or absence of a food reward

To investigate whether the orexin system is sensitive to the presence of Cheerios as a food reward in the NIH arena, activation (i.e. co-expression of c-Fos in orexin-positive cells) was investigated when Cheerios were present or absent from the food bowl at the centre of the NIH arena. The results showed that the presence of Cheerios produced an increase in orexin cell activation relative to activation in the absence of Cheerios, in the neonatally-injured males re-injured in adulthood ('FW+FW' animals). The level of activation in the absence of Cheerios was the same as that of baseline levels of activation (~15%). This result suggests that the orexin system is activated in the presence of a food reward, which is in line with literature showing that cues associated with, and motivation to seek, a food reward are linked to orexin signalling (Borgland et al., 2009; Choi et al., 2010; Sharf et al., 2010).

Behavioural results from the previous chapter showed that FW+FW animals spent less time in the centre of the NIH arena, and reared more, during the 5-10 minute time bin when Cheerios were absent. Correlating the behavioural outcome measures with the percentage of orexin cells co-expressing c-Fos showed that rearing behaviour was positively correlated, i.e. as orexin cell activation increased, so did the number of rears. This is in direct contrast to the correlation seen in the same group of animals when the Cheerios are present (see section 4.5.4).

This result was intriguing, as it shows that, despite the orexin system being insensitive to whether an animal eats or not (see section 4.5.3), it is sensitive to the presence or absence of a reward in the NIH arena. Furthermore, the positive and negative correlations seen between orexin cell activation and rearing behaviour when the Cheerios are absent or present suggests that the presence of a food reward is impacting upon the orexin system. When Cheerios are absent, the orexin system may be reflecting exploratory drive as seen by increased rearing behaviours. However, when Cheerios are present, exploratory drive may be confounded by the motivational aspect of the presence of a food reward, hence the decrease in rears as orexin cell activation increases.

## 4.5.6 Significant results are seen in male FW+FW animals only

As in the previous chapter investigating reward-related behaviours in the NIH arena, it is after an animal receives an injury in adulthood that behavioural differences are highlighted between treatment groups. Orexin cell activation changes were only seen in neonatally-injured males, re-injured as adults. Why is it that only these animals display alterations in orexin signalling?

It has already been shown that these 'FW+FW' animals are subjected to injury during a developmental time point when interference can cause long-term changes in nociceptive processing (see La Prairie and Murphy, 2010) and development of the HPA axis (Nunez et al., 1996). The long-term changes in nociceptive sensitivity caused by repeated neonatal surgery have been confirmed within this thesis, as the previous chapter showed sensory withdrawal thresholds were altered in neonatally-injured animals. Long-term changes in nociceptive processing have been linked to baseline hypoactivity of the endogenous opioid system, which becomes hyper-responsive after an adult injury (La Prairie and Murphy, 2009). Perhaps noxious interference during a critical period of development is causing a similar shift in responsiveness of the orexin system, leading to hyper-responsiveness of the system after adult re-injury and driving increased reward-motivated behaviours. 'Critical periods', during which interference with a developing system causes long-term changes, have been shown in the nociceptive system (Walker et al., 2009b), the stress-responsivity system (Enthoven et al., 2008), and the visual system (Wiesel, 1982). Furthermore, evidence that the orexin system is present and functional from birth (van den Pol et al., 2001) places it in a position whereby its development could be affected by outside interference.

This proposed model of shifted orexinergic tone after neonatal injury could explain why increased activation of the orexin system after NIH exposure is only seen in neonatally-injured animals when they are re-injured as adults, much like the enhanced hyperalgesia seen in neonatally-injured animals following re-injury (see La Prairie and Murphy, 2010). Further experiments would be needed to investigate the effect of orexin antagonism during development, and any resulting long-lasting changes on reward function.

An alternative theory to explain why a re-injury in adulthood causes an increase in orexin cell activation is explained by a speculative argument that links drug withdrawal to pain states.

Orexin mRNA is increased when an animal enters into a drug-withdrawal state, and c-Fos expression is seen in LH orexin cells in this state (Georgescu et al., 2003; Zhou et al., 2006; Zhou et al., 2008). Morphine withdrawal also results in increased c-Fos expression in the orexin cells of the PFA-DMH region in mice (Sharf et al., 2008), most likely as withdrawal is aversive and arousing for an animal, and the stress of withdrawal activates the HPA axis (Zhou et al., 2006; Corominas et al., 2010). The resulting CRF release will activate medial hypothalamic structures, as CRF-containing fibres synapse directly onto orexin cells (Winsky-Sommerer et al., 2005).

Withdrawal in humans induces states of dysphoria, irritability, anxiety, and anhedonia, in addition to physiological symptoms (see Koob and Volkow, 2009). Animal models of withdrawal have tried to model the psychological effects seen in humans, using behavioural paradigms such as the elevated plus maze and forced swim tests to measure anxiety-like and depression-like behaviours (Castilho et al., 2008; Perrine et al., 2008). Interestingly, chronic pain states in humans can produce symptoms similar to those of withdrawal i.e. anxiety and anhedonia (Meyer-Rosberg et al., 2001) and treatments include anxiolytics and antidepressants (Dworkin et al., 2007), linking reward withdrawal and pain states.

 It is possible to speculate that neonatally-injured animals are being placed in an anhedonic, withdrawal-like state by re-injury during adulthood, and this state causes increases in orexin cell activation similar to those seen during withdrawal, leading to increased reward-seeking motivational drive to explore the novel NIH arena (as seen by increased rearing behaviour) and approach a food reward (as seen by increased time spent around the food-bowl). To confirm this speculation, it would be interesting to block orexin signalling with an antagonist administered prior to NIH testing, and see if behaviour was unaltered, and orexin signalling unchanged in this situation.

### 4.5.7  Technical considerations

In the experiments in this chapter, expression of c-Fos protein within orexin-positive cells was used as a marker of cell activation. C-Fos is the protein product of the immediate early gene *c-fos*, and has become one of the most widely used functional anatomical markers of activated neurons for a number of reasons. It shows low basal expression levels, is induced by a variety of signals including ions, neurotransmitters, growth factors and drugs, shows a transient response, and can be easily combined with markers for other things (see Kovács, 2008). Presence of c-Fos protein has been widely used in the central nervous system as a marker of system activation (Herrera and Robertson, 1996) as well as in the reward literature to study motivation, addiction, and withdrawal (Georges et al., 2000; Georgescu et al., 2003; Aston-Jones et al., 2010).

However, c-Fos is not a perfect marker of cell activation in these studies. The DAB reaction is performed during immunostaining to visualise c-Fos protein, and strength of staining relies on the length of time the tissue is left in the DAB solution by the experimenter. This can vary each time it is done, depending on the rapidity of the DAB reaction. Faint staining over the whole section can result from poor quality perfusions, degradation of the quality of the c-Fos primary antibody (as multiple freezing and thawing of the stored antibody can have a detrimental effect on its binding), or the freshness of the DAB solution. All of these will require a longer period in the DAB solution. To minimise the risk of false negative cell counts due to faint staining, tissue was preferentially stained more darkly. However, this in turn increases background staining, and could have potentially led to false positives in some instances.

In addition, orexin is located in the cytoplasm, so the orexin stain occasionally occluded the c-Fos nuclear stain, especially when the nucleus was in a focal plane lower than, and behind, the cytoplasm. Whilst special attention was paid to cells in these circumstances, it was not always clear whether the nucleus of an orexinergic cell was stained for c-Fos, again potentially leading to false positive or false negative cell counts. Whilst efforts were made to minimise these confounds and produce clearly stained tissue, there will be inherent variability within the cell counts due to these issues.

## *4.6  Summary*

Differences in activation of orexin cells were only seen between groups of animals in which behavioural differences in the NIH arena were also seen, in particular the neonatally-injured males re-injured in adulthood. Correlations between activation and behavioural outcome measures were also only seen in groups of animals that showed altered behaviour compared to controls, suggesting that orexin is a neurobiological correlate of reward-related behaviours. This shows that orexin is a potential biomarker of animal reward behaviour in a specific subset of animals, and that orexin system activation is seen in groups of animals that showed shifted behaviour in the NIH paradigm, fulfilling the hypotheses of this chapter.

Two possible explanations are presented to explain why orexin cell activation is only shifted in neonatally-injured animals, re-injured as adults. One states that the neonatal injuries occurred during a critical period of development, causing a long-term shift in orexin system reactivity, and the other suggests that animals may be in a state similar to drug withdrawal. In both cases, activation of the orexin system is proposed to be driving motivation to seek a reward.

The correlations between cell activation and behaviour do not confer any information on cause. It is unclear from these experiments whether orexin activation is driving behaviour, or whether orexin cells are activated in response to behaviour. To answer this question, further experiments would need to be done that interfere with orexin function, i.e. via administration of the orexin antagonist SB. Decreases in orexin cell activation, coupled with behavioural alterations towards a food reward, would help determine whether orexin is driving, or reflecting, animal behaviour in the NIH arena.

# 5 Discussion

This thesis has aimed to test the hypothesis that repeated early pain altered adult reward-related processing in the rat. To do this, I first tested the functionality of the reward pathways of the neonatal rat with morphine (Chapter 2), then tested the long-term effect of a repeated neonatal foot wound upon reward behaviours in the adult (Chapter 3), and finally investigated the orexin system as a potential neurobiological correlate of reward behaviours (Chapter 4).

## 5.1 Chapter 2 – Activation of neonatal reward pathways

Activation of reward pathways by morphine was measured using induction of pERK, a marker of cell activation, within the dopaminergic (tyrosine hydroxylase-positive) cells of the ventral tegmental area (VTA). Projections from the VTA form the basis of the mesolimbic dopaminergic pathway, heavily implicated in reward processing (Wise, 2004).

The experiments in Chapter 2 showed that a single systemic morphine injection activates the reward pathways of the P7, P21 and adult rat to the same extent, such that the percentage of dopaminergic (DAergic) cells co-expressing pERK was increased from ~10% to ~30%, relative to control groups, at all three ages. This result proves that the neonatal reward pathways are functional within the first week of life, and that morphine will activate these pathways.

Systemic morphine injections repeated over 5 days produced an age-dependent response in activation of dopamine cells. In the adult, the percentage of DA cells activated after the 5$^{th}$ injection was decreased to baseline/control levels, suggesting that the adults develop cellular tolerance after repeated morphine challenge. However, the preweanling animals continued to show increased DA cell activation in comparison with baseline/control groups, suggesting that these young animals do not develop the same level of cellular tolerance to morphine as the adult.

Analgesic tolerance, widely documented in the literature as a decrease in the effects of morphine upon repeat administration (see Christie, 2008), was not measured in these

experiments, although general sedation and analgesia were observed and recorded in each age group after the final morphine dose.

Potential explanations for the different response to repeated morphine in preweanling animals include suggestions that the young animal has immature tolerance systems compared to those of the adult. For example, younger animals may not display 'superactivation' of the adenylyl cyclase signalling cascade after chronic morphine (Avidor-Reiss et al., 1996), or may not have mature 'anti-opioid' systems that downregulate opioid response in the adults (see Mao, 1999; Christie, 2008). A further explanation suggests that the young animals have yet to develop mature functional connectivity to prefrontal cortical areas, which can regulate the activation of the VTA in the adult (see McCutcheon and Marinelli, 2009).

In summary, these results showed that rat pups have functional reward pathways in the first week of life. As noxious stimulation during this time has lasting effects in the rat, and nociception and reward processing are closely linked, this provides a strong framework for the hypothesis that early pain will cause long-lasting shifts in reward processing in the rat.

## 5.2 Chapter 3 – Early pain affects adult reward-related behaviours

To investigate the long-term effects of neonatal pain on adult reward behaviours, rat pups were subject to a repeated foot wound within the first three weeks of life. This is a time window of rapid functional maturation in the pain system and a critical period within which noxious stimulation causes long-lasting effects (see Fitzgerald, 2005; Walker et al., 2009b). Adult reward behaviours were investigated using a novelty-induced hypophagia (NIH) paradigm. Novelty-induced hypophagia refers to the inhibition of feeding in the rodent resulting from exposure to a novel environment, and the NIH arena designed for these investigations was a classic conflict paradigm, where the animal had to overcome the inherent behavioural drive to stay 'safe' near the edges of an aversive bright circular arena, in order to reach a palatable food treat placed in the centre of the arena. Control experiments removing the food treats from the centre of the arena confirmed that the treats were encouraging approach behaviours.

Results showed that neonatally-injured females displayed a baseline decrease in sensitivity to noxious stimulation, i.e. hypoalgesia, which is consistent with literature showing that females are more vulnerable to the long-term effects of neonatal injury (La Prairie and Murphy, 2007). However, at baseline NIH testing in adulthood, neither males nor females displayed any altered exploratory behaviours in comparison to anaesthetised controls. Neonatally-injured males were less likely to consume food treats, suggesting that motivational drives, mediated by reward pathway activation, were shifted in these animals. Overall, these experiments showed that early pain experience did not alter baseline activity in reward pathways.

Re-injury in adulthood of a previously injured animal highlighted differences in reward approach behaviours. Animals with a prior history of injury showed increased anxiety-like behaviours in the NIH arena, as indexed by defecation and decreased rearing behaviour. In addition, the number of approaches to the food treats and time spent around them increased, suggesting that the motivational drive to approach the food reward was higher in animals with a history of prior injury, despite the approach inducing an anxiogenic state. These results are consistent with literature from animal and human studies showing that the effects of neonatal injury are most pronounced after a re-injury later in life (Bhutta et al., 2001; Peters et al., 2005; La Prairie and Murphy, 2009).

Furthermore, these results show that a repeated neonatal injury can cause long-lasting alterations in behaviour towards a reward in adulthood. This is, to date, the first experimental data to explicitly investigate the effect of early pain on later reward behaviours. Bhutta et al (2001) investigated the long-term effects of repeated formalin injection upon adult pain thresholds, and found that alcohol preference and amphetamine response was decreased in neonatally-inflamed animals, however, this study did not investigate the effect of adult re-inflammation.

## 5.3 Chapter 4 – A neurobiological correlate of reward-related behaviours

The orexin system, originating in the lateral hypothalamus, has been heavily implicated in reward and addiction due to its direct influence on the mesolimbic dopamine pathway (Fadel and Deutch, 2002; Narita et al., 2006; see Aston-Jones et al., 2010). In Chapter 4, the orexin system was identified as a candidate system to reflect the behavioural changes seen in Chapter 3, and activation of orexin cells (seen via c-Fos expression) was studied in animals after testing in the NIH arena.

Results showed that, in line with the behavioural results from Chapter 3, orexin cell activation was the same as control activation levels in animals with a history of neonatal foot wound when tested at baseline, but that this changed when the animals were re-injured as adults. In re-injured adults, orexin cell activation upon NIH testing was increased to around ~30%, doubling the activation. Furthermore, cell activation correlated with behavioural outcome measures – rearing behaviour was negatively correlated to orexin cell activation. This is consistent with literature investigating the role of orexin in reward and addiction, showing the same correlative phenomenon (Harris et al., 2005; Harris et al., 2007b; Aston-Jones et al., 2009).

Control experiments in the absence of food treats confirmed orexin signalling as sensitive to food reward, as cell activation was decreased to baseline levels when food treats were absent from the food bowl. Again, this decrease was correlated with behavioural outcome measures, as rearing behaviour was positively correlated with orexin cell activation, further confirming the orexin system as a neurobiological substrate capable of reflecting reward behaviours. Other outcome measures such as approach to the food reward and time spent around it did not show any correlations with cell activation. Whilst Meaney et al (2001) showed that repeated maternal deprivation as a neonate could cause increases in drug responses in the adult, this is some of the first data to explicitly link repeated neonatal pain to shifts in adult reward seeking.

## 5.4 Discussion of possible mechanisms

The results described above show that a repeated injury during the neonatal period causes changes in reward-related behaviours in adulthood, and that these altered

behaviours are correlated with changes in activation of neurobiological systems involved in processing reward and addiction. Possible mechanisms involved in mediating these changes are presented below.

To begin, repeated injury within the neonatal period results in excessive afferent input from the periphery to the brain, through pathways that are not yet fully mature (see Fitzgerald, 2005). This excessive nociceptive input causes long-term alterations in pain sensitivity (see Grunau et al., 2006) by a variety of potential mechanisms. Neonatal skin wounds and inflammation cause excessive sprouting of nociceptor nerve terminals in the dorsal horn of the spinal cord, both of which contribute to enhanced excitability of spinal nociceptive circuits and therefore an increased pain response upon re-injury (Ruda et al., 2000; Walker et al., 2003b). Neonatal injury also causes changes in adult spinal cord gene expression (Ren et al., 2005), developmentally-mediated shifts in excitatory spinal cord transmission (Li et al., 2009), and expansion of receptive field size of dorsal horn neurons (Ririe et al., 2008), all of which could lead to enhanced neuronal excitability in neonatally injured adult animals that would help to explain the hypersensitivity of the nociceptive system to painful stimuli.

Central sensitisation may be initiated by neonatal inflammation, whereby spinal nociceptive neurons are sensitised by peripheral tissue damage, leading to continued and enhanced spinal pain transmission. This is suggested to share similar mechanisms to long-term potentiation (LTP) in higher brain regions, in that insertion of AMPA receptors and 'unmasking' of previously 'silent' NMDA synapses strengthens synaptic connectivity and could in turn further increase nociceptor excitability (see Ji et al., 2003). In addition to the immature balance between excitatory and inhibitory spinal processing (see Fitzgerald, 2005) and immature descending inhibitory pathways before three weeks of life in the neonatal rat (Fitzgerald and Koltzenburg, 1986; Hathway et al., 2009), all of these factors could strongly influence developing nociceptive circuitry and cause the changes in pain response seen in these experiments that persist well into adulthood.

Alterations in pain sensitivity in adulthood after neonatal injury are also speculated to involve upregulation of endogenous opioidergic tone in areas involved in descending modulation of pain – La Prairie and Murphy (2009) suggest that repeated injury may be

causing tonic opioid release within the PAG, aimed at dampening consistent spinal nociceptive signalling. The complex link between opioid and dopamine signalling could therefore mean that chronic increases in opioid release affect dopamine signalling. For example, the balance of tonic and phasic dopamine release can be altered by stress, meaning that subsequent phasic dopamine release upon rewarding stimuli is attenuated and dopaminergic responses altered (see Wood, 2006). It is possible that repeated neonatal surgery, which is inherently stressful, is also altering dopaminergic tone in the developing organism.

The upregulation of endogenous opioids, produced by repeated neonatal surgery, could 'reset' the level of descending control during a critical period of development, and is suggested to persist into adulthood (La Prairie and Murphy, 2009). This resetting could explain the baseline decreases in sensitivity i.e. hypoalgesia seen in both animals and humans with a history of injury (Ren et al., 2004; Hermann et al., 2006; see La Prairie and Murphy, 2010) - as endogenous opioid levels are raised, a net overall inhibition of spinal nociceptive processing results. In addition, a blunted stress response within the hypothalamic-pituitary-adrenal (HPA) axis is seen in the adult after neonatal stress, which could contribute to decreased baseline pain sensitivity (Nunez et al., 1996). Furthermore, the stress of early injury could cause increases in tonic dopamine release, which inhibit phasic dopamine responses to external stimuli, producing a dampened dopaminergic response system.

Changes in nociceptive circuitry (such as increased afferent input to the dorsal horn of the spinal cord and hyperinnervation of wounded skin, described above) induced by repeated neonatal injury lead to a system that is sensitised to respond when a new insult occurs, resulting in exaggerated hyperalgesic behavioural responses to a new injury (see Fitzgerald, 2005). Excessive hyperalgesia is seen in both animals and humans upon a later re-injury (Peters et al., 2005; Walker et al., 2009b; Taddio et al., 2009). In addition, if phasic dopamine release is decreased in neonatally injured animals due to tonic stress-related dopamine release during infancy, then it is possible to speculate that motivational drives for reward will be increased in these animals, in order to compensate for a dampened phasic dopaminergic response to reward. This speculation is supported by evidence from this thesis that orexin cell activation upon presence of a food reward is greater in previously injured animals when re-injured as adults, as well

as evidence that the orexin system is directly linked to reward motivation and activation of the dopaminergic system (Narita et al., 2006; Borgland et al., 2009).
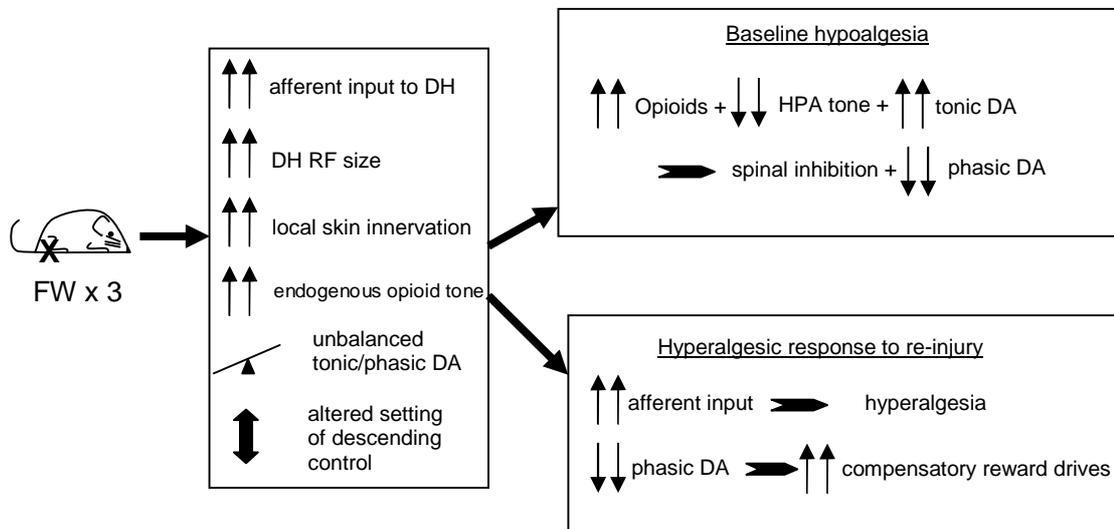


**Figure 5-1 – A schematic diagram to represent possible mechanisms of the long-term effects of repeated neonatal foot wound (FW x 3) on adult behaviours. Repeated injury causes changes in spinal nociceptive circuitry, alterations in endogenous descending control, and a possible stress-induced imbalance in tonic/phasic dopamine release. At baseline in adulthood, hypoalgesia results due to increased circulating opioids, a blunted HPA response, and higher tonic levels of dopamine, leading to net decrease in spinal excitability. Upon re-injury, increased spinal afferent input leads to behavioural hyperalgesia. In addition, decreased phasic DA release, in turn caused by enhanced tonic release, causes an increase in compensatory motivational drives, reflected in increased reward drives in previously injured animals.**

## 5.5 Speculating on the link between reward behaviours and orexin activation

Whilst the mechanisms that could be involved in causing altered reward behaviours are outlined above, the precise linkage between behavioural results and activation of the orexin system observed in this thesis remain unclear at this point. In this section, I speculate as to how the results presented in chapters 3 and 4 are related and how they fit into a model of early pain experience-dependent reward behaviours.

Cheerios have been shown to be driving behaviour, as removing them decreases approach behaviours (fewer central zone entries and less time spent around the food bowl) compared to when Cheerios are present. That the most obvious behavioural

differences are seen in the neonatally-injured animals when re-injured as adults ('FW+FW' animals) suggests that these animals have, as described above, alterations in spinal and supraspinal nociceptive processing that influences adult responses to pain. These animals may also show disturbed HPA axis regulation (see Grunau et al., 2006) and therefore be more anxious, as indexed by increased defecation and decreased rearing behaviours. However, these animals also display increased time spent around the central food bowl which could explain the decrease in rearing behaviours, as animals are engaged in contemplating the consumption of the palatable food reward instead of exploring the arena. The fact that animals do not eat more Cheerios than controls during NIH testing is puzzling.

Another possibility is that these FW+FW animals are incapable of making clear decisions regarding a reward. In the presence of Cheerios, the animal is exposed to a true conflict situation - to avoid the open, aversive environment, or to approach the palatable reward. This faulty decision-making could be related to the shifted nociceptive processing seen, and is based upon the linkage of the pain and reward networks and the possible concept of an overlap in the critical period in development of reward pathways and of nociceptive pathways such that early nociceptive interference affects both later pain and reward processing. The results from Chapter 2 showing a lack of tolerance to morphine in young animals supports the suggestion of immature prefrontal connectivity at this age, as does research showing that medial prefrontal cortical connectivity, which is crucial for decision-making, is immature in preweanling animals (see McCutcheon and Marinelli, 2009), which matches the critical period suggested for pain processing (La Prairie and Murphy, 2009; Walker et al., 2009b).

The correlations between rearing and orexin cell activation are intriguing and not easily explained. When the Cheerios were present, a negative correlation was seen i.e. the greater the rearing, the less the orexin cell activation. It could be that the less anxious animals are rearing more frequently and therefore their motivational drives lie with exploration of the aversive environment rather than contemplation of the food reward. Rearing in this cohort of FW+FW animals was generally decreased compared to controls which suggests that under normal circumstances (i.e. non re-injured animals) rearing and exploration is inherently rewarding, which is in line with literature on exploration as rewarding for an animal (see Hughes, 2007) and supports the speculation

that these animals display faulty reward-related decision-making. The lack of correlations between orexin activation and any other outcome measure was unexpected, and could be due to small group numbers, large variability within groups, and the inherent difficulties in interpreting the meaning of behavioural outcome measures in animal experiments (Stanford, 2007b).

When Cheerios were absent, rearing behaviour was generally increased in the 'FW+FW' group, as animals engaged in more exploratory behaviours and spent less time around the central zone. Under these circumstances, the greater the orexin cell activation the greater the rearing behaviour, so it could be argued that when the food reward was absent, exploration was in itself rewarding. This provides further support that animals within the FW+FW cohort are less capable of making decisions relating to a specific palatable reward, as the presence of Cheerios reward showed an inverse correlation to rearing behaviour.

However, at this point, these suggestions are speculative only, and would need further investigation and careful experimental design to fully elucidate the links between reward behaviours and neurobiological activation in the adult after repeated neonatal injury.

## 5.6 The Motivation-Decision model

The behavioural results can also be viewed in the light of Fields' Motivation-Decision model (2006). The motivation-decision model states that the decision to respond to a stimulus is based upon the premise that anything that is potentially more important for survival than pain should exert anti-nociceptive effects. In the case of testing in the NIH arena, animals that have been injured as adults display anti-nociceptive effects, as they show greater behavioural responses to reward than animals with no adult injury, despite a recent foot wound. This suggests that nociceptive events (i.e. ongoing input from primary afferent nociceptors) are being ignored, and the more salient event (i.e. presence of the food treats) is being attended to. In the absence of food, the motivation to explore becomes the more salient behavioural drive.

The motivation-decision model is based on the reward/cost decision by classic brain 'reward' associated areas, and the resulting behaviour to pain dictated by opioidergic

descending modulation (Fields, 2004; 2006). Results from the literature and from the experiments presented in this thesis suggest that early repeated injury affects opioidergic tone (La Prairie and Murphy, 2009) and shifts orexin-mediated reward pathway activation (Chapter 4), resulting in altered behaviours towards reward in adulthood and a system primed to respond to further painful stimuli (Chapter 3; Walker et al (2009b)). This data extends the motivation-decision model, as pain experienced during vulnerable periods of development takes on a more salient motivating role in adulthood, in order to promote survival. The increased saliency of nociceptive input is reflected in the enhanced hyperalgesia to an adult injury. Motivation for a reward becomes accordingly increased when pain becomes more salient, and is reflected here in an increased motivational drive to approach food reward, decrease in exploratory rearing, plus corresponding orexin system activation, seen in the previously injured animals.

Interestingly, evidence to support the motivation-decision model is found in work on rat pups younger than ten days old, as Kehoe and Blass (1986d) found that distress upon maternal separation produced analgesia to a noxious stimulus, suggesting that the motivating survival drive of behavioural distress i.e. ultrasonic vocalisation, over-rides pain responses, as the model predicts. This also supports Fields' assumption that the 'decision' to respond to stimuli is mainly unconscious (Fields, 2004; 2006), as rat pups in the first week of life do not have functionally mature prefrontal connectivity (see McCutcheon and Marinelli, 2009) and are arguably not conscious of their behavioural drives. This in turn fits with data showing that sucrose 'analgesia' in young rats is mediated by the brainstem i.e. does not require a cerebral cortex (Anseloni et al., 2005).

## 5.7  Further work

The results from the experiments in this thesis provide intriguing evidence on the long-term effects of repeated early pain on reward function in the adult. However, further work is necessary to confirm these results and to clearly elucidate the mechanisms causing these results.

Firstly, it would be interesting to repeat the experiments using a classical reward paradigm, such as conditioned place preference, as competing motivational drives (i.e. approach/avoidance of the food reward) add complexity to the interpretation of

behaviour within the NIH arena. It would also be interesting to see if repeated neonatal pain affected intracranial self-stimulation and/or intracranial self-administration of drug in a part of the brain critical for reward processing, for example the nucleus accumbens. These experiments could provide robust evidence for the effect of early pain on reward processing. In addition, the effect of repeated morphine administration during infancy upon adult reward behaviours would be an interesting avenue of research, to model the clinical situation where morphine is routinely used as an analgesic and for sedation (Anand, 2007).

Ultimately, basic pain research is aimed at reducing pain in humans. To transfer the research described in this thesis to the clinic, longitudinal studies of human infants and the long-term consequences of neonatal pain on attitude towards a reward would be necessary. Currently, much research is underway to study neonatal pain mechanisms, but longitudinal research on children and adults with prior pain history is still relatively rare. Difficulties with longitudinal studies include the fact that clinical records from infancy are incomplete, or not available. However, newer cohorts of post-NICU children such as the UK EpiCure cohort come complete with more comprehensive clinical notes, and therefore provide the basis for helpful epidemiological studies in the future (Wood et al., 2005; Walker et al., 2009a). Over the years, it would be interesting to monitor children in these cohorts for drug abuse and/or addiction problems, and more subtle reward-related dopaminergic personality traits such as sensation-seeking and extroversion. In addition, existence of these cohorts provides researchers with many potential candidates for imaging studies, using techniques such as fMRI to investigate BOLD responses to pain and reward, or positron emission tomography (PET) to investigate dopamine receptor binding in adulthood. Both of these have been used to study adult pain and reward responses, but have not yet investigated the consequences of pain during childhood (see Leknes and Tracey, 2008; Volkow et al., 2009).

A major challenge if these results are to be tested in infants would be to find an outcome measure of reward in young infants. The question of how to measure a subjective 'reward' in young infants is confounded by both technical and ethical difficulties, but the quality of the infant-caregiver bond might provide insights into reward processing in young children. Infant attachment i.e. the bond between offspring and primary caregiver (usually the mother) is rewarding for both child and mother (Bowlby, 1978; Insel,

2003). Animal studies have shown that separation causes distress in young rat pups and is mediated by opioid signalling (Kehoe and Blass, 1986d; Blass et al., 1987). Mu opioid receptor knock-out mouse pups develop disordered attachment to the dam (Moles et al., 2004), and mice lacking the dopamine β-hydroxylase gene fail to nurture their offspring (see Palmiter, 2008). In humans, activation of the mesolimbic dopamine pathway from the VTA is seen in mothers when viewing their infants (Noriuchi et al., 2008), and human imaging studies have begun to identify a neurobiological substrate for infant attachment, based in reward processing areas such as the nucleus accumbens, VTA and medial prefrontal cortices (see Parsons et al (2010) for a comprehensive review). The attachment of infant to mother lasts throughout life, and can affect adult relationships, self-esteem, response to stress and mental health in later life (see Rees, 2005).

Attachment style and quality have been linked to pain experience in humans, with disordered attachment styles linked to chronic pain and problems in coping with pain (Porter et al., 2007). For example, chronic pain patients with high levels of avoidant attachment self-scored pain intensity more highly, and patients with fearful attachment styles displayed increased levels of pain catastrophising (Ciechanowski et al., 2003; Meredith et al., 2006a). In volunteers with no history of chronic pain, acute pain induced by the cold-pressor test (which involves holding the arm in iced water for as long as possible) was scored as less intense, less anxiogenic and more personally controllable in subjects with secure attachment styles (Meredith et al., 2006b).

The suggestion of attachment as rewarding, together with evidence on the effect of attachment style on pain experience, produced the idea that monitoring the infant-caregiver attachment upon a painful stimulus (i.e. routine immunisation), information regarding reward processing in young children could be gleaned.

## 5.8 Summary and conclusions

To summarise, this thesis aimed to test the hypothesis that repeated pain during early infancy can cause long-term changes in adult reward sensitivity. Studies that performed repeated foot wound surgery to neonatal rat pups showed that reward behaviours were different in the adult animal when the animals received a new injury. These behavioural

differences were reflected in activation of the orexin system, providing a neural substrate for reward behaviours. Testing the integrity of early reward pathways with morphine showed the functionality of reward signalling in the first week of the rat's life, but illustrated quantitatively different responses after repeated morphine in comparison to the adult, suggesting that, similar to nociceptive processing, reward processing does not reach maturity until the third week of postnatal life.

The existence of a critical period of nociceptive development, plus the extensive overlap between reward and pain processing, suggests that reward pathways may also be vulnerable to interference during this period. In turn, this could inform clinical practice, enabling more appropriate treatment of infants exposed to repeated painful procedures during early life, and helping to minimise the long-term consequences of early pain on both later pain sensation and reward processing.

# 6  Appendix 1 - SOLUTIONS

## 6.1.1  **Phosphate buffers**

All are based on dilutions of 0.2M phosphate buffer (pH 7.4), made in the following manner (for a 5L yield):

$\qquad$ 4,050ml 0.2M $Na_2HPO_4$ (28.4g/L $dH_2O$)

$\qquad$ 950ml 0.2M $NaH_2PO_4$ (27.6g/L $dH_2O$)

### 6.1.1.1  0.1M

1:1 dilution with $dH_2O$ of 0.2M phosphate buffer -  i.e. for 1L, add 500ml $dH_2O$ to 500ml 0.2M PB.

### 6.1.1.2  0.01M

Dilution of 0.1M PB -  i.e. for 1L, add 100ml 0.1M PB to 900ml $dH_2O$.

### 6.1.1.3  0.6M PB (for perfusions)

For 5L stock:

$\qquad$ 1.2L $NaH_2PO_4$ (dissolve 99.34g in 1.2L $dH_2O$)

$\qquad$ 4.2L $Na_2HPO_4$ (dissolve 357.7g in 4.2 $dH_2O$)

## 6.1.2  **TTBS**

To make 1L:

$\qquad$ 3ml Triton X100 (Sigma Aldrich, Steinheim, Germany)

$\qquad$ 50ml 1M Tris solution (Sigma Aldrich, Steinheim, Germany)

$\qquad$ 8.7g NaCl (Fisher Scientific, Leicester, UK)

## 6.1.3  **PFA**

To make 5L stock 12% PFA:

$\qquad$ 500g PFA powder (Fisher Scientific)

$\qquad$ 4.166L $dH_2O$

$\qquad$ ~few ml NaOH

To make 1L 4% PFA immediately prior to perfusion:

      333ml 12% stock PFA

      167ml 0.6M PB

      500ml dH$_2$O

Add 50ml NaF if staining for pERK.

## 6.1.4 <u>Sucrose and azide</u>

Add 5% or 30% (w/v) to 0.1M PB. Add 1μl/ml 20% sodium azide. For example:

### 6.1.4.1 30%

    1L 0.1MPB

    300g saccharose (VWR, Leuven, Belgium)

    1ml azide

### 6.1.4.2 5%

    1L 0.1MPB

    50g sucrose

    1ml azide

## 6.1.5 <u>Blocking serum</u>

      300μl serum i.e. horse, goat, etc

      300μl 10% Triton X-100

      both per ml 0.1M PB

For DAB staining, add 200μl hydrogen peroxide ~30% solution (VWR, Leuven, Belgium) per ml 0.1M PB.

# 7 Appendix 2 - Chapter 2: Graphing the time course of reward behaviours

The results presented below describe the time course of behaviours either over the course of 5 days post-surgery (sensory testing), or during the 15 minute testing period in the NIH arena, split for gender.

## *Sensory testing*

## Mechanical withdrawal thresholds – 5 day time course

## Male

There are no significant differences (2-way ANOVA) in mechanical withdrawal threshold between ipsilateral and contralateral foot, nor between treatment group (foot wounded and anaesthesia), in adult males at baseline.
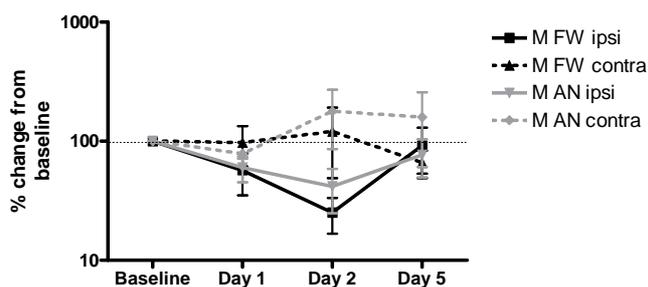


**Graph 7-1– A line graph to show how male mechanical withdrawal thresholds change from baseline at 1, 2 and 5 days after a single adult foot wound. Both injured (ipsi) and uninjured (contra) paws are shown for previously injured (M FW) and uninjured (M AN) animals.  The Y axis is a logarithmic scale as the vFh increase in force (in grams) between each hair number is logarithmic. Percentage change from baseline was plotted to show changes in sensitivity more clearly. M FW n=3, M AN n=3.**

## Female

After the adult females are exposed to a single foot wound, mechanical withdrawal thresholds do not change significantly over the course of 5 days post-injury (2-way Repeated measures ANOVA). However, Graph 7-2 shows a trend for the ipsilateral paw in the FW animal to decrease from baseline, i.e. withdrawal threshold to decrease; and the contralateral paw to increase from baseline i.e. withdrawal threshold to increase. Both paws in the AN animals appear to stay around baseline levels.
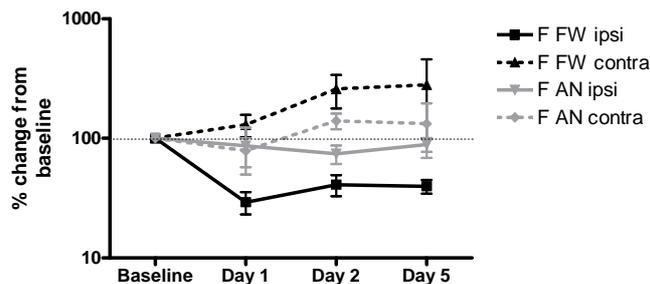
**Graph 7-2 - A line graph to show how female mechanical withdrawal thresholds change from baseline at 1, 2 and 5 days after a single adult foot wound. Both injured (ipsi) and uninjured (contra) paws are shown for previously injured (M FW) and uninjured (M AN) animals. The Y axis is a logarithmic scale as the increase in force (in grams) between each von Frey hair is logarithmic. Percentage change from baseline was plotted to show changes in sensitivity more clearly. F FW n=3, F AN n=3.**

## Thermal withdrawal thresholds – 5 day time course

## Male

Graph 7-3 shows the time course of changes in thermal withdrawal thresholds after a single adult foot wound. General trends show that the ipsilateral paws of both previously injured and uninjured animals show a drop in threshold, i.e. their sensitivity increases, until the thresholds return towards baseline levels at day 5 post-surgery.
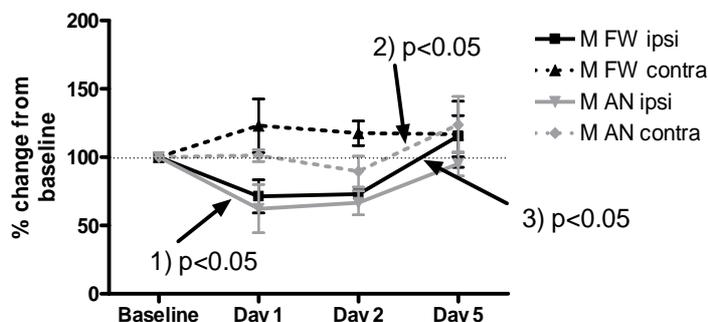


**Graph 7-3 – A line graph to show that thermal withdrawal thresholds change after single adult injury. 1) There is a significant decrease in threshold in the AN animal's ipsilateral paw one day after injury; 2) There is a significant increase between days 2 and 5 in the AN contralateral paw's threshold and 3) there is a significant increase between days 2 and 5 in the FW ipsilateral paw's threshold (p<0.05 for all; 2-way Repeated measures ANOVA with Bonferroni's post-tests). M FW n=3, M AN n=3.**

## Female

General trends show that the FW ipsilateral paw shows a drop in threshold i.e. an increase in sensitivity after adult foot wound that begins to return to baseline by 5 days post-injury. The contralateral paw and both paws in the AN animals show a significant increase in threshold over 5 days ($p<0.05$; 2-way Repeated measures ANOVA with Bonferroni's post-tests).
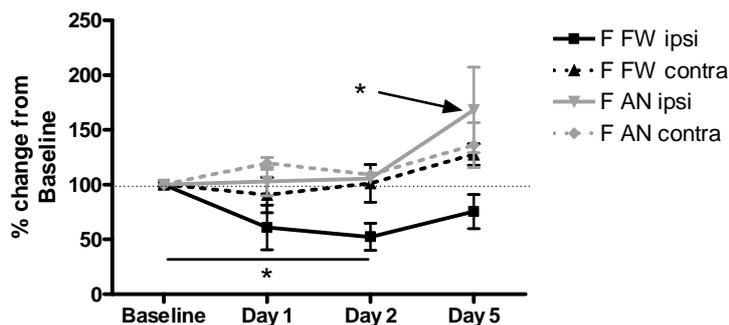


**Graph 7-4 – A line graph to show that thermal withdrawal thresholds change after single adult injury. The arrow indicates that the AN ipsilateral paw's threshold is significantly higher at day 5 than at all other time points ($p<0.05$; 2-way Repeated measures ANOVA with Bonferroni's post-tests). The lower line and asterisk show that the FW ipsilateral paw's threshold is significantly lower at day 2 than at baseline ($p<0.05$; 2-way Repeated measures ANOVA with Bonferroni's post-tests). F FW n=3, F AN n=3.**

## *Baseline behavioural testing*

The graphs below describe behaviour in the novelty-induced hypophagia test in animals with or without a history of neonatal injury. Graphs describe the time course of behaviours during the 15 minute test period, separated into 5 minute time bins. M FW group n=19, M AN group n=13, F FW group n=19, F AN group n=11.

## Exploratory behaviours: Rearing, central zone entries, and percentage of time spent in the central zone

## Number of rears decrease over the testing period

### *Males*

Graph 7-5 shows how rearing significantly decreases over the 15 minute testing period ($p=0.028$; 2-way Repeated measures ANOVA with Bonferroni's post-tests). There were

no differences at any time point between the injured and uninjured groups at any time point (Multivariate ANOVA).
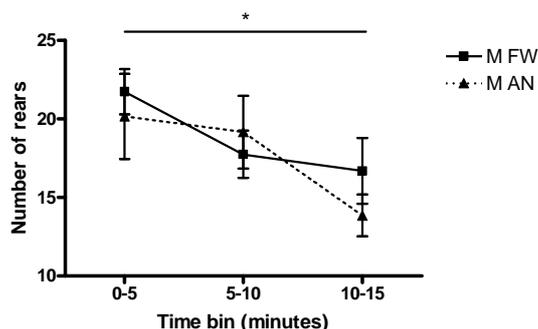


**Graph 7-5 – a line graph to show the number of rears that male foot wounded and anaesthesia-only animals performed, time-binned into 5 minutes bins. \*p=0.028; 2-way Repeated measures ANOVA with Bonferroni's post-tests.**

## *Females*

The number of rears that injured females made significantly decreased over the testing period (p=0.006; 2-way Repeated measures ANOVA with Bonferroni's post-tests). There were no significant differences between treatment groups (Multivariate ANOVA). Graph 7-6 illustrates this data:
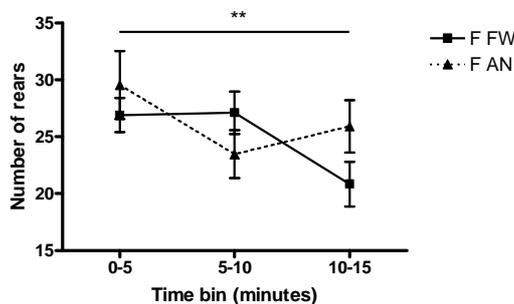


**Graph 7-6 – A line graph to show the number of rears that female foot wounded and anaesthesia-only animals performed, time-binned into 5 minutes bins. \*\*p=0.006; Repeated measures ANOVA with Bonferroni's post-test.**

# Central zone entries decrease over the testing period

## *Males*

The number of entries that the anaesthesia group made into the central zone decreased significantly over the 15 minute time period (p=0.009; 2-way Repeated measures ANOVA with Bonferroni's post-tests). There is a trend for the injured group to show

fewer entries, but this was not significant. There were no significant differences between injured and uninjured animals (Multivariate ANOVA).
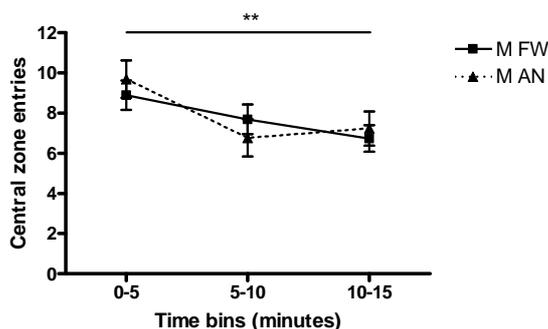


**Graph 7-7 – a line graph to show the number of central zone entries that male foot wounded and anaesthesia-only animals performed, time-binned into 5 minute bins. \*\*p=0.009; 2-way Repeated measures ANOVA with Bonferroni's post-tests.**

## *Females*

The number of central zone entries drops over the testing period in both injured and uninjured females (F FW: p=0.001, F AN: p=0.009; 2-way Repeated measures ANOVA with Bonferroni's post-tests). There were no differences between treatment group (Multivariate ANOVA).
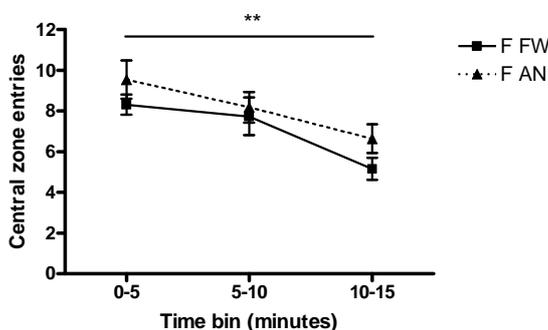


**Graph 7-8 – A line graph to show the number of central zone entries that female foot wounded and anaesthesia-only animals performed, time-binned into 5 minute bins. \*\*p=0.009; 2-way Repeated measures ANOVA with Bonferroni's post-tests.**

# Time spent in central zone increases over the testing period

## *Males*

The time that the injured animals spent in the central zone increased between the first and last time bins (p=0.037; 2-way Repeated measures ANOVA with Bonferroni's post-tests). There was no significant difference between treatment groups (Multivariate ANOVA).
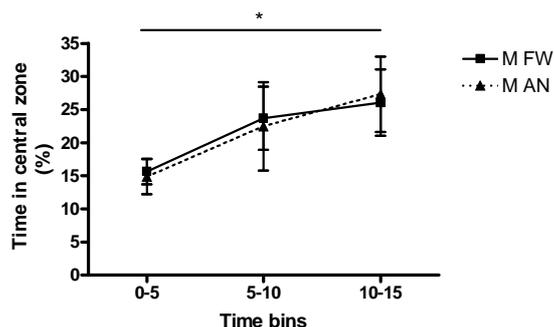
**Graph 7-9 – a line graph to show how male injured and anaesthesia-only animals increase their percentage of time spent in the central zone of the NIH arena over the 15 minute testing period. *p=0.037; 2-way Repeated measures ANOVA with Bonferroni's post-tests.**

## *Females*

The time that the injured females spent in the central zone does not significantly increase over the testing period (2-way Repeated measures ANOVA). There was no significant difference between treatment groups (Multivariate ANOVA).
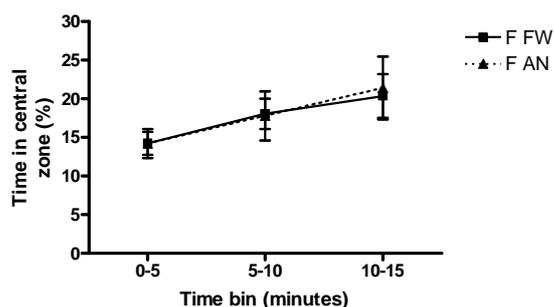


**Graph 7-10 – A line graph to show the percentage of time spent female animals spent in the central zone of the NIH arena in each time bin of the 15 minute testing period.**

## Appetitive behaviour

## The number of Cheerios eaten differs between time bin

### *Males*

The number of Cheerios eaten was time-binned. The results show that there is a difference between FW (n= 12) and AN (n=10) groups in the 10-15 minute time bin (p<0.05, 2-way Repeated measures ANOVA with Bonferroni's post-tests).

Graph 7-11 also shows that there is a difference in the number of Cheerios eaten between the 0-5 and both 5-10 and 10-15 time bins for both injured and uninjured animals (M FW: 0-5 vs. 5-10, p<0.001, 0-5 vs. 10-15, p<0.01; M AN 0-5 vs. 5-10,

p<0.01, 0-5 vs. 10-15, p<0.001; 2-way Repeated measures ANOVA with Bonferroni's post-tests).
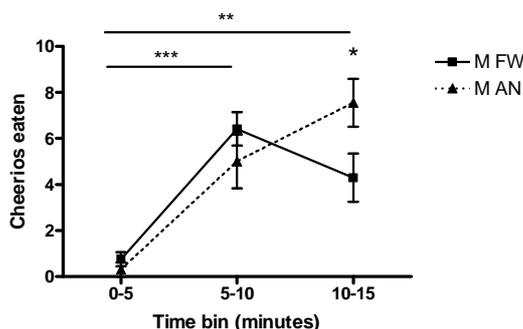


**Graph 7-11 – A line graph to show how the number of Cheerios eaten differs between time bins. The lines above the graph show the data from the male FW group only, although the AN group also show similar differences. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; 2-way Repeated measures ANOVA with Bonferroni's post-tests**

### Females

The time that the injured females (n=16, F AN n=10) spent in the central zone does not significantly increase over the testing period (2-way Repeated measures ANOVA). There was no significant difference between treatment groups (Multivariate ANOVA).
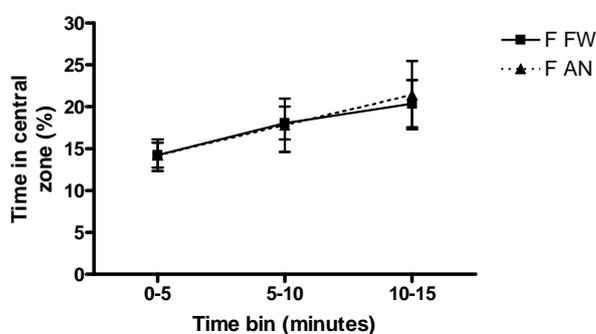


**Graph 7-12 – A line graph to show the percentage of time spent female animals spent in the central zone of the NIH arena in each time bin of the 15 minute testing period.**

## *The effect of single acute foot wound in adulthood on reward related behaviours in animals with no prior history of injury*

The graphs below describe behaviour in the novelty-induced hypophagia test in animals with or without a history of neonatal injury. Graphs describe the time course of behaviours during the 15 minute test period, separated into 5 minute time bins.

## Exploratory behaviours – rearing, central zone entries, and percentage of time spent in the central zone

## Rears are decreased in adult-injured animals compared to controls

*Males*

Graph 7-13 illustrates how male animals that receive a single adult foot wound (n=14) rear less than those with a similar background (repeated neonatal anaesthesia, n=13). This effect is significant in the 5-10 minute time-bin (p=0.016; Multivariate ANOVA with Tukey's HSD post-tests). Animals that are acutely injured in adulthood show a drop in rearing over the first ten minutes (as shown by the arrow, p<0.01) that is still significantly lower by 15 minutes (indicated by the line and asterisk at the bottom, p<0.05; both from Repeated measures ANOVA with Tukey's Multiple Comparison Test).
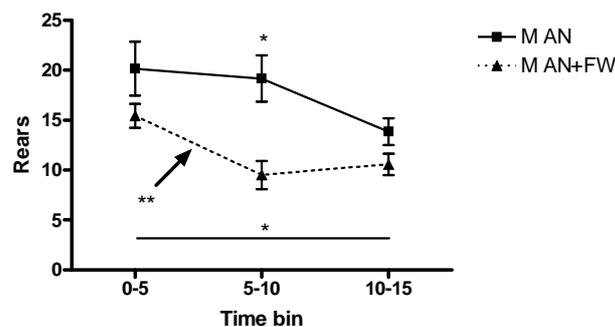


**Graph 7-13 – A line graph to show rearing behaviour in each time bin, in males after a single adult foot wound in animals with no history of prior injury. The arrow and line below the graph are explained in the text. *p<0.05; **p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparison Test**

*Females*

Graph 7-14 illustrates how female animals that receive an adult foot wound (n=13) rear less than those with a similar background (repeated neonatal anaesthesia, n=11). This effect is significant in the 0-5 and 10-15 minute time-bins (0-5: p=0.002; 10-15: p=0.013; Multivariate ANOVA with Tukey's HSD post-tests).
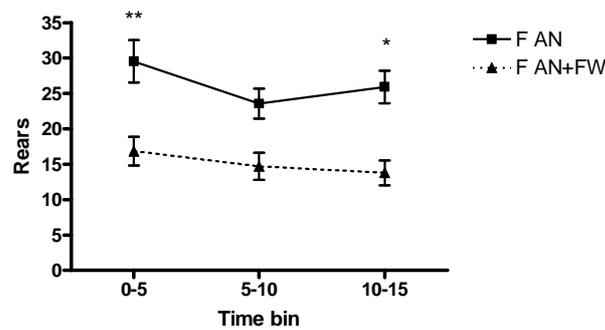
**Graph 7-14 - A line graph to show rearing behaviour in each time bin, in females after a single adult foot wound in animals with no history of prior injury. *p<0.05; **p<0.01. Multivariate ANOVA with Tukey's HSD post-tests.**

## Central zone entries decrease over the testing period

### *Males*

Graph 7-15 shows how rearing behaviour decreases over the 15 minute testing period in males with no history of injury ($p<0.05$ to $p<0.001$; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). There were no differences between treatment groups (Multivariate ANOVA).
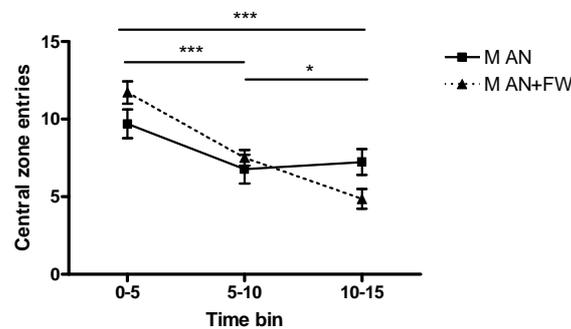


**Graph 7-15 – A line graph to show central zone entries in each time bin of the testing period, in males after a single adult foot wound. *p<0.05; **p<0.01; ***p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparison Test.**

### *Females*

Central zone entries decrease between time bins over the testing period. This is a significant decrease ($p<0.05$ to $p<0.001$; Repeated measures ANOVA with Tukey's Multiple Comparisons test). There were no differences between treatment groups (Multivariate ANOVA).
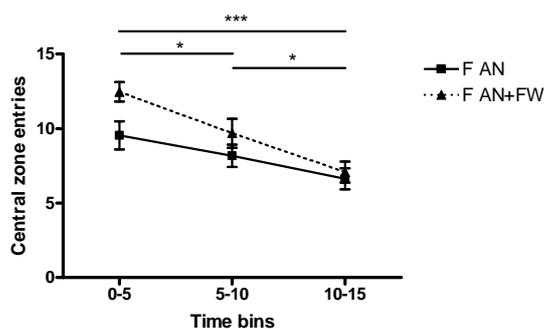
**Graph 7-16 - A line graph to show central zone entries in each time bin of the testing period, in females after a single adult foot wound. Significant differences are shown for the F AN+FW group only, although the F AN group showed a similar significant decrease over the total 15 minute test session (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001. Repeated measures ANOVA with Tukey's Multiple Comparison Test).**

## Percentage of time spent in central zone increases during the testing period

### *Males*

Graph 7-17 shows that the amount of time spent in the central zone increases after the first 5 minute time bin, but this is only a significant increase in the AN+FW group (p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). There were no significant differences between treatment groups (Multivariate ANOVA).
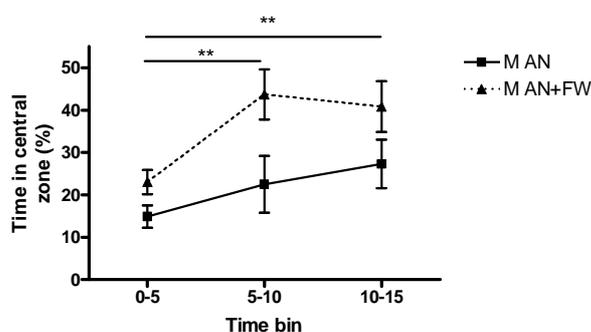


**Graph 7-17 – A line graph to show the amount of time males spent in the central zone in each time bin of the testing period, in males after a single adult foot wound. \*\*p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparison Test.**

### *Females*

Female animals that received a single adult foot wound spend more time in the central zone, in comparison to animals with the same background (repeated neonatal anaesthesia) that were not injured as adults (5-10 minute time bin: p=0.007; 10-15

minute time bin: p=0.001; Multivariate ANOVA with Tukey's HSD post-tests). The females that were injured as adults (F AN+FW) show an increased amount of time in the central zone over the 15 minute testing period ($p<0.05$ to $p<0.001$; Repeated Measures ANOVA with Tukey's Multiple Comparisons Test), which was not seen in the uninjured control group (F AN).
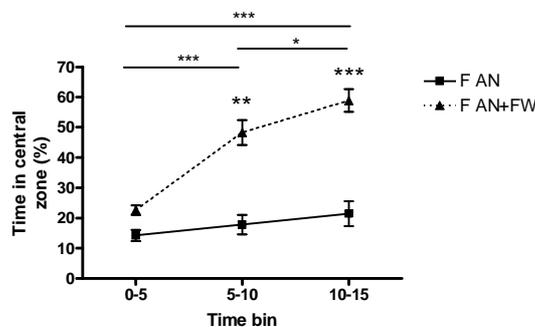


**Graph 7-18 – A line graph to show the amount of time females spent in the central zone in each time bin of the testing period, in females after a single adult foot wound. Lines above the graph indicate \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated Measures ANOVA with Tukey's Multiple Comparisons Test. Asterisks above the F AN+FW triangles indicate \*\*p=0.007; \*\*\*p=0.001; Multivariate ANOVA with Tukey's HSD post-tests.**

## Appetitive behaviours

## Cheerios are eaten after the first 5 minutes of the testing period

### *Males*

Eating behaviour does not begin until 5 minutes of testing, as shown in Graph 7-19. There were no significant treatment group differences (M AN n=10, M AN+FW n=12; Multivariate ANOVA).
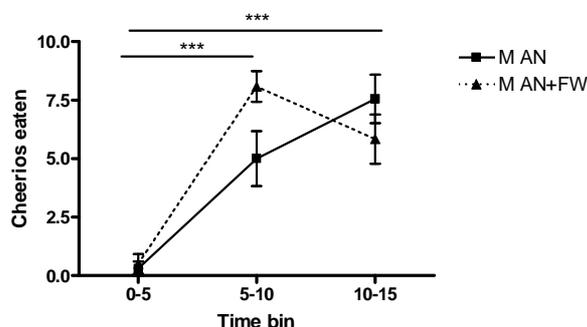


**Graph 7-19 - A line graph to show the Cheerios eaten per time bin in males with no prior history of foot wound, after a single adult foot wound. Significant data shown for M AN+FW group only for clarity, although the M AN group showed similar differences (\*\*\*p<0.001.; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).**

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*Females*

Eating behaviour begins after 5 minutes, and increases over the 15 minute time period in the adult injured females (F AN+FW: p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). Injured females (n=10) eat significantly fewer Cheerios than uninjured controls (n=13) in the 10-15 minute time bin (p<0.01; 2-way Repeated measures ANOVA with Bonferroni post-tests).
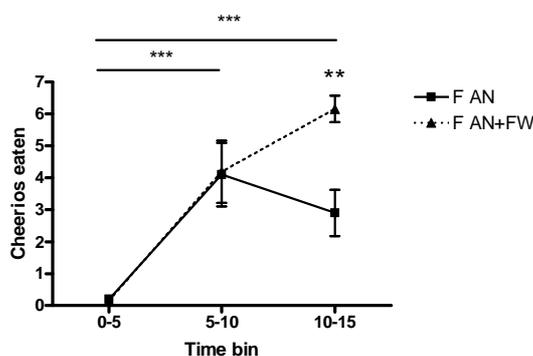


**Graph 7-20 – A line graph to show the Cheerios eaten per time bin in females with no prior history of foot wound, after a single adult foot wound. Lines above the graph indicate \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test. Asterisks above the F AN+FW triangle indicate \*\*p<0.01; 2-way Repeated measures ANOVA with Bonferroni post-tests.**

## Removing the food reward alters exploratory behaviours in males and females with no history of injury

To investigate the effect that the presence of the food reward had in general on exploratory behaviours, the Cheerios were removed from the central bowl and separate litters of animals who received a single adult foot wound were tested. Genders are grouped in the following results. M AN+FW Present n=14; M AN+FW Absent n=5; F AN+FW Present n=13; F AN+FW Absent n=4.

## Exploratory behaviours do not show the same increases or decreases in frequency when the Cheerios are absent.

*Rearing behaviour is affected by the absence of Cheerios in females*

As already reported, rearing in males decreases between time bins over the testing period. This decrease is lost when the Cheerios are removed.

Removing the Cheerios has the effect of increasing rearing in the female animals at the 5-10 minute time bin (see Graph 7-21 – p=0.003; Multivariate ANOVA with Tukey's HSD post-tests).
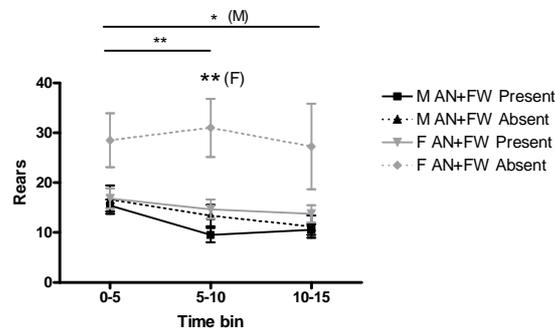


**Graph 7-21 – A line graph showing rearing behaviour in animals with no history of injury, when Cheerios are either present of absent. Rearing behaviour is increased in females when the Cheerios are absent. (M) and (F) refer to the gender of the effect, for both Present and Absent groups. (M) *p<0.05; **p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test. (F) **p=0.003; Multivariate ANOVA with Tukey's HSD post-tests.**

## *Entries into the central zone do not decrease significantly when Cheerios are absent.*

As already reported, the number of entries by males and females into the central zone decreases between time bins over the course of the testing period. This decrease is not significant when the Cheerios are absent.
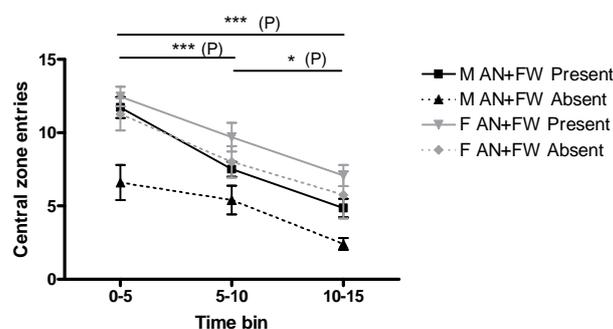


**Graph 7-22 – A line graph showing that entries into the central zone decrease over the testing period in animals with no history of neonatal injury. (P) refers to the significant results in the presence of Cheerios. *p<0.05; **p<0.01; ***p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## *Percentage of time spent in the central zone does not increase over the testing period, and is lower in females when Cheerios are absent.*

There are significantly fewer central zone entries by females in the final 5 minute time bin when Cheerios are absent (p=0.031; Multivariate ANOVA with Tukey's HSD post-tests). As previously reported, the number of entries into the central zone increases between the first and last time bins when Cheerios are present. This is not the case when the Cheerios are absent (see Graph 7-23 )
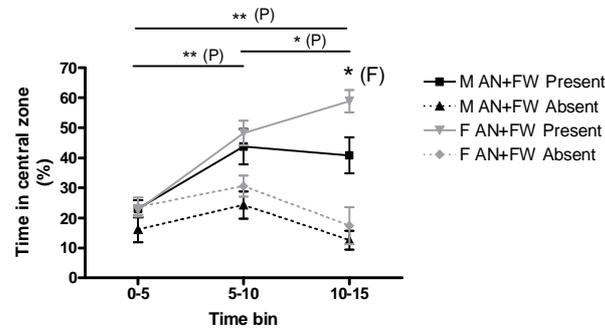


**Graph 7-23 – A line graph to show the effect of absence of Cheerios on percentage of time spent in the central zone. (F) refers to significance in female animals only. (P) refers to significant results in the presence of Cheerios. (P) \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test; (F)\*p=0.031; Multivariate ANOVA with Tukey's HSD post-tests.**

## Moving the food-bowl within the arena does not alter reward-related behaviours in animals with no history of injury

The effect of moving the food-bowl from the centre to a less anxiogenic quadrant was investigated. Litters of animals who received a single adult foot wound were tested with the food-bowl in an 'alternate quadrant' close to the edge of the arena (see Methods) and compared to the results from litters with the food-bowl in the centre. The central zone was defined as circling the food-bowl, regardless of its position. M AN+FW Central group, n=14; M AN+FW AltQuad group, n=3; F AN+FW Central group, n=13; F AN+FW AltQuad group, n=11.

## Exploratory behaviours are minimally affected by the alternate placement of the food-bowl

### *Rearing behaviour is not affected by the placement of the Cheerios*

As previously shown, the number of male rears decreases over the recording period. This is not significant in females, nor for groups where the food-bowl is in the alternate quadrant.
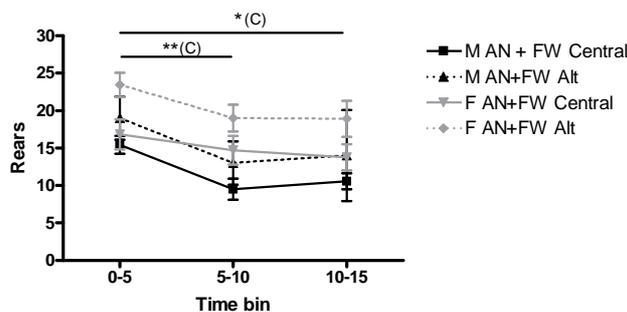
**Graph 7-24 - A line graph showing rearing behaviour in animals with no history of injury, when the Cheerios are moved to an alternate quadrant. (C) denotes significant results when the Cheerios are centrally placed. *p<0.05; **p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test)**

### Central zone entries do not differ when the food-bowl is alternatively placed

As previously shown, the number of central zone entries decreases between time bins over the total testing time for both genders. This trend is similar when the food-bowl is in the alternate quadrant, but is only significant in females (p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Tests).
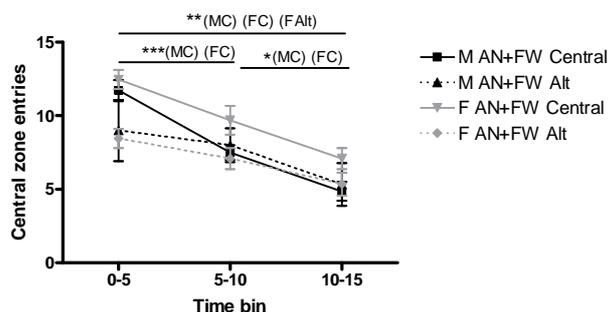


**Graph 7-25 – A line graph to show the number of central zone entries in each time bin over the 15 minute testing period. (MC) refers to male results when the food-bowl was centrally placed; (FC) refers to females results when the food-bowl was centrally placed; (FAlt) refers to female results when the food-bowl was in the alternate quadrant. *p<0.05; **p<0.01; ***p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

### Percentage of central zone time is the same wherever the food-bowl is placed

The percentage of time spent in the central zone increases during the testing period. This trend is not significant (except for females between the first 2 time bins) when the food-bowl is in the alternate quadrant.
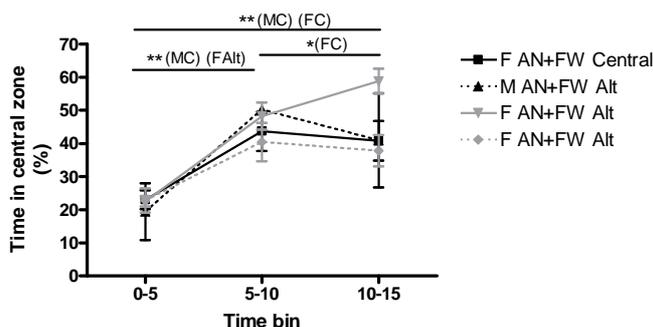
**Graph 7-26 – A line graph to show the percentage of time spent in the central zone when the Cheerios are moved to an alternate quadrant. (MC) refers to male results when the Cheerios are centrally placed; (FC) refers to female results when the Cheerios are centrally placed; (FAlt) refers to female results when the food-bowl was in the alternate quadrant. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## Appetitive behaviours

### *Cheerios eaten*

The number of Cheerios eaten peaks in the 5-10 minute time bin, as previously shown. This trend is the same when the Cheerios are moved within the arena, and there are no significant differences between Cheerio placement groups (Multivariate ANOVA).
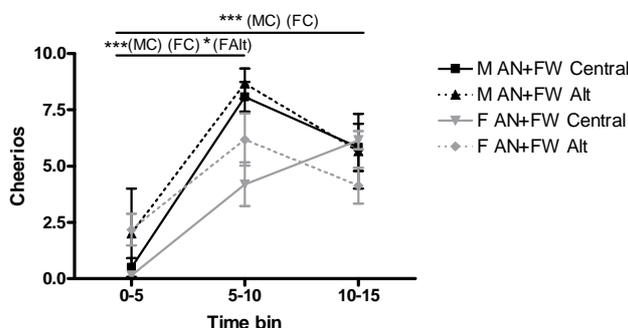


**Graph 7-27 – A line graph to show the number of Cheerios eaten per time bin over the test period. (MC) refers to male results when the Cheerios are centrally placed; (FC) refers to female results when the Cheerios are centrally placed; (FAlt) refers to female results when the food-bowl was in the alternate quadrant. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## *The effect of re-injury to animals with a history of repeated neonatal foot wounds on reward-related behaviours*

The results presented in this section will describe the effect that a single adult foot wound has on an animal with a prior history of surgery. Animals all had repeated neonatal foot wound surgery within the first 21 days of life.

As above, the behaviours were measured during a 15 minute testing period in the novelty-induced hypophagia paradigm (see Methods); outcome measures have been classified into anxiety-related measures (faeces and urine), exploratory behaviours (rearing, central zone entries and time spent in central zone) and appetitive behaviours (time to first Cheerio and total number of Cheerios); and were time-binned accordingly. Genders have been analysed separately. M FW group, n=19; MFW+FW group, n=14; F FW group, n=19, F FW+FW group, n=12.

## Exploratory behaviours – rearing, central zone entries, and percentage of time spent in the central zone

## Rears are near-significantly decreased in males with a history of neonatal injury

### *Males*

Number of rears decreases over the testing period in the re-injured animals only ($p<0.001$; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). Males that were re-injured as adults (M FW+FW) reared near-significantly fewer times in the 5-10 minute time bin compared to control animals (M FW) – $p=0.06$; Multivariate ANOVA with Tukey's HSD post-tests.
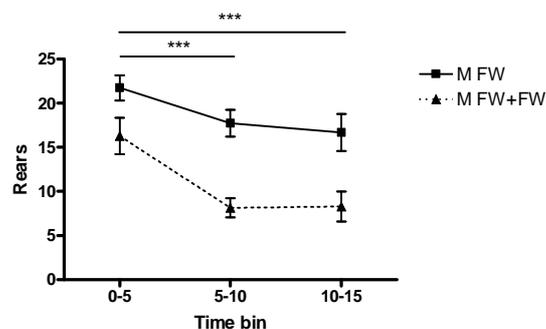


**Graph 7-28 – A line graph to show the number of rears per time bin in males with a history of neonatal repeated injury. \*\*\*$p<0.001$; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

### *Females*

Number of rears decreases over the testing period in the uninjured-as-adult animals only ($p<0.01$; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). Females that were re-injured as adults (F FW+FW) reared significantly fewer times in

the first two time bins compared to control animals (0-5: p=0.016; 5-10: p=0.000; Multivariate ANOVA with Tukey's HSD post-tests).
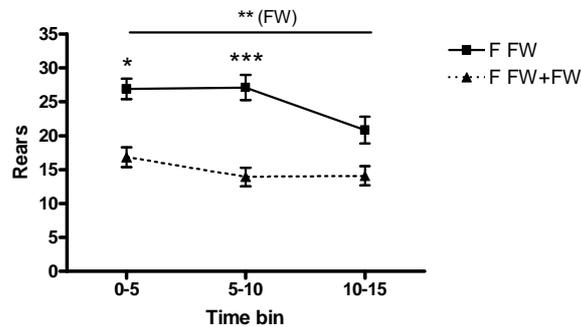


**Graph 7-29 - A line graph to show the number of rears per time bin in females with a history of neonatal repeated injury. \*\*(FW) refers to results in the FW group. \*\*p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test. Larger asterisks indicate treatment group differences. \*p=0.016; \*\*p=0.000; Multivariate ANOVA with Tukey's HSD post-tests.**

## Central zone entries decrease during the testing period, regardless of adult treatment

### *Males*

The number of entries into the central zone decreased between time bins in re-injured males only (p<0.05 to p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). There were no differences between treatment groups at any time point (Multivariate ANOVA).
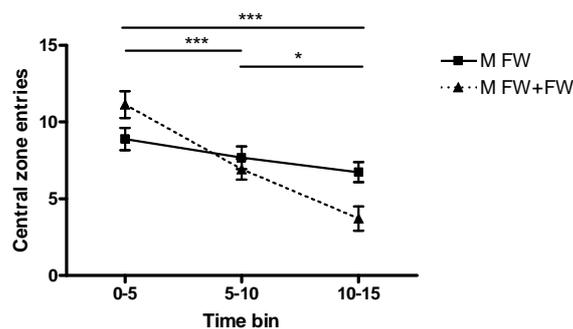


**Graph 7-30 – A line graph to show the number of entries into the central zone per time bin in males with a history of repeated neonatal injury. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

*Females*

The number of entries in the central zone was significantly higher in the first two time bins in females that were re-injured as adults (p=0.000 and p=0.012; Multivariate ANOVA with Tukey's HSD post-tests).

Central zone entries also decreased significantly over the testing period for both adult uninjured and adult re-injured animals (p<0.05 to p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).
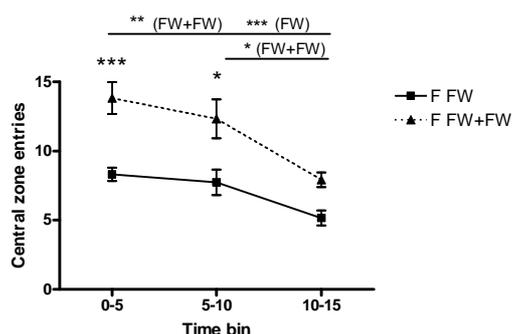


**Graph 7-31 – A line graph to show the number of entries into the central zone per time bin in females with a history of repeated neonatal injury. (FW) and (FW+FW) refer to the treatment group. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test. The larger asterisks denote treatment group differences: \*\*\*p=0.000; \*p=0.012; Multivariate ANOVA with Tukey's HSD post-tests.**

# Percentage of time in the central zone increases further in re-injured animals than controls.

*Males*

The percentage of time spent in the central zone increase between time bins, particularly in males that were re-injured as adults (see **Graph 3-30**). There was a near-significant treatment effect of adult re-injury on the central zone time percentage in the final 10-15 minute time bin (p=0.073; Multivariate ANOVA with Tukey's HSD).
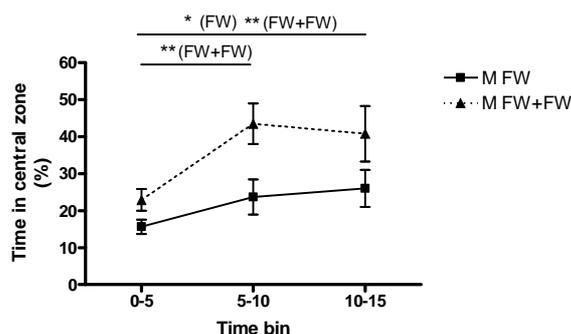
**Graph 7-32 – A line graph to show the percentage of time spent in the central zone in each time bin for males with a history of neonatal injury. (FW) refers to adult-uninjured animals. (FW+FW) refers to animals that were re-injured as adults. \*p<0.05; \*\*p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).**

*Females*

Females that were re-injured as adults spent significantly more time in the central zone in the final time bin (p=0.007; Multivariate ANOVA with Tukey's HSD).

The percentage of time spent in the central zone significantly increases between time bins in females that were re-injured as adults.
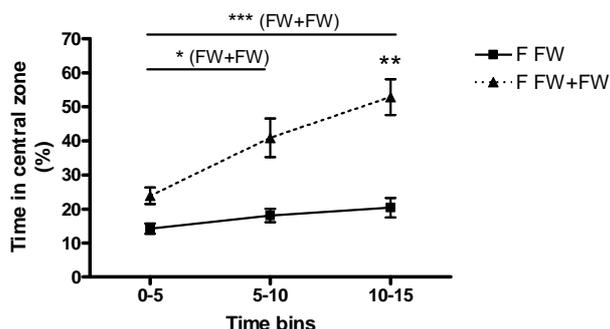


**Graph 7-33 - A line graph to show the percentage of time spent in the central zone in each time bin for females with a history of neonatal injury. (FW+FW) refers to animals that were re-injured as adults. \*p<0.05; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test. The larger asterisk denotes a significant effect of treatment group. P=0.007; Multivariate ANOVA with Tukey's HSD.**

## Appetitive behaviours

## Cheerio consumption is not affected by an adult re-injury

*Males*

Cheerio consumption does not begin until the second time bin, and peaks in the 5-10 minute time bin. For both the uninjured (n=12) and injured (n=12) adults, there was a

significant increase in Cheerio consumption after the first time bin and over the total period (p<0.001 and p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).
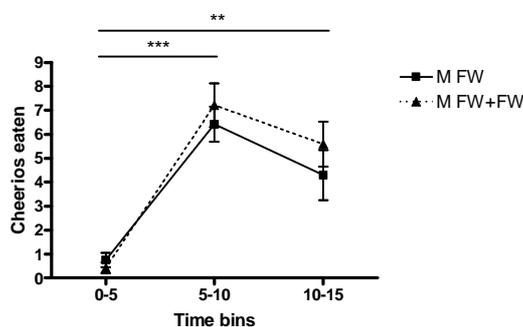


**Graph 7-34 – A line graph to show the number of Cheerios eaten per time bin in males with a history of foot wound. \*\*\*p<0.001; \*\*p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## *Females*

Cheerio consumption significantly increases between time bins and over the total period in both uninjured (n=13) and adult re-injured (n=11) females (p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). There was no significant effect of treatment (Multivariate ANOVA).
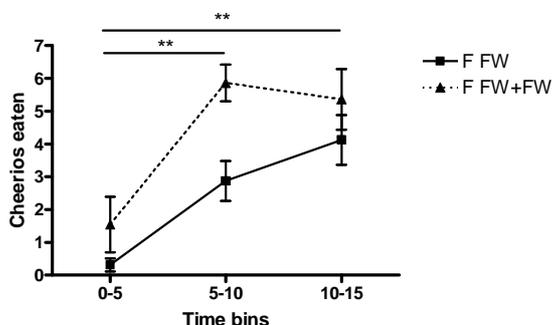


**Graph 7-35 – A line graph to show the number of Cheerios eaten per time bin in females with a history of foot wound. \*\*p<0.01; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## Removing the food reward does not alter exploratory behaviours in males and females with a history of injury

To investigate the effect that the presence of the food reward had in general on exploratory behaviours, the Cheerios were removed from the central bowl and separate litters of animals with a history of foot wound that were re-injured as adults were tested.

M FW+FW Present group, n=19; M FW+FW Absent group, n=5; F FW+FW Present group, n=12; F FW+FW Absent group, n=4.

## Exploratory behaviours – rearing, central zone entries and percentage of time spent in central zone

### *Rearing behaviour is affected by the presence of Cheerios in females with a history of injury*

Females that were re-injured as adults showed an increased number of rears when the Cheerios were absent (p=0.045; Multivariate ANOVA with Tukey's HSD post-tests). The number of rears decreased over the testing period for both groups of males (p<0.01 to p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).
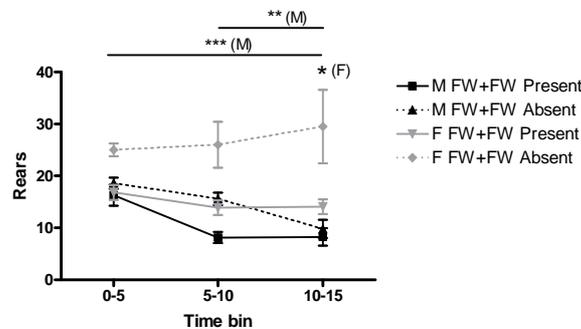


**Graph 7-36 – A graph to show the number of rears of both genders per time bin when Cheerios were present or absent in animals with a history of foot wound. (M) refers to results that were significant in males only \*\*p<0.01; \*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test. The larger asterisk refers to results for females only (F)\*p=0.045; Multivariate ANOVA with Tukey's HSD post-tests.**

### *Central zone entries are not affected by the presence or absence of Cheerios in animals with a history of injury.*

The number of entries into the central zone decreased between time bins. This effect was similar for all groups, regardless of the presence of Cheerios.
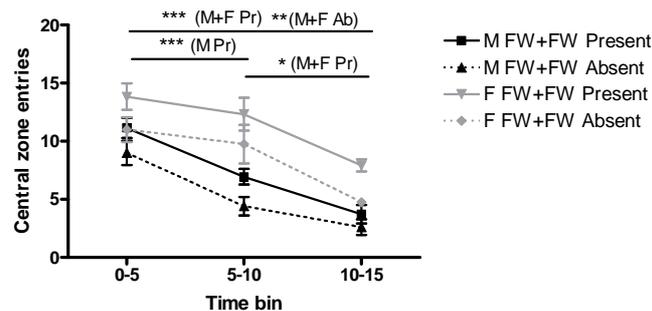
**Graph 7-37 – A line graph to show the number of central zone entries per time bin when Cheerios are present/absent in animals of both gender with a history of neonatal repeated injury. (M) or (F) refer to gender, (Pr or Ab) refer to presence of absence of Cheerios. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).**

### *Percentage of time spent in the central zone does not show any increase over the testing period when the Cheerios are removed*

The percentage of time spent in the central zone increases in both genders during the testing period, as previously shown. This effect is lost in both genders when the Cheerios are removed.
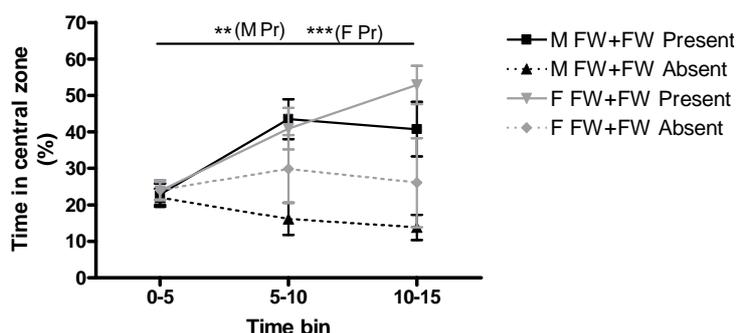


**Graph 7-38 – A graph to show the percentage of time spent in the central zone per time bin for both genders in animals with a history of repeated foot wound. (M) and (F) refers to the gender of the result. (Pr) refers to the result in the presence of Cheerios. \*\*p<0.01; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test).**

## Moving the food-bowl within the arena slightly alters reward-related behaviours in animals with a history of foot wound

The effect of moving the food-bowl from the centre to a less anxiogenic quadrant was investigated. Litters of animals with a history of foot wound that were re-injured as adults were tested with the food-bowl in an 'alternate quadrant' close to the edge of the arena (see Methods), and compared to results from litters with the food-bowl in the centre. The central zone was defined as circling the food-bowl, regardless of its position. Genders are grouped in the following results. MFW+FW Centre n=14; MFW+FW AltQuad n=10; F FW+FW Centre n=12; F FW+FW AltQuad n=5.

## Exploratory behaviours – rearing, central zone entries and percentage of time spent in central zone

### *Rears are not affected by the placement of the food-bowl in animals re-injured as adults*

As described previously, the number of rears that males perform decreases over the 15 minute testing period. This trend is the same for males when the bowl is in the alternate quadrant (p<0.05; Repeated measures ANOVA with Tukey's Multiple Comparisons Test). There is no effect on female rearing behaviour.
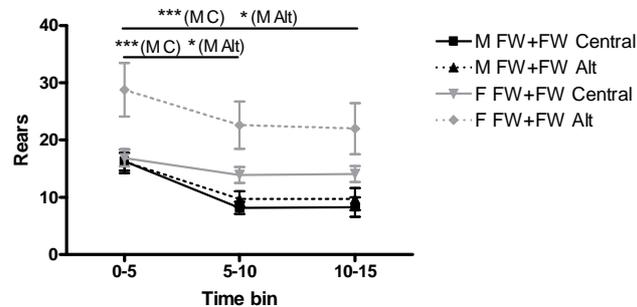


**Graph 7-39 – A graph to show the number of rears per time bin in animals of both gender with a history of foot wound that have been re-injured. (M C) refers to male results from Cheerios centrally placed, (M Alt) refers to male results from Cheerios in the alternate quadrant. \*p<0.05; \*\*\*p<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## *Central zone entries are not affected by the placement of the Cheerios in re-injured animals with a history of injury.*

As shown previously, males and females that are re-injured as adults show a decrease in central zone entries over successive time bins. This effect is replicated in situations where the Cheerios are absent. There are no effects of placement of the Cheerios.
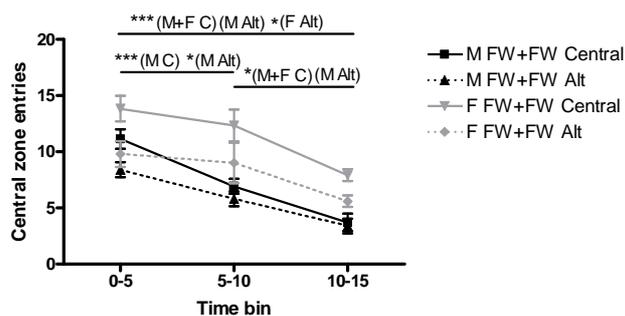


**Graph 7-40 – A graph to show the number of central zone entries per time bin for males and females that are re-injured as adults, when the Cheerios are present or absent. (M) or (F) refers to the gender within which the results are seen. (C) or (Alt) refers to whether the Cheerios were placed centrally or in the alternate quadrant. \*p<0.05; \*\*p<0.01;\*\*\*P<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

***The increase in percentage of time spent in the central zone is lost in females re-injured as adults when the food-bowl is moved from the centre***

As previously shown, the percentage of time spent in the central zone increased between time bins, for both males and females re-injured as adults. This result is lost in females re-injured as adults when Cheerios are moved to an alternate quadrant.
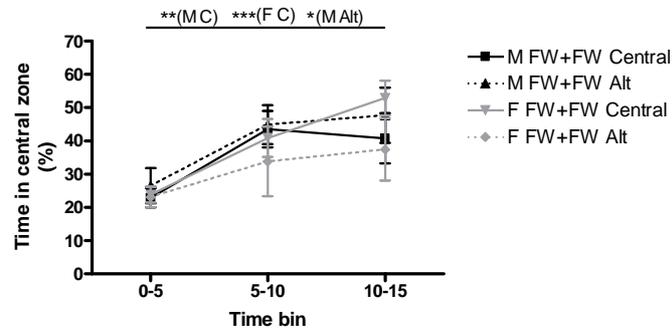


**Graph 7-41 – A graph to show the percentage of time spent in the central zone per time bin for animals that are re-injured as adults. (M) and (F) refer to the gender in which the results are seen. (C) and (Alt) refers to whether the Cheerios were placed centrally or in the alternate quadrant. \*p<0.05; \*\*p<0.01;\*\*\*P<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

## Appetitive behaviours

***Number of Cheerios eaten is unaffected by the placement of the food-bowl in re-injured animals***

As previously shown, Cheerio consumption begins after the first time bin (see Graph 7-34 and Graph 7-35). This pattern is the same when the Cheerios are moved from the centre of the arena to the edge (alternate quadrant).
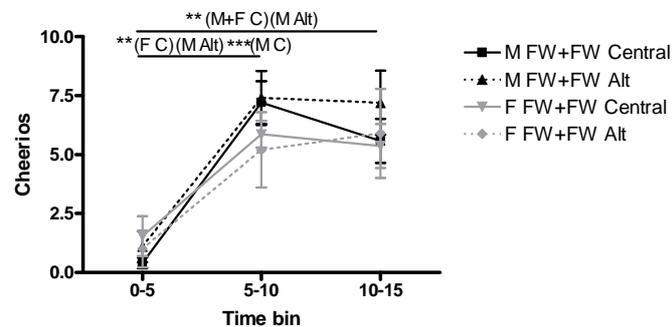


**Graph 7-42 – A graph to show the number of Cheerios eaten per time bin by animals of both gender re-injured as adults. (M) and (F) refer to the gender in which the results are seen. (C) and (Alt) refers to whether the Cheerios were placed centrally or in the alternate quadrant. \*\*p<0.01;\*\*\*P<0.001; Repeated measures ANOVA with Tukey's Multiple Comparisons Test.**

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