Evaluation of the Neurokinin-1 receptor knockout mouse model of Attention Deficit Hyperactivity Disorder

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

Abstract

Attention Deficit Hyperactivity Disorder (ADHD) is a common neurobehavioural disorder in children and often persists into adulthood. Current treatments lack efficacy in a large number of patients, and the aetiology of ADHD needs to be further elucidated in order to develop alternative treatments. The neurokinin-1 receptor knockout (NK1R-/-) mouse has recently been proposed as an animal model of ADHD; it is hyperactive and this is reduced by psychostimulants, polymorphisms in the TACR1 gene (the human NK1R gene) have been found in patients with ADHD, and NK1R-/- mice have deficits in monoamine transmission, consistent with the pathophysiology of ADHD. Finally, the NK1R-/- mouse exhibits inattentiveness, impulsivity and perseveration in the 5-Choice Serial Reaction-Time Task (5-CSRTT).

The aim of this thesis was to further validate the NK1R-/- mouse model of ADHD by studying the effects of *d*-amphetamine on its performance in the 5-CSRTT, and subsequently to test alternative drugs that could be therapeutically beneficial. However, over the course of this work, the hyperactivity of NK1R-/- mice was not stable when tested acutely. Therefore, telemetry was used to monitor the activity of mice in their homecage over many days. Also, the breeding method was expanded to include heterozygous matings in order to minimise genetic drift. Whilst hyperactivity in this test was robust in both colonies, the impulsivity seen in NK1R-/- mice bred from homozygous matings was not present in NK1R-/- mice bred from heterozygotes. Furthermore, there was a reduction in choline acetyltransferase-positive cells in the striatum of homozygously-bred but not heterozygously-bred NK1R-/- mice.

These findings highlight important interactions between early environment, NK1R function and the central cholinergic system. They also support evidence suggesting that abnormal cholinergic transmission is involved in ADHD, and could underlie impulsivity. Finally, these findings could have important implications for future research on impulse control disorders.

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5-CSRTT	5-Choice Serial Reaction-Time Task
5-HT	Serotonin
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of variance
BCA	Biocinchoninic
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
CNS	Central nervous system
CO_2	Carbon dioxide
CPP	Conditioned place preference
CPT	Continuous Performance Task
DA	Dopamine
DAB	3,3'-Diaminobenzidine
DAG	Diacyl glycerol
DAT	DA transporter
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
FITC	Fluorescein isothiocyanate
G-protein	Guanine nucleotide binding protein
GABA	Gamma-amino butyric acid

GANC	Gancyclovir
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horse radish peroxidase
i.p.	Intraperitoneal
IP_3	Inositol 1,4,5-trisphosphate
ITI	Inter-trial interval
LDEB	Light/Dark Exploration Box
LH	Limited hold
LITI	Long inter-trial interval
mAChR	muscarinic acetylcholine receptors
MSN	medium spiny neuron
NA	Noradrenaline
nAChR	nicotinic acetylcholine receptors
NGF	Nerve growth factor
NI	No injection
NK1	Neurokinin-1
NK1R	Neurokinin-1 receptor
NK1R-/-	Neurokinin-1 receptor knockout mouse
NK2	Neurokinin-2
NK3	Neurokinin-3
PB	Phosphate buffer
PFA	Paraformaldehyde

RIPA	Radioimmunoprecipitation assay
RTH	Resistance to thyroid syndrome
SD	Stimulus duration
SHR	Spontaneously hypertensive rat
TACR1	Tachykinin-1 receptor
TR-β1	Thyroid hormone receptor β1 transgenic mouse
TTBS	Tris-Triton Buffered Saline
VITI	Variable inter-trial interval

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1 Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is a common neurobehavioural disorder in children and persists into adulthood in many cases (Wender et al., 2001). Although abnormal catecholamine transmission is thought to be central to the disorder, its aetiology remains unclear. Furthermore, there is concern over the efficacy and long-term effects of the most commonly prescribed treatments, the psychostimulants *d*-amphetamine and methylphenidate. Animal models play an important role in furthering our understanding of human disorders. In this thesis, the validation of a new animal model of ADHD, the neurokinin-1 receptor knockout mouse (NK1R-/-) is discussed.

1.1 Attention Deficit Hyperactivity Disorder

1.1.1 Clinical diagnosis

The worldwide prevalence of ADHD in children is estimated to be between 5.9% and 7.1%, and the disorder is more frequent in males than females (Willcutt, 2012). ADHD persists into adulthood in one to two-thirds of patients (Wender et al., 2001). As defined in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), three core symptoms define ADHD: hyperactivity, impulsivity and inattention and they have to be present for at least 6 months prior to diagnosis. Within this, three subtypes have been defined: Predominantly Hyperactive/Impulsive Type (for patients presenting with 6 or more hyperactive and/or impulsive symptoms), Predominantly Inattentive Type (for patients presenting with 6 or more inattentive symptoms) and Combined Type (for patients presenting with 6 or more symptoms from each of the other two subtypes). More female patients than male patients are diagnosed with the Predominantly Inattentive Type, while male patients are more likely to have the Combined Type (Willcutt, 2012).

Currently, the most widely prescribed treatments are psychostimulants such as methylphenidate (e.g. Ritalin) and *d*-amphetamine (e.g. Adderall®). However, in many patients these treatments are not effective, and there is also concern over the

safety of long-term stimulant use. In order to identify new treatments the pathophysiology of the disease needs to be understood.

1.1.2 Pathophysiology

Imaging studies indicate that a disruption of normal fronto-striatal circuits is linked to ADHD. Executive function, such as the effective manipulation of working memory and response inhibition, is mediated by this network, and deficits in these processes are seen in patients with ADHD. In line with this, a hypoactive fronto-striatal circuit has been found in these patients (Dickstein et al., 2006). Abnormal activation of mesolimbic circuitry, which mediates the response to reward, is also found in patients with ADHD (Ströhle et al., 2008).

Dopaminergic, noradrenergic and serotonergic neurons innervate fronto-striatal circuits, and abnormal transmission of these monoamines is thought to underlie the pathophysiology of ADHD. The long-standing theory of ADHD is the dopamine (DA) hypothesis, stating that a deficit in DA within fronto-striatal circuits causes ADHD (Levy, 1991). This is supported by the fact that psychostimulants are the most widely prescribed treatments for the disorder. However, these stimulants block the reuptake, and increase the release of not just DA, but also noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT) (Kuczenski and Segal, 1989; Wilens, 2006). Furthermore, the selective NA reuptake inhibitor atomoxetine is prescribed for the treatment of ADHD (Michelson et al., 2003, 2001), suggesting that ADHD is not caused exclusively by a deficit in DA transmission. As seen in section 1.1.3.1, the hypothesis that abnormal monoamine transmission is involved in ADHD has been supported by genetic studies.

There is increasing evidence to suggest that cholinergic dysregulation may be involved in the pathophysiology of ADHD. Maternal smoking is a risk factor for ADHD (Linnet et al., 2003), yet there is also evidence that symptoms of ADHD are improved by nicotine (Levin et al., 1996), which could explain why smoking is highly comorbid with ADHD (McClernon and Kollins, 2008). Also, within the striatum, acetylcholine and DA have a reciprocal relationship: DA receptors modulate the firing of striatal cholinergic interneurons, nicotinic acetylcholine receptors located on DA axons modulate DA release (Rice and Cragg, 2004) and

muscarinic acetylcholine receptors modulate DA receptor activation on striatal medium spiny neurons (Zhou et al., 2003). These mechanisms are further discussed in chapter 6 and suggest that acetylcholine may play a role in the pathophysiology of ADHD.

1.1.3 Cause

The underlying cause of ADHD is still widely debated, and understanding it is important for the development of new effective treatments (Biederman and Faraone, 2005). Various aetiologies have been proposed and are outlined below.

1.1.3.1 Genetics

The heritability of ADHD is around 75%, suggesting a genetic basis for the disorder (Faraone et al., 2005). Due to the disruption of monoamine transmission in patients with ADHD, studies have focussed on genes relating to DA, 5-HT and NA transmission. Polymorphisms in genes relating to DA have been found in patients with ADHD, including the DA transporter gene DAT1 (Cook et al., 1995) and dopamine D4 and D5 receptors (Daly et al., 1999; LaHoste et al., 1996). In addition, polymorphisms in the 5-HT transporter gene and the 5-HT1B receptor gene have been identified (Hawi et al., 2002; Seeger et al., 2001). Polymorphisms in NA transporter and receptor genes have also been associated with ADHD (Comings et al., 2003, 2000), although these were found in Tourette's syndrome patients with comorbid ADHD; in other studies looking only at ADHD, no polymorphisms were found (Faraone et al., 2005). Animal models have also guided the search for the genetic basis of ADHD. For example, the coloboma mouse is hyperactive and this is reduced by *d*-amphetamine. It also has a deletion of the SNAP25 gene, which is involved in exocytotic neurotransmitter release (Hess et al., 1996). Subsequently, an association between polymorphisms of the SNAP25 gene and ADHD has been described (Barr et al., 2000). Animal models of ADHD are further discussed in section 1.1.4.

1.1.3.2 Environmental factors

Hypoxia during childbirth, caused by a range of complications including pre-eclampsia (hypertension during pregnancy) or foetal distress, has been linked to ADHD (Biederman and Faraone, 2005). Hypoxia disrupts normal DA function in

the brain (Boksa and El-Khodor, 2003) which fits with the dopaminergic hypothesis of ADHD. Maternal smoking during pregnancy also increases the risk of ADHD (Linnet et al., 2003). Animal studies suggest this could be caused by changes in the number of DA receptor binding sites as compensation for a loss of nicotinic receptors following pre-natal nicotine exposure (Fung and Lau, 1989; Slotkin et al., 1987).

1.1.4 Animal models of ADHD

Investigations into the aetiology of ADHD have been aided by the development of animal models of the disorder. As well as providing insight into the causes of a disease, animal models can also help develop new treatments. A good animal model will fulfil three criteria: face, construct and predictive validity. In the context of ADHD, face validity means that the animal has the symptoms of the disorder, such as deficits in attention, impulsivity and hyperactivity. Construct validity means that the symptoms must relate to the pathophysiology of the disorder, such as a disruption of fronto-striatal circuits. Finally, the model needs good predictive validity, meaning that it can predict new treatments and identify previously unknown causes of the disorder. The first step in verifying the predictive validity of a model is often to test the effects of currently available treatments. In ADHD, this could mean, for example, testing whether psychostimulants reduce the animals' deficits.

Various animal models of ADHD have been developed, and can be broadly divided into two categories: genetic models and pharmacological models. These are discussed below.

1.1.4.1 Genetic models

<u>The spontaneously hypertensive rat</u>

The spontaneously hypertensive rat (SHR) was initially developed to study hypertension by inbreeding hypertensive Wistar-Kyoto rats (Okamoto and Aoki, 1963). However, following the finding that the SHR is hyperactive (Moser et al., 1988), it has become the most widely studied model of ADHD. The SHR has deficits in sustained attention and motor impulsivity, thus satisfying the criteria of face validity (Sagvolden, 2000). It also has a deficit in DA and NA in fronto-striatal

circuits (Russell et al., 2000, 1995). However, while *d*-amphetamine has been shown to alleviate hyperactivity (Myers et al., 1982), methylphenidate did not improve hyperactivity, attention or impulsivity (Van den Bergh et al., 2006). Another issue with the SHR is that the control animals used are Wistar-Kyoto rats. These rats show cognitive deficits when compared to Wistar rats, such as hypoactivity (Paré, 1989). Therefore, studies comparing SHRs and Wistar-Kyoto rats should be interpreted with caution.

Table 1.1 Summary of genetic and pharmacological animal models of ADHD. Hyp: hyperactivity; Imp: impulsivity; Att: attention. Adapted from Sontag et al. (2010).

The dopamine-transporter knock-out mouse

The DA transporter (DAT) clears extracellular DA and is the main target for *d*-amphetamine and methylphenidate. Alterations in the DAT gene have been found in patients with ADHD (Cook et al., 1995), and for these reasons the DAT knock-out mouse was investigated as a model of ADHD. This mouse is hyperactive and this hyperactivity is reduced by *d*-amphetamine and methylphenidate (Gainetdinov et al., 1999). It also shows deficits in spatial memory (Gainetdinov et al., 1999) and a decrease in intracellular DA storage and release, presumably to compensate for the loss of DAT (Jones et al., 1998). However, because these mice are lacking DAT, the effects of psychostimulants are presumably mediated by other monoamine transporters (see: Van der Kooij and Glennon, 2007). In patients with ADHD, DAT will still be present even if its function is abnormal. This brings into question the predictive validity of this model.

The thyroid hormone receptor β1 transgenic mouse

Around 70% of patients with resistance to thyroid syndrome (RTH) meet the diagnostic criterion for ADHD. RTH results in elevated thyroid hormone, and the thyroid hormone receptor β1 mutant mouse (TR-β1) has been investigated in relation to ADHD (Siesser et al., 2006). While mice lacking the thyroid hormone receptor gene have normal behaviour (Forrest et al., 1996), the TR-β1 mouse has a mutation in the ligand-binding domain of this gene and is hyperactive, impulsive and inattentive, and has deficits in DA turnover. Furthermore, it shows increased activity which is reduced by methylphenidate (Siesser et al., 2006). However, the main issue with this model of ADHD is that while patients with RTH can exhibit symptoms of ADHD, there is no evidence linking thyroid abnormalities to patients with ADHD.

Other genetic models

The models discussed above are those that currently satisfy to the greatest extent the face, construct and predictive validity. Other models have also been studied, but they fail to meet all three criteria. These include the coloboma mutant mouse, which has a mutation in the SNAP25 gene. As mentioned in section 1.1.3.1,

polymorphisms in SNAP25 have been found in patients with ADHD (Barr et al., 2000). The coloboma mouse is hyperactive and has abnormal DA and NA function (Jones et al., 2001). However, while the hyperactivity is reduced by *d*-amphetamine, it is increased by methylphenidate, questioning its predictive validity (Hess et al., 1996). Other models, such as the Naples high-excitability rat and mice lacking alpha-synuclein proteins have also been studied. The face, construct and predictive validity of all of these genetic models of ADHD are summarised in Table 1.1.

1.1.4.2 Pharmacological models

Due to the involvement of DA in ADHD, rats with neonatal lesions to DA-containing neurons have been studied in relation to ADHD. These rats are hyperactive and this is reduced by *d*-amphetamine and methylphenidate (Luthman et al., 1989). As well as changes in the DA system, these lesions also increase 5-HT in the striatum (Towle et al., 1989). Another pharmacological model that has been studied is neonatal hypoxia in rats. As mentioned in section 1.1.3.2, hypoxia during childbirth is a risk factor for ADHD. Rats that have experienced hypoxia are hyperactive and this is reduced by *d*-amphetamine (Speiser et al., 1988). DA, NA and 5-HT transmission is also altered in these rats (Dell'Anna et al., 1993). These two models therefore have some face, construct and predictive validity. Further studies need to establish whether there are also deficits in impulsivity or attention in these rats. Other models have also been suggested, and are summarised in Table 1.1.

Although there are many animal models of ADHD, none completely satisfy the criteria for face, construct and predictive validity. Recently, a new animal model of ADHD has been proposed: the NK1R-/- mouse. In the next section, the neurokinin-1 receptor (NK1R) and its preferred ligand, substance P, are described.

1.2 Substance P and the neurokinin-1 receptor

1.2.1 Substance P

Substance P is a neuropeptide neurotransmitter expressed by subsets of neurons found throughout the central nervous system (CNS) and the peripheral nervous system. It was first discovered in 1931 in equine brain and intestine tissue by Von Euler and Gaddum (1931) but was not named until 1934 (Gaddum and Schild,

1934). The 'P' is thought to derive either from 'preparation' or 'power'(Otsuka and Yoshioka, 1993). Despite many attempts to purify and characterise substance P, it was not until forty years later that its amino acid structure was determined as an undecapeptide (11 amino acid sequence): H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Chang et al., 1971).

The role of substance P as a sensory transmitter was confirmed with the demonstration that substance P is present in concentrations around 10-20 times higher in dorsal than ventral roots of bovine spinal cord (Takahashi et al., 1974) and that substance P exerts an excitatory effect on frog (Konishi and Otsuka, 1974a) and rat (Konishi and Otsuka, 1974b) neurons in the spinal cord. Subsequently, research into substance P focused on its localisation within the spinal cord, specifically within the primary afferent terminals of the dorsal horn. However, as well as its role in sensory transmission, substance P has been implicated in a range of functions, due to its wide distribution in the CNS (for review see: Otsuka and Yoshioka, 1993).

Substance P belongs to a family of tachykinins, which derives from the word 'tachy', meaning rapid, and 'kinin', which is the name given to various polypeptides involved in vasodilation and smooth muscle contraction. Two other mammalian tachykinins have also been discovered: neurokinin A (or substance K) and neurokinin B (or neuromedin K). Substance P and neurokinin A are encoded by alternative splice variants of the preprotachykinin A gene, and neurokinin B is encoded by the preprotachykinin B gene (Kotani et al., 1986; Nawa et al., 1984, 1983) (Figure 1.1).

Figure 1.1 Illustration of the synthesis of mammalian tachykinins. Numbered boxes represent gene exons. PPT: preprotachykinin; SP: substance P; NKA: neurokinin A; NKB: neurokinin B. Adapted from Otsuka and Yoshioka (1993).

1.2.2 Tachykinin receptors

Each mammalian tachykinin preferentially binds to a different receptor subtype. These receptors were first discovered in the 1980s (Buck et al., 1984; Iversen et al., 1982; Laufer et al., 1986; Lee et al., 1982), and have since been renamed neurokinin-1 (NK1), neurokinin-2 (NK2) and neurokinin-3 (NK3). They are the

preferential binding sites for substance P, neurokinin A and neurokinin B, respectively (Mantyh et al., 1989; Regoli et al., 1987). However, all three tachykinins have some degree of binding affinity for each receptor (Maggi and Schwartz, 1997).

The cloning of NK1Rs from the rat revealed that they are guanine nucleotide binding protein (G-protein) coupled receptors (Hershey and Krause, 1990; Yokota et al., 1989). The human receptor is 94.5% homologous to the rat receptor (Gerard et al., 1991; Takeda et al., 1991). Substance P binding to NK1Rs activates three G-protein subunits: $G_{q/11}$, $G_{\alpha s}$ and $G_{\alpha o}$, which are predicted to activate three second messenger pathways (Garcia et al., 1994; Khawaja and Rogers, 1996; Merritt and Rink, 1987; Nakajima et al., 1992; Quartara and Maggi, 1997; Roush and Kwatra, 1998; Takeda et al., 1992). $G_{q/11}$ activates phospholipase $C_{\beta,}$ which in turn induces the formation of inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 increases intracellular calcium concentration, while DAG activates protein kinase C. $G_{\alpha s}$ activates adenylyl cyclase, which synthesises cyclic adenosine monophosphate (cAMP). cAMP is involved in various processes including intracellular signal transduction, the activation of protein kinases and the regulation of ion channels. Finally, $G_{\alpha o}$ activates phospholipase A_2, which causes the release of arachidonic acid by the hydrolysis of membrane phospholipids. Arachidonic acid regulates signalling enzymes, including phospholipase C and protein kinase C (Figure 1.2).

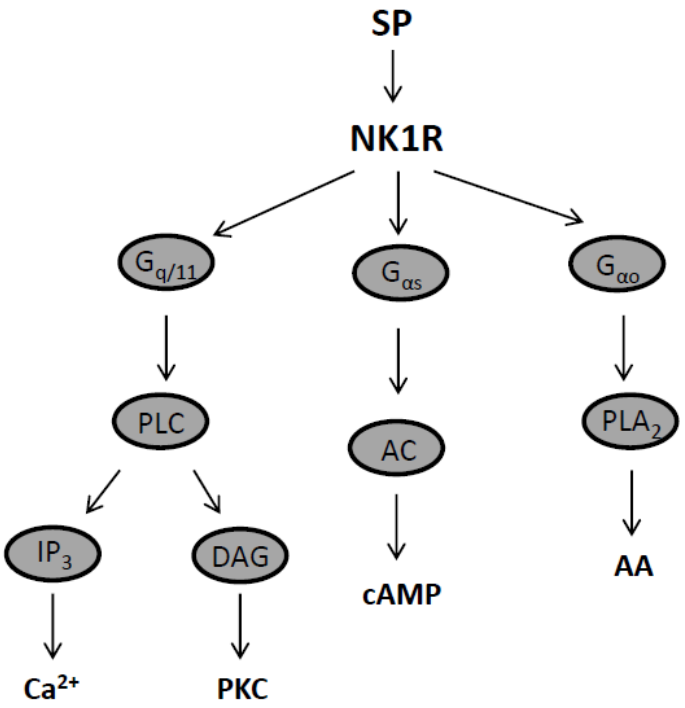

Figure 1.2 Illustration of NK1R signaling. Substance P binding to NK1R activates 3 G-protein subunits and subsequently 3 second messenger pathways. PLC: phospholipase C_β; AC: adenylyl cyclase; PLA_2: phospholipase A_2; IP_3: inositol 1,4,5-trisphosphate; DAG: diacyl glycerol; cAMP: cyclic adenosine monophosphate; AA: arachidonic acid; PKC: protein kinase C.

1.2.3 Distribution of substance P and NK1R

Substance P and NK1Rs are located throughout the peripheral and central nervous systems (for review see: Otsuka and Yoshioka, 1993; Quartara and Maggi, 1997). In this thesis, the focus will be on their distribution within the mouse brain. Levels of substance P and NK1R are particularly low within the cortex or hippocampus, being located mainly within subcortical areas in rats (Nakaya et al., 1994) and mice (Weir, 2012).

Telencephalon

NK1Rs are densely expressed in the striatum, within both the caudate-putamen and the nucleus accumbens (Nakaya et al., 1994). Substance P expression is also high in the striatum, where it is found in the dynorphin-positive, gamma-amino butyric acid (GABA)-ergic medium spiny neurons that project to the globus pallidus, the substantia nigra and the ventral pallidum (Anderson and Reiner, 1990), as well as onto striatal cholinergic interneurons (Bolam et al., 1986), which all express NK1R (Aubry et al., 1994; Gerfen, 1991). The striatum is involved in habit formation, reward and action selection and suppression (Gage et al., 2010;

Pennartz et al., 2009). The strong presence of substance P and NK1R in the striatum suggests they may play an important role in these processes.

Substance P fibres are also located in the medial and central nuclei of the amygdala (Emson et al., 1978), as are NK1R (Nakaya et al., 1994). The amygdala is involved in affective disorders (Phelps and LeDoux, 2005) and, as discussed later in this chapter, the anti-depressant effects of NK1R antagonists are thought to be mediated, in part, by this structure (Kramer et al., 1998).

Within the brain, the highest levels of substance P are found within the substantia nigra (Davies and Dray, 1976). The substantia nigra forms part of the direct and indirect basal ganglia loops, where it plays a key role in modulating motor actions (Bolam et al., 2000). However, there is almost no NK1R expression in this region (Nakaya et al., 1994). It has been suggested that this mismatch between substance P and NK1R expression is caused by striatal substance P-positive neurons that project to the substantia nigra (Gerfen, 1991). These projection neurons have a local axon collateral that project onto other striatal neurons expressing NK1R. Other neurotransmitters, such as GABA, are found in these projection neurons and have binding sites in the substantia nigra. This allows neurotransmitters to act in different regions depending on the type of receptor expressed. Alternatively, substance P could be binding to other tachykinin receptors in the substantia nigra, such as NK3 (Dam et al., 1990; Keegan et al., 1992).

Diencephalon

The habenula nucleus has one of the highest expression levels of NK1R in the brain. This structure is thought to be involved in affective processes such as reward and stress, and it has recently been suggested that it is involved in suppressing motor responses to aversive or non-rewarding stimuli (Hikosaka, 2010). Substance P-containing fibres that project to the ventral tegmental area also originate in the habenula nucleus (Emson et al., 1977). This projection is involved in a range of processes, including stress responses and locomotor activity (Elliott et al., 1992; Stinus et al., 1978).

There is an abundance of substance P within the hypothalamus (Iversen et al., 1976) and NK1Rs are also expressed throughout the hypothalamus (Nakaya et al.,

1994). Substance P release within this structure is thought to control hormone release from the pituitary gland (Aronin et al., 1986; Lasaga and Debeljuk, 2011) and is also involved in aggressive behaviour (Haller, 2012).

Mesencephalon and Metencephalon

Substance P and NK1R are expressed in the locus coeruleus (Guyenet and Aghajanian, 1977; Nakaya et al., 1994). NA is synthesised within the locus coeruleus, and NK1R are found on noradrenergic neurons in this structure (Chen et al., 2000; Santarelli et al., 2001). This suggests that substance P and NK1R in this region may play a modulatory role on the major noradrenergic projections to the CNS.

Substance P and NK1Rs are also expressed in the dorsal raphe nucleus. The major serotonergic projections originate in this nucleus, and while NK1R are not located on serotonergic neurons, they can modulate serotonergic activity in the dorsal raphe nucleus due to their presence on glutamatergic neurons (Liu et al., 2002).

1.2.4 The role of substance P and NK1R in clinical disorders

Due to the presence of substance P and NK1Rs in subcortical areas related to emotion, stress and reward, their role in affective disorders and addiction has been widely studied. This section will review substance P and NK1R agonist and antagonist studies in relation to these disorders.

1.2.4.1 Stress and anxiety

The link between the substance P/NK1R system and stress has been extensively studied. For example, using microdialysis, an increase in substance P release has been found in the medial amygdala following immobilisation stress and exposure to an elevated platform (Ebner et al., 2004). Radioimmunoassays have also found an increase in substance P in limbic areas after foot shock (Siegel et al., 1984) and after restraint stress or a saline injection (Rosén et al., 1992). However, a decrease in substance P and NK1R binding has also been found following restraint stress (Takayama et al., 1986). In order to help clarify the role of substance P and NK1R in stress and anxiety responses, the effects of agonists and antagonists have been widely studied. Substance P and NK1R agonists tend to induce an anxiogenic phenotype in a variety of tests, such as the elevated-plus maze (Baretta et al., 2001;

Bassi et al., 2007; Gavioli et al., 1999) and conditioned place avoidance (Elliott, 1988). In contrast, NK1R antagonists induce an anxiolytic phenotype in paradigms including the elevated-plus maze (Santarelli et al., 2001; Teixeira et al., 1996) and stress-induced vocalisations (Kramer et al., 1998; Rupniak et al., 2000).

However, other studies have found an opposite role for substance P and NK1R in stress. For example, substance P injections into the nucleus basalis magnocellularis induced an anxiolytic phenotype in rats tested in the elevated-plus maze (Hasenöhrl et al., 1998b; Nikolaus et al., 1999). Also, ablation of NK1R-expressing neurons in the amygdala of mice increased anxiety behaviour in the elevated-plus maze (Gadd et al., 2003). These discrepancies could be due to the method of agonism or antagonism, the behavioural paradigm used, or the dose. For a full review of the role of substance P and NK1R in stress and anxiety, see Ebner & Singewald (2006).

1.2.4.2 Depression

Stress is a risk factor for depression, and stress responses are often studied in animal models of depression (Nestler and Hyman, 2010). Therefore, the potential efficacy of NK1R antidepressants in depression has also been widely studied. Elevated levels of substance P have been found in the cerebrospinal fluid of depressed patients (Rimón et al., 1984) although this was not replicated in another study (Berrettini et al., 1985). An antidepressant role for NK1R antagonists was first proposed following the finding that stressed-induced vocalisations and vocalisations induced by a substance P agonist are virtually abolished by the antidepressants fluoxetine and imipramine, the anxiolytics diazepam and buspirone as well as by the NK1R antagonists L733 060 and L760 735 (Kramer et al., 1998). Furthermore, the NK1R antagonist MK 869 (Aprepitant) reduced symptoms of depression in patients to the same extent as the anti-depressant paroxetine, but with fewer side-effects (Kramer et al., 1998). This anti-depressant effect of NK1R antagonism was later replicated with another compound, L759 274 (Kramer et al., 2004).

Antidepressants enhance 5-HT, NA and DA transmission. The antidepressant effects of NK1R antagonism were originally thought to be independent from

monoamine systems (Kramer et al., 1998). However, it was later shown that these effects may rely on an interaction between NK1Rs and monoamines (Adell, 2004; Gobbi and Blier, 2005). Treatment with the NK1R antagonist L760 735 increases burst firing of NA neurons in the locus coeruleus of guinea pigs (Maubach et al., 2002). The NK1R antagonists CP 96345 and RP 67580 increase firing of 5-HT neurons in the dorsal raphe nucleus (Gobbi et al., 2007; Haddjeri and Blier, 2001), but this was not seen after NA depletion, highlighting an interaction between NA, 5-HT and NK1R (Gobbi et al., 2007; Haddjeri and Blier, 2008). NK1R antagonists also have an effect on DA: the NK1R antagonist GR205 171 increases the firing rate of DA neurons in the ventrotegmental area that project to the frontal cortex, thus increasing DA. It was suggested that the increase in firing rate was caused by blockade of NK1R located on inhibitory GABAergic neurons (Lejeune et al., 2002).

Despite NK1R antagonists showing promise as a new class of antidepressants, a subsequent phase III clinical trial found no effect of Aprepitant on the symptoms of major depressive disorder (Keller et al., 2006). This failure may be due to species-dependent binding affinities of NK1R antagonists. Although the rat, mouse and human NK1R are 94.5% homologous (Takeda et al., 1991), and the guinea pig NK1 receptor is 96.6% homologous to the human receptor (Gorbulev et al., 1992), minor variations in the receptor sequence can produce large pharmacological differences (Fong et al., 1992). Alternatively, initial trials may have recruited a subgroup of patients that preferentially respond to substance P blockade (Keller et al., 2006). Indeed, depression is a multifaceted disorder and may be underlain by different biological mechanisms.

1.2.4.3 Reward and addiction

As well as being involved in stress and depression, substance P and NK1R have been investigated in relation to reward. This is due to their location in brain areas associated with reward, such as the mesolimbic pathway projecting from the ventral tegmental area to the nucleus accumbens. Many studies have looked at the effect of substance P and NK1R in a conditioned place preference (CPP) paradigm, in which animals learn to associate a chamber with a rewarding drug. Control animals will spend significantly more time in the chamber that was previously associated with reward. Following microinjections of substance P into the nucleus

basalis magnocellularis, the globus pallidus, the amygdala or the caudate-putamen, mice spend more time in the substance P-associated chamber compared to the saline-associated chamber (Hasenöhrl et al., 1998a; Kertes et al., 2010, 2009; Krappmann et al., 1994). In line with this, ablation of NK1R neurons in the amygdala abolishes CPP to morphine (Gadd, 2003), and the NK1R antagonists L733 060 and L703 606 abolish brain self-stimulation of morphine (Robinson et al., 2012). The rewarding effects of substance P may be mediated in part by the endogenous opioid system, because the opioid antagonist naloxone abolishes CPP to substance P (Hasenöhrl et al., 1991). They also may be partially mediated by the DA system, because substance P injections increase DA release within the neostriatum and nucleus accumbens (Boix et al., 1992).

Due to the involvement of substance P in reward, the effects of NK1R antagonists in addiction have also been studied. NK1R antagonism with L703 606 reduces free-choice alcohol consumption (Thorsell et al., 2010) and the NK1R antagonist L822 429 abolishes stress-induced alcohol seeking (Schank et al., 2011). In addition, a clinical trial has shown that the NK1R antagonist LY686 017 reduces alcohol craving in patients (George et al., 2008). Recently, the NK1R antagonist Aprepitant has also been suggested as a potential treatment for opioid addiction (Walsh et al., 2012).

1.2.4.4 Other clinical disorders

One of the most widely studied roles of substance P is in relation to pain. As mentioned in section 1.2.1, substance P is concentrated in the primary afferent nociceptors which terminate in the dorsal horn (Todd, 2002). Intrathecal injections of substance P increases thermal pain sensitivity (Moochhala and Sawynok, 1984; Yasphal et al., 1982), while NK1R antagonists reduce mechanical hypersensitivity induced by nerve ligation in rats (Cumberbatch et al., 1998). Furthermore ablation of NK1R-expressing neurons in the spinal cord results in reduced mechanical pain sensitivity (Mantyh et al., 1997; Nichols et al., 1999), although this effect may not be NK1R-mediated, since ablation of these neurons would also cause other receptor subtypes to be lost. However, NK1R antagonists failed to reduce pain in human patients (Rupniak and Kramer, 1999), perhaps because in animals they are reducing stress, rather than the pain response (Hill,

2000). It is also possible that NK1R antagonists would be effective at treating a different subset of patients that have visceral pain, rather than neuropathic pain, since NK1R antagonists have also been shown to alleviate visceral pain in rats (Gaudreau and Plourde, 2003).

A clinical success for NK1R antagonists has been in the treatment of emesis. NK1R antagonists reduce vomiting in ferrets (Bountra et al., 1993; Gardner et al., 1995), and the NK1R antagonist Aprepitant is now licenced to treat emesis in patients undergoing chemotherapy for cancer (Martin et al., 2003). Aprepitant has also shown promise as an anti-tumor drug for the treatment of cancer (Muñoz and Rosso, 2010). Finally, the NK1R antagonist CJ12 255 can prevent post-operative tissue adhesion in patients (Reed et al., 2008).

Overall, this highlights the role of substance P and NK1R in a variety of central and peripheral mechanisms. In order to further establish their precise role in these disorders, mice lacking functional NK1R have also been widely studied.

1.3 NK1R knockout mice

Genetically modified mice are an invaluable tool in science for understanding the function of individual genes. A knock-out mouse is created by rendering the protein coding for a specific gene non-functional. Comparison of knockout mice to wildtype mice whose genetic background differs only for the gene of interest enables the function of that gene to be further understood. In this section, the methodology behind creating a knockout mouse will be explained, following which the creation and phenotype of the NK1R-/- mouse will be described.

1.3.1 Creation of a single-gene knock-out mouse

The first step in creating a knockout mouse is to insert an engineered deoxyribonucleic acid (DNA) cassette into the gene of interest that prevents the gene from coding for the usual protein (e.g. NK1R). The cassette contains flanking DNA and a selection marker, such as an antibiotic resistance gene. This cassette is then inserted into embryonic stem cells through electroporation. Through naturally occurring homologous recombination, some stem cells will contain the new DNA. Following injection of an antibiotic into the growth medium, the selection marker contained within the cassette means that only the embryonic

stem cells that have incorporated the new DNA survive. These cells are subsequently injected into a blastocyst and implanted into a mother, who gives birth to chimeric mice. These chimeric mice are mated with wildtype mice and, if the new DNA sequence has been taken up by the germ cells of the chimeric mouse, mice that are heterozygous for the gene of interest are born. Heterozygous mice can then be mated to create wildtype, heterozygous and knockout mice.

1.3.2 Issues with knockout mice

1.3.2.1 Breeding methods

In order to minimise animal wastage and cost, homozygous mice are often bred separately, in order to generate litters that contain all wildtype or all knockout mice. However, an issue with this is that *de novo* genetic mutations can occur that are passed down to future generations, thus causing the wildtype and NK1R-/- mouse colonies to drift (Crusio et al., 2009). If colonies are maintained homozygously, these issues can be minimised by occasionally backcrossing the mice onto an identical background strain. Another issue with maintaining homozygous colonies is that early environment can affect mouse phenotype (Crusio, 2004). Indeed, it is possible that wildtype and knockout mice provide different maternal care, which can cause changes in the genotype and phenotype of their offspring (e.g. Meaney, 2001). This can be prevented by breeding mice from heterozygous parents. These issues are further discussed in chapter 5.

1.3.2.2 Background strain

The phenotype of a knockout mouse is not necessarily solely due to loss of the gene of interest. Indeed, different mouse strains vary genetically, and depending on the background strain on which the mutation is created, the phenotype can be very different (Crawley et al., 1997). Differences between mouse strains have been found for a variety of behaviours including locomotor activity, learning and memory, and parental behaviour (for review see: Crawley et al., 1997). An interaction between background strain and NK1R has previously been shown (McCutcheon et al., 2008): NK1R-/- mice bred on a mixed C57BL/6 X 129/Sv background are more sensitive to morphine and show increased neurogenesis in the hippocampus compared to wildtype mice. However, NK1R-/- mice bred on a

pure C57BL/6 background do not show these differences. This highlights the importance of choosing the right strain when creating a knockout, and also suggests that the effects of gene deletion should be studied on more than one background.

1.3.2.3 Flanking genes

During the creation of a knockout mouse, the embryonic cells are often generated from a different background to that in which they are subsequently implanted (for example, 129/Sv blastocysts are often implanted into a C57Bl/6 foster mother). There is an inverse relationship between genetic recombination and distance between the loci of genes. This makes it highly probable that mice containing the mutation from the 129/Sv blastocysts will share the 129/Sv alleles that are close to the gene of interest. Equally, C57BL/6 mice will tend to share C57BL/6 alleles. Therefore, differences between wildtype and knockout mice may be caused by the alleles close to the gene of interest, and not by the gene itself (Gerlai, 1996). One of the ways of overcoming this issue is to 'backcross' the offspring onto a single background strain, although even this will not completely eliminate the problem (Crusio, 2004). For this reason, it is important to compliment knockout mouse studies with other techniques, such as pharmacological antagonism of the gene of interest.

1.3.3 Creation of the NK1R-/- mouse

Initial investigations into the effects of genetic manipulation of NK1R on the nervous system used an NK1R-/- mouse on a C57BL/6 X 129/Sv background. This was achieved by implanting the vector cassette into 129/Sv blastocysts, and subsequently implanting the blastocysts into a C57BL/6 foster mother (De Felipe et al., 1998). The offspring were later crossed onto an MF1 strain, in order to minimise the influence of the 129/Sv component. 129/Sv mice perform poorly in various behavioural tests, for example showing increased anxiety in the elevated-plus maze (EPM) compared to C57BL/6 mice (Hagenbuch et al., 2006; Homanics et al., 1999). Another group created NK1R-/- mice on a pure 129/SvEv background (Santarelli et al., 2001). NK1R-/- mice have also been bred by taking the original 129/Sv X C57BL/6 mice and backcrossing them onto a C57BL/6 strain for 10 generations (McCutcheon et al., 2008). In this thesis, NK1R-/- mice on a 129/Sv X

C57BL/6 background crossed with an MF1 strain were used. A full description of their creation is given in section 2.1.1.

1.3.4 Behavioural studies with NK1R-/- mice

As discussed throughout section 1.2.4, substance P and NK1R have been studied in relation to pain, affective disorders and reward. In this section, the behaviour of NK1R-/- mice in paradigms modelling symptoms of these disorders is described.

1.3.4.1 Affective disorders

Investigations with NK1R-/- mice suggest that they have blunted stress responses: they do not develop stress-induced analgesia and are less aggressive in the resident intruder test (De Felipe et al., 1998). Also, in wildtype pups, the stress of maternal separation causes an increase in vocalisations. This is reduced by anxiolytic and antidepressant drugs, as well as NK1R antagonists, and NK1R-/- mice show a reduction in vocalisations compared to wildtype mice (Rupniak et al., 2000; Santarelli et al., 2001). NK1R-/- mice also show a reduction in risk assessment behaviours in the Light/Dark Exploration Box (Fisher et al., 2007; Herpfer et al., 2005). In the forced swim and tail-suspension tests, wildtype mice treated with the antidepressant fluoxetine show an increase in struggle duration, and this is also seen in NK1R-/- mice (Rupniak et al., 2001). However, there have also been discrepancies in the anxiolytic profile of NK1R-/- mice. Santarelli et al. (2001) found that NK1R-/- mice spent more time in the open arms of the EPM, but this increase was not seen in other studies (De Felipe et al., 1998; Murtra et al., 2000a; Rupniak et al., 2001). As mentioned in section 1.3.3, this difference is likely to be due to the background strain used, since the 129/Sv wildtype mice used by Santarelli et al. (2001) have an anxiogenic profile in the EPM that is not seen in C57BL/6 wildtype mice (Hagenbuch et al., 2006).

1.3.4.2 Pain

The role of NK1R in pain is complex. NK1R-/- mice do not show stress-induced analgesia, but show normal responses to acute pain (De Felipe et al., 1998). However, other studies have shown that NK1R-/- mice do not develop hyperalgesia to capsaicin administered viscerally or into the paw (Laird et al., 2001, 2000). These different findings may relate to a dissociation between pain

and stress responses, and as discussed in section 1.2.4.4, this may be the reason for the failure of NK1R antagonists in the clinic (Hill, 2000).

1.3.4.3 Reward and addiction

The response to morphine is blunted in NK1R-/- mice. They fail to show increased locomotion in response to acute or chronic morphine, they do not self-administer or develop CPP to morphine, and they also have a blunted response to naloxone-induced opiate withdrawal (Murtra et al., 2000a; Ripley et al., 2002). The rewarding effects of alcohol are also absent in NK1R-/- mice: they consume less alcohol than wildtype mice in a free-choice drinking paradigm, and do not develop CPP to alcohol (George et al., 2008). These effects are not due to a global disruption of reward pathways, since the behavioural responses to food and cocaine are normal.

1.3.4.4 Locomotor hyperactivity

NK1R-/- mice show increased locomotor activity in the open field and in the Light/Dark Exploration Box (LDEB) after a vehicle injection (Fisher et al., 2007; Herpfer et al., 2005) and this is blunted by *d*-amphetamine (Yan et al., 2010). As discussed in section 1.3.6.1, this is the initial finding that led to the NK1R-/- mouse being proposed as a model of ADHD. However, initial studies with NK1R-/- mice found no differences in activity in the open field (De Felipe et al., 1998). The hyperactive phenotype of the NK1R-/- mouse, and the discrepancies in activity found between studies are fully discussed in chapter 5.

1.3.5 Neurochemical phenotype of NK1R-/- mice

As discussed in section 1.2.4, the substance P/NK1R system interacts with brain monoamines. In this section, abnormalities in monoamine transmission found in NK1R-/- mice are discussed.

1.3.5.1 Serotonin

Similar to the effects seen after administration of NK1R antagonists in wildtype mice, NK1R-/- mice have an increase in the firing rate of serotonergic neurons in the dorsal raphe nucleus (Santarelli et al., 2001). Surprisingly, the authors found very little overlap between NK1R and serotonergic neurons. However, in the locus coeruleus, NK1R are co-localised with NA neurons (Santarelli et al., 2001), and the

locus coeruleus modulates firing of 5-HT neurons in the dorsal raphe nucleus (Peyron et al., 1996). Radioligand binding of an agonist and antagonist for 5-HT1A autoreceptors in the dorsal raphe nucleus was reduced in NK1R-/- mice. This, combined with a decrease in 5-HT1A autoreceptor mRNA, suggests that 5-HT1A autoreceptors are downregulated in NK1R-/- mice (Froger et al., 2001). This was further supported by the finding that in the frontal cortex, although baseline 5-HT efflux does not differ between genotypes, the selective 5-HT reuptake inhibitor (SSRI) paroxetine induces a 4-fold increase in 5-HT efflux in NK1R-/- mice compared to wildtype mice (Froger et al., 2001). Finally, administration of substance P into the dorsal raphe nucleus decreases 5-HT efflux in the frontal cortex of wildtype mice, but this decrease is not seen in NK1R-/- mice. This indicates that an increase in 5-HT is normally reduced by substance P binding to NK1R (Guiard et al., 2007).

1.3.5.2 Noradrenaline

Anaesthetised NK1R-/- mice have increased basal NA in the prefrontal cortex compared to wildtype mice (Herpfer et al., 2005). This is not due to a difference in NA reuptake, since the NA reuptake inhibitor desiprimine increases NA to the same extent in both genotypes. Furthermore, Western blots revealed no difference in the amount of NA transporter protein (Fisher et al., 2007; Herpfer et al., 2005). This suggests that the increase was due to an increase in NA release. However, whereas the local infusion of an α_2-autoreceptor antagonist into the prefrontal cortex did not reveal any genotype differences in NA efflux, when administered systemically, NA efflux was increased in wildtype but not NK1R-/- mice. There was a reduction in radioligand binding to α_2-autoreceptors in the locus coeruleus of NK1R-/- mice, suggesting that this difference is caused by the desensitisation of α_2-autoreceptors (Fisher et al., 2007). Since NA neurons project from the locus coeruleus to the prefrontal cortex, this desensitisation could also explain the increased basal NA seen in NK1R-/- mice. However, a further study found that a depolarising pulse of potassium infused via the dialysis probe increased NA efflux to the same extent in wildtype and NK1R-/- mice, but a second pulse increased NA efflux only in NK1R-/- mice. This effect was calcium-dependent, indicating that it was caused by exocytosis. This suggests that differences in NA efflux are caused by

both abnormal autoreceptor regulation and increased release of NA in the prefrontal cortex of NK1R-/- mice (Yan et al., 2009).

1.3.5.3 Dopamine

NK1R-/- mice have abnormal DA transmission. In the prefrontal cortex of freely-moving NK1R-/- mice, there is a 50% decrease in DA efflux compared to wildtype mice. While there is no difference in basal DA efflux within the dorsal striatum, *d*-amphetamine increases DA efflux in wildtype mice but does not affect efflux in NK1R-/- mice (Yan et al., 2010). These abnormalities are also seen in wildtype mice treated with the NK1R antagonist RP 67580, suggesting that DA transmission in the prefrontal cortex, as well as the striatal DA response to *d*-amphetamine, is mediated by NK1R (Yan et al., 2010).

The neurochemical and hyperactive phenotype of NK1R-/- mice described in this section has led to the proposal that the NK1R-/- mouse could be a new animal model of ADHD. Below, the face, construct and predictive validity of the NK1R-/- mouse are summarised.

1.3.6 NK1R-/- mice and ADHD

1.3.6.1 Face and predictive validity

NK1R-/- mice and wildtype mice injected with the NK1R antagonist RP 67580 are hyperactive (Herpfer et al., 2005; Yan et al., 2010), and this hyperactivity is reduced by *d*-amphetamine and methylphenidate (Yan et al., 2010). Recently, these mice have also been shown to exhibit impulsivity and inattention in the 5-Choice Serial Reaction-Time Task (5-CSRTT) (Yan et al., 2011). This behavioural test was developed in order to simultaneously test motoric impulsivity and visual attention in rodents (Carli et al., 1983; Humby et al., 2005). These findings are further discussed in chapter 3.

1.3.6.2 Construct validity

NK1R-/- mice have abnormalities in DA, NA and 5-HT transmission, which fits with the theory that abnormal monoamine transmission is central to ADHD. The human equivalent of the NK1R gene is the tachykinin-1 receptor (TACR1). Genetic association studies in patients with ADHD have identified four single nucleotide

polymorphisms in the TACR1 gene that are positively associated with ADHD (Yan et al., 2010). This suggests that the NK1R-/- mouse has good construct validity.

1.4 Aims

In this thesis, the NK1R-/- mouse is further investigated as a model of ADHD by testing the effects of *d*-amphetamine on impulsivity and attention in the 5-CSRTT. The L-type calcium channel blocker nifedipine is also tested in NK1R-/- mice in the 5-CSRTT as an alternative treatment for ADHD. Next, work establishing conditions under which hyperactivity is robust in NK1R-/- mice is presented. Finally, due to potential issues with breeding mice from homozygotes, the behavioural deficits and molecular phenotype of mice bred from heterozygotes are also investigated.

2 Materials and methods

2.1 Animals

All experiments in this thesis were licensed under the *Animals (Scientific Procedures) Act 1986.*

2.1.1 Creation of the NK1R-/- mouse

NK1R-/- mice were created based on the method outlined in section 1.3.1, in De Felipe et al. (1998) and in Figure 2.1. A vector cassette containing an internal ribosome entry site, the coding sequence *lacZ* and a neomycin-resistance gene for positive selection were inserted into the *Stu*I site in exon1 of the NK1R gene. Two copies of the HSV-*tk* gene were also inserted at the 5' end for negative selection. The vector cassette was electroporated into embryonic stem cells from 129/Sv mice and following homologous recombination, the stem cells that did not contain the new DNA (and therefore did not have the neomycin-resistence gene) were killed by injecting the antibiotic geneticin (G418). Gancyclovir (GANC) was injected to remove cells containing the HSV-*tk* gene due to random integration of the vector, rather than at the specific locus. Cells containing the targeted vector were injected into blastocysts from C57BL/6 mice and implanted into a C57BL/6 mother. Chimeric male offspring were mated with C57BL/6 female wildtype mice, and subsequently mice heterozygous for the mutation were mated leading to the birth of wildtype, heterozygous and NK1R-/- mice on a 129/Sv X C57BL/6 background. Later, these mice were crossed once with mice from an MF1 background, in order to dilute the 129/Sv component. All mice used in this thesis are on a 129/Sv X C57BL/6 background crossed with an MF1 strain. It is important to note that the C57BL/6 mice are from Harlan (Bicester, UK), and that these mice lack the gene coding for α-synuclein (Specht and Schoepfer, 2001). This has been linked to a decrease in impulsivity in the 5-CSRTT (Peña-Oliver et al., 2012). However, because the background is identical in wildtype and NK1R-/- mice, this should not affect any genotype differences.

2.1.2 Breeding method

During initial work in this thesis, wildtype and NK1R-/- mice were obtained by homozygous breeding, meaning that NK1R-/- and wildtype mice were bred separately. Due to concerns about genetic drift and early environment, discussed in section 1.3.2 (Crusio, 2004; Crusio et al., 2009), mice were later bred from heterozygous matings, so that each litter could in theory contain wildtype, NK1R-/- and heterozygous mice. Male and female mice were mated between the age of 2 and 8 months, after which they were replaced with new breeding pairs. Offspring were weaned at 3 weeks.

2.1.3 Housing

Mice were housed in a central facility at University College London. Due to the location of the equipment, mice used in the telemetry experiment in chapter 5 were housed in a different central facility at University College London, but the housing conditions were identical in both facilities. Mice were kept in cages containing bedding and environmental enrichment in groups of 2-5. Cages were cleaned twice weekly. Water and food were available *ad libitum* apart from during the 5-CSRTT experiment (2.4.1.2). The rooms were kept at 21±2°C and 45±5% humidity. Lights were on between 08.00 and 20.00, apart from during some of the later telemetry experiments (see chapter 5 for details), when lights were on between 07.00 and 19.00. The light/dark cycle was consistent for all mice from birth and throughout the experiment. Mice that were a minimum of 6 weeks old were used for each experiment. Only male mice were used because in humans ADHD is more prevalent in males than females (Willcutt, 2012). Also, sex differences in 5-CSRTT performance have been found in rats (Bayless et al., 2012) . Future investigations into the NK1R-/- mouse should look at whether any deficits present in males are also present in females.

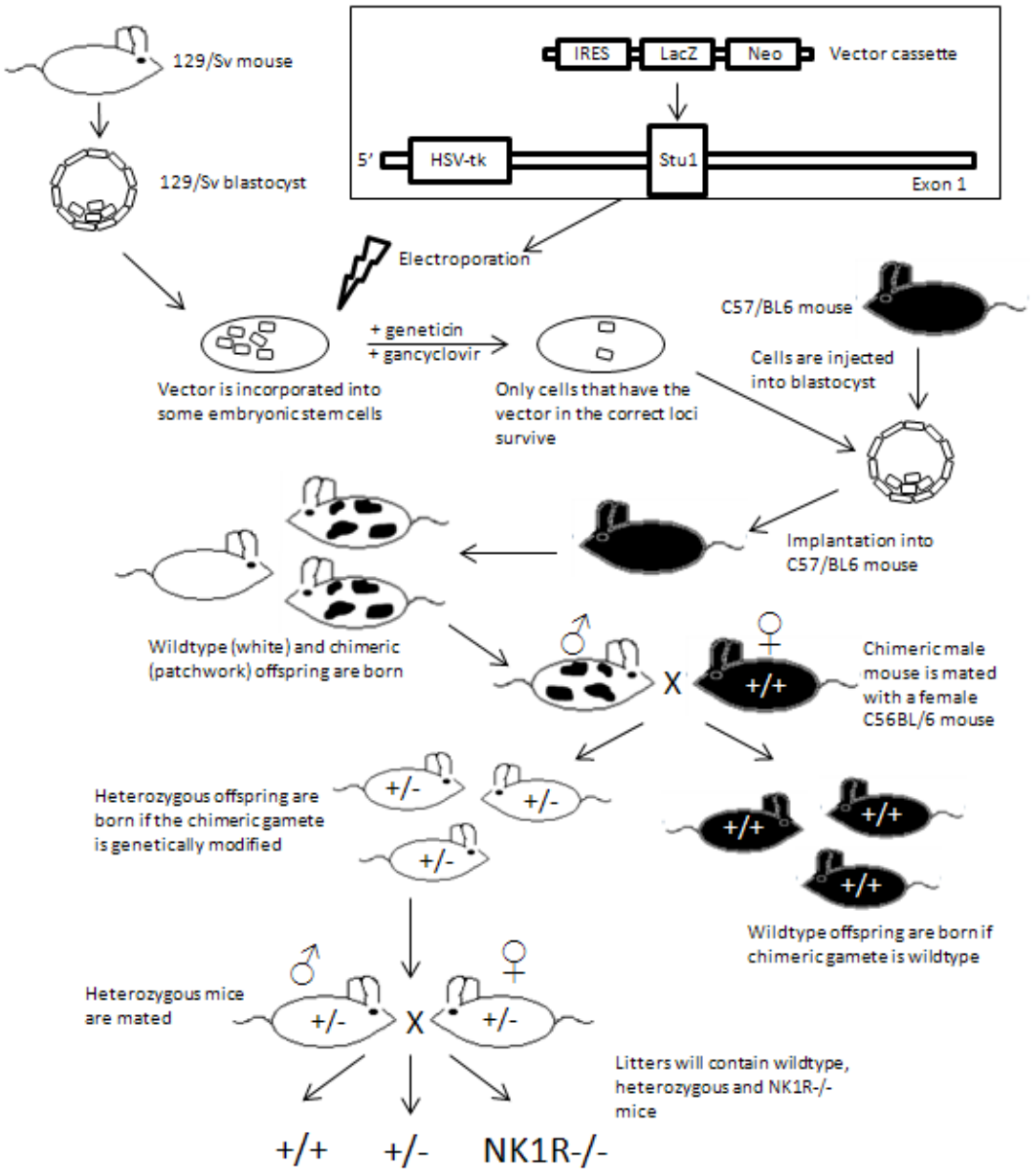

Figure 2.1 Illustration of the method used to create the NK1R-/- mouse.

2.2 Genotyping

2.2.1 Verification with polymerase chain reaction

2.2.1.1 Ear punching

Ear clippings were taken with a 2mm ear punch from mice aged at least 2 weeks, placed into a 0.5mL eppendorf tube, and kept on ice until digestion.

2.2.1.2 Digestion

75μL of alkaline lysis reagent (25mM NaOH, 0.2mM disodium EDTA, dissolved in ultra-pure water) were added to the sample-containing tubes and they were heated at 95°C for 30mins before being cooled at 4°C. 75μL of neutralising reagent (40mM Tris-HCl dissolved in ultra-pure water) was then added to each tube and they were kept at -20°C until amplification.

2.2.1.3 Polymerase chain reaction

While samples were defrosting, the polymerase chain reaction (PCR) master mix was made, containing primers for the NK1R gene (NK1-F and NK1-R), as well as a primer for the neomycin-resistance gene inserted into the vector cassette (Neo-F). 19μL of master mix was added to 0.5mL eppendorfs containing 6μL of each sample. The master mix for each tube contained:

- 43.86μL Thermophilic DNA Polymerase 10X Reaction Buffer, Mg-Free (Promega)
- 9.418μL dNTP mix (Promega)
- 28.322μL 25 mM magnesium chloride
- 23.375μL NeoF: 5'-GCAGCGATCGCCTTCTATC-3'
- 23.375μL NK1-F: 5'-CTGTGGACTCTAATCTCTTCC-3'
- 23.375μL NK1-R: 5'-ACAGCTGTCATGGAGTAGATAC-3'
- 169.388μL nuclease-free water
- 1.887μL *Taq* DNA polymerase (Promega)

The master mix was also added to three control tubes containing a sample from a wildtype mouse, an NK1R-/- mouse, and nuclease-free water. The tubes were then

placed in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) for DNA amplification (Table 2.1).

Table 2.1 DNA amplification steps.

Step	Temperature	Duration	
1	95°C	5mins	
2	60 °C	30s	Cycle
3	72 °C	30s	35 times
4	94 °C	30s	
5	60 °C	30s	
6	72 °C	5min	

2.2.1.4 Electrophoresis

Following DNA amplification and cooling at 4°C, 4μL of loading buffer was added to each tube. Loading buffer contained 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol. 14μL from each tube was loaded into wells on a 2% agarose gel (2g agarose dissolved in 100ml 0.5M Tris-borate-EDTA (TBE) buffer (National Diagnostics, Hull, UK)) with 8μL ethidium bromide. 5μL of ladder was loaded into the first well (Bioline, London, UK) and samples were run in duplicate. The gel was electrophoresed by adding 500mL of TBE buffer and 2μL of ethidium bromide to the tank and running the gel at 115V for approximately 1h with a FEC105 Voltage Power Pack. The gel was visualised and photographed on an ultraviolet transilluminator plate (UVP Ltd, Cambridge, UK). For wildtype mice, a band was seen at 350 bases and for NK1R-/- mice a band was seen at 260 bases. Heterozygous mice had a band at both of these bases (Figure 2.2).

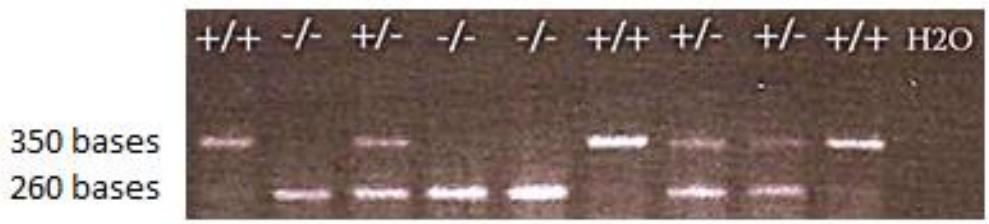

Figure 2.2 Genotyping using Polymerase Chain Reaction. Example of a gel showing bands for wildtype (+/+), NK1R-/- (-/-) and heterozygous (+/-) mice. One well was loaded with H_2O instead of a DNA sample to control for contamination.

2.2.2 Verification with immunohistochemistry

Following PCR, genotypes can be further verified with immunohistochemistry (see section 2.5). This was done in chapter 6 with an antibody against NK1R. Because wildtype and heterozygous mice both show strong staining for NK1R, this method must be used in conjunction with PCR.

2.3 Culling

2.3.1 Transcardial perfusion

Mice were terminally anaesthetised with an i.p. injection of 0.3mL sodium pentobarbital (Euthatal; Merial, Harlow, UK). Once breathing had stopped and reflexes were absent, mice were intracardially injected with 20 mL of heparinised 0.9% saline followed by 20mL of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). The brain was removed and put into 4% PFA overnight and then transferred into 30% sucrose (in 0.1M PB containing 0.02% sodium azide) for at least 24h to cryoprotect the brains.

2.3.2 CO_2 asphyxiation

Mice were placed into a chamber into which carbon dioxide (CO_2) was infused until mice were unconscious. Cervical dislocation was subsequently performed to ensure death.

2.4 Behavioural paradigms

2.4.1 5-CSRTT

2.4.1.1 Apparatus

Four operant chambers (21.6 x 17.8 x 12.7 cm) placed within ventilated sound-attenuating boxes (55.9 x 38.1 x 35.6 cm) were used to train and test the mice (Med Associates, St.Albans, VT, USA). On one wall there were five equally spaced apertures (1cm diameter) containing an infrared laser beam to detect nose pokes and a stimulus light. On the opposite wall there was an aperture for the magazine (2.2cm diameter) containing an infrared laser beam to detect nose pokes, a light and a milk dipper (Figure 2.3). A milk tray placed under the dipper enabled the delivery of 0.01 mL of 30% condensed milk (diluted in H_2O). Delivery was signalled by illumination of the magazine. A house light was mounted above the

magazine and the floor was a metal grid under which a tray of sawdust was placed in order to facilitate cleaning. Stimulus presentation and nose poke recording was recorded by a Smart Control Package 8IN/16OUT with an interface for Windows by MED-PC (Med Associates, St.Albans, VT, USA).

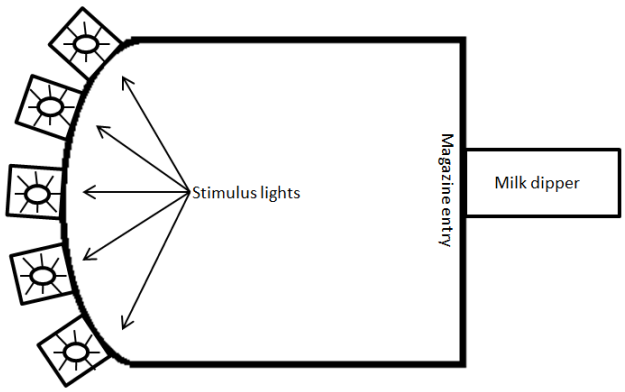

Figure 2.3 Illustration of the 5-CSRTT apparatus.

2.4.1.2 Procedure

Wildtype and NK1R-/- mice were housed in cages of four. Wildtype and NK1R-/- mice from homozygous matings were housed separately, while those from heterozygous matings were housed together. At 6-8 weeks of age they were put on a restricted food diet in order to reduce their weight to 90% of their free-feeding weight. Water was available *ad libitum*. All mice were brought to the lab at 09.00, weighed and habituated to the experimental room for 30mins. 6 wildtype and 6 NK1R-/- mice were tested between 10.00 and 12.00 and 6 wildtype and 6 NK1R-/- mice were tested between 13.00 and 15.00, Monday to Friday.

Habituation

For the first three days, mice were placed in the boxes with all the lights illuminated. A nose poke into the magazine hole led to delivery of the reward for 10s. Condensed milk was placed into the stimulus holes on the first two days to encourage the animals to nose poke.

Following this, the mice had to perform a non-spatial version of the task. When placed into the box, the house light and magazine light were illuminated. A trial was initiated by a nose poke into the magazine hole, following which the magazine light was extinguished and all five stimulus lights were illuminated. A nose poke

into any of the stimulus light apertures led to delivery of the reward. Eating the reward triggered the beginning of the next trial. Mice had to receive a total of 50 reinforcers in 30mins on 2 consecutive days to pass to the first training stage. If this did not happen after 10 sessions, mice passed to the first training stage anyway.

Training stages

All training sessions lasted 30mins or 100 trials (after subtracting premature trials), whichever came first. Mice were trained on six stages of increasing difficulty and were required to meet certain response criteria for 2 consecutive days before passing to the next stage (apart from at stage 6 where the criteria had to be met for 3 consecutive days, and mice were kept on stage 6 for at least 7 days before passing to the testing stages). The parameters and passing criteria of each stage are presented in Table 2.2.

Table 2.2 Stimulus parameters and progression criteria for the 5-CSRTT training stages. SD: Stimulus duration; LH: limited hold; ITI: inter-trial interval. Adapted from Yan et al. (2011).

At the beginning of each session, mice were placed in the box with the house light and magazine light illuminated. A nose poke into the magazine would initiate a trial. One of the five stimulus lights would be illuminated after a fixed amount of time (inter-trial interval, ITI), and would remain illuminated for a certain amount of time (stimulus duration, SD). Finally, mice had a fixed amount of time in which to respond to the stimulus light (limited hold, LH). If they made a correct response, the reward would be delivered. Consumption of the reward initiated the next trial.

If the mice poked into the wrong stimulus hole (incorrect response), failed to respond within the limited hold (omission), or responded before the stimulus appeared (premature response), the house light was extinguished for 5s (time out). Any responses made during the time out reset the 5s. Following the time out, the house light and magazine were re-illuminated and mice had to poke into the magazine hole without receiving a reward in order to start the next trial. If mice made a correct response and continued to poke in the stimulus holes, this was not punished but each poke was recorded as a perseverative response.

Testing

Once the mice passed stage 6, they were challenged once weekly with tests to promote inattention and impulsivity: the long inter-trial interval (LITI) and the variable inter-trial interval (VITI). In the LITI, mice had to wait 7s before stimulus onset. In the VITI, they had to wait 2, 5, 10 or 15s before stimulus onset, delivered on a random schedule. The duration of the session was increased to 45mins in these tests. Details of the testing schedule and drug administration are given in the corresponding chapters.

2.4.1.3 Scoring

The following behaviours were recorded and analysed:

- *Total number of trials*: Total number of trials completed during the testing period, minus any premature responses
- *%Accuracy*: Total number of correct responses / (total number of correct responses + total number of incorrect responses)

- *%Omissions*: Total number of omissions / (total number of correct responses + total number of incorrect responses + total number of omissions)
- *%Premature responses*: Total number of premature responses / (total number of correct responses + total number of incorrect responses + total number of omissions + total number of premature responses)
- *Latency to correct response*: Time to make a correct response (s)
- *Latency to collect the reward*: Time to collect the reward after a correct response (s)
- *Perseverative responses*: Total number of responses into the correct stimulus hole after a correct response

2.4.2 LDEB

2.4.2.1 Apparatus

Two boxes were used for testing. They measured 45 x 20 x 25 cm and were divided into two zones: a light zone with white walls and floor (30 cm long, 20 lux) and a dark zone with black walls and floor (15 cm long, 4 lux). Line markings on the floor divided both zones into 5 x 5 cm squares and in between the two zones there was a guillotine-style door (Figure 2.4).

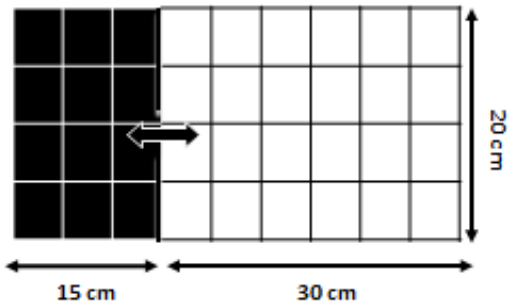

Figure 2.4 Illustration of the LDEB apparatus.

2.4.2.2 Procedure

Animals were brought to the experimental room at 10.00 and left to habituate until 13.00. One wildtype and one NK1R-/- mouse were tested during each session. They were placed individually into the dark zone to habituate for 90mins. When injections were given, they were administered after 60mins before replacing the

mice into the dark zone for a further 30mins. After this, the mice were transferred with minimal handling to the centre of the light zone, the guillotine door was removed and behaviour was recorded on camera for 30mins.

2.4.2.3 Scoring

The following behaviours were scored blind in 5min time bins and analysed:

- *Total number of line crosses*
- *Activity in the light/unit of time*
- *Total time spent in the light zone (s)*
- *Number of transitions to the light zone*

2.4.3 Activity chamber

2.4.3.1 Apparatus

A transparent Perspex box (30 x 19 x 18 cm) was covered in white paper and placed in an activity meter (41 x 41 cm, Columbia Instruments, USA). The activity meter emitted parallel infrared laser beams spaced 27mm apart and 28mm from the floor. Light in the chamber measured 100 lux.

2.4.3.2 Procedure

Mice were habituated to the experimental room for 3 days prior to testing, and for 30mins on the testing day. They were then placed in the centre of the activity chamber. Their behaviour was recorded for 10mins.

2.4.3.3 Scoring

An ambulatory activity count was recorded by the activity meter every time the mice made two consecutive beam breaks. The total ambulatory activity was recorded at the end of each 5min time bin.

2.4.4 Telemetry

2.4.4.1 Apparatus

A radiotelemetry probe weighing 1.6g and measuring 1.1cm^3 (TAF-10, Data Sciences International, USA) was implanted into a mouse and output an activity parameter. This is calculated based on the strength of the probe signal relative to an antenna implanted in the centre of the telemetry mat. The activity count

therefore depends on the distance moved, the orientation of the animal, and how much it moves during the testing period. Each mouse cage is placed on a telemetry mat and as the mouse moves about the cage, the signal received by the antenna varies in strength based on the distance and orientation of the mouse. A Data Exchange Matrix (Data Sciences International, USA) monitors the changes in signal (16 samples/s) and transfers these data as digital pulses to the computer. This is translated into an activity count by the Dataquest A.R.T.™ software (Data Sciences International, USA).

2.4.4.2 Surgery

Mice were placed in a chamber and anaesthesia was induced with 5% isoflurane. Once the animals were unconscious they were placed on a heated pad and maintained with 2% isoflurane and oxygen at 0.5L/minute administered through a nose cone. The mice were placed on their back and the limbs were loosely taped down. Hair was shaved from the ventral area and the incision site was scrubbed with disinfectant and alcohol. A 2cm incision was made through the skin on the ventral abdomen. A subcutaneous pocket was made along the lateral flank with blunt dissection and the transmitter probe was inserted. The skin was closed with 6-0 non-absorbable sutures and an analgesic was administered. Following removal of the anaesthetic, mice were kept on heated pads until they were moving normally. They were left to recover for 7 days before measuring their activity.

2.4.4.3 Procedure

Mice were housed individually post-surgery. After recovery, each cage was placed on a telemetry mat and the probes were turned on with a magnet. During the light phase the cages were lit at 100 lux. Activity was recorded by the computer every 15mins for 2mins throughout the experiment. In all experiments, baseline activity was monitored for 48h. In experiments where mice were injected, they subsequently received an injection 30mins before the dark phase every other day. On completion of the experiment, mice were culled by CO_2 asphyxiation (see section 2.3.2), the probe was cut out of the pocket and the probe was turned off with a magnet. Activity counts for each 2min time bin were transferred into Microsoft Excel for analysis.

2.5 Immunohistochemistry

2.5.1 Sectioning

Following transcardial perfusion and brain removal (see section 2.3.1), brains were placed on a frozen platform and cut coronally at 40μm and placed in 5% sucrose with 0.02% sodium azide in 0.1M PB in sets of six (every sixth section was placed in the same well). All staining was performed on free-floating sections. 0.1M PB contained 190 mM monosodium phosphate and 810 mM Disodium hydrogen phosphate (BDH Laboratory Supplies, Poole, UK).

2.5.2 Blocking

In order to reduce non-specific binding, sections are blocked for 1h in 0.03% Triton X-100 (BDH Laboratory Supplies, Poole, UK) and 3% normal serum in 0.1M PB. Where possible, the host serum was from the same species as the secondary antibody. For 3,3'-Diaminobenzidine (DAB) immunohistochemistry, hydrogen peroxide was added to the blocking solution to eliminate any endogenous peroxidase activity.

2.5.3 Antibodies

After being blocked, sections are placed in the primary antibody prepared in Tris-Triton Buffered Saline (TTBS, containing 0.05 M Tris base, 0.3% Triton X-100 and 0.9% sodium chloride) and left on a rocking platform at room temperature overnight, or at 4ºC for three nights. They were then washed three times in 0.1M PB for 10mins and incubated in the secondary antibody for either 1h30 (fluorescent staining) or 2h (DAB staining). Full details of the antibodies and concentrations used are given in chapter 6.

2.5.4 Amplification

2.5.4.1 Avidin-Biotin

Avidin is a glucoprotein that has high affinity for the vitamin biotin, which is present in all living cells. When biotin is conjugated to the secondary antibody, the avidin complexes will bind to it. For fluorescent staining, a marker such as fluorescein isothiocyanate (FITC) is conjugated to avidin. For DAB staining, horse radish peroxidase (HRP) is conjugated to avidin (ABC complex, Vectastain kit,

Vector Labs). As explained in section 2.5.5, this allows the antigen of interest to be visualised.

2.5.4.2 Tyramide signal amplification

Tyramide is activated by HRP, and binds to electron-rich regions adjacent to the HRP-binding sites. Used in conjunction with the ABC complex, this amplifies the signal by increasing the number of binding sites that can be visualised.

Details of the amplification methods used in each immunohistochemistry experiment are given in chapter 6.

2.5.5 Visualisation

2.5.5.1 DAB

DAB staining was visualised with the DAB substrate kit (Vector Labs). The substrate solution contains peroxide. DAB reacts with HRP in the presence of peroxide to form an insoluble brown polymer. During the amplification step, HRP is conjugated to avidin (ABC complex, Vectastain kit, Vector Labs), which binds to the biotinylated secondary antibody. This allows the antigen of interest to be visualised.

2.5.5.2 Fluorescence

Fluorescent dyes emit light at a certain wavelength. These can bind directly to the secondary antibody, or can bind to avidin, which will then bind to the biotinylated secondary antibody. In this thesis, two different dyes were used: Alexa Fluor 594, which fluoresces red (617 nm) when excited at 590 nm and FITC, which fluoresces green (515 nm) when excited at 495 nm. Because different dyes emit light at different wavelengths, this method is commonly used to simultaneously detect more than one antigen.

2.5.6 Mounting

Sections were placed in 0.01M PB and mounted onto gelatinised slides. For DAB staining, the slides were dehydrated by placing them in gradually increasing concentrations of alcohol. DPX mounting medium (BDH Laboratory Supplies, Poole, UK) was then used for cover-slipping. For fluorescent staining, the slides are

cover-slipped with Fluoromount aqueous mounting medium (Sigma-Aldrich, Poole, UK).

2.5.7 Imaging

DAB sections were visualised and images were captured with a Nikon E4500 camera attached to a Nikon Eclipse E800 microscope. Fluorescent sections were visualised and images were captured with a CCD camera (C-4742-95; Hamamatsu Photonics, Hamamatsu, Japan) attached to a Leica DMR microscope (Leica Imaging Systems Ltd., Cambridge, UK). Details of cell quantification are given in chapter 6.

2.6 Western blots

The following solutions were used for western blotting:

Radioimmunoprecipitation assay (RIPA) buffer

100 mM sodium chloride

100 mM sodium fluoride

20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

5 mM ethylenediaminetetraacetic acid (EDTA)

1 mM sodium orthovanadate

1% NP-40

Loading buffer

50% glycerol

10% sodium dodecyl sulphate (SDS, Sigma-Aldrich)

0.5% bromophenol blue (Sigma-Aldrich)

MOPS running buffer

0.05 M 3-(N-morpholino) propane sulphonic acid (MOPS) (Invitrogen, Oregon)

0.05 M Tris base

3.5 mM SDS

1 mM EDTA

Transfer buffer

48 mM Tris base

39 mM glycine

0.037% SDS

10% methanol

2.6.1 Tissue preparation

2.6.1.1 Dissection

Mice were killed by CO_2 asphyxiation (see section 2.3.2) and their brains were removed and frozen on dry ice. Brains were stored at -80°C until dissection. Frozen brains were cut into 1mm coronal sections with an acrylic mouse brain matrix (Zivic Instruments, PA, USA). Using a 1.5mm diameter tissue punch, sections were taken from the dorsal striatum and frozen at -80°C.

2.6.1.2 Homogenisation

Tissue was kept on ice throughout the procedure to avoid protein denaturation. Protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktails 1 & 2 (Sigma-Aldrich) were added to RIPA buffer and this was added to each tissue sample. Samples were homogenised in a FastPrep Biopulveriser (MP Biomedicals Europe, France) for 10s, and then kept on ice for 1-2h. Samples were then centrifuged at 12 000 RPM for 15mins and the supernatant was transferred into a new Eppendorf tube.

2.6.1.3 Protein determination

Protein was measured with a biocinchoninic (BCA) kit. Protein standards of known concentration were loaded in duplicate to the first row of a 96 well plate. Tissue samples were added in duplicate to subsequent rows. Bicinchoninic acid and 4% copper sulphate was added to all wells. The plate was incubated at 37ºC for 30mins and protein concentration was determined with a colorimeter.

2.6.2 Protein transfer

Loading buffer was added to samples containing 15µg of protein to make a volume of 19µL. They were boiled for 5mins to denature the proteins then cooled on ice. A 10% gel was covered with running buffer (see section 2.6) and 15 µL of each sample was loaded to the wells. Proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 50mins, 180V). Meanwhile, a polyvinylidine difluoride membrane (Bio-Rad, UK) was prepared by briefly soaking it in 100% methanol and then in transfer buffer. The gel and membrane were then placed in the transfer tank between filter paper and sponges. An ice block was added to the tank to prevent the gel from sticking to the membrane and the gel was electroblotted onto the membrane (1h, 100V).

2.6.3 Immunoblotting

Details of antibodies and concentrations are given in chapter 6. The membrane was blocked for 1h in 4% milk diluted in 0.1 M phosphate-buffered saline and 0.1% Tween 20 (PBS-Tween). It was then incubated overnight at 4ºC on a roller in the antibody diluted in the same milk solution. The next day, the membrane was washed 3 times for 10mins in PBS-Tween and then placed in the secondary antibody on a roller for 45mins. Following 3 more washes, it was placed in 0.1 M PBS before being developed with enhanced chemiluminescence western blotting detection reagents (GE Healthcare) and visualised with a ChemiDoc-XRS molecular imaging system (Bio-Rad, UK).

2.6.4 Normalising

In order to normalise each sample, the membrane was incubated for 90mins in glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is an enzyme involved in glycosis and is found in high levels in almost all tissues and cells. It is therefore useful for protein normalisation. The membrane was washed again and placed in the secondary antibody for 45mins, washed and rinsed in PBS without Tween before being developed, as above.

2.6.5 Analysis

Photographed membranes were analysed using Image J software (NIH, USA). The ratios for the band densities of the antibody of interest/GAPDH were calculated.

The mean ratio for the wildtype control samples was calculated and all samples were expressed as a percentage of this mean ratio. Further details are given in chapter 6.

2.7 Statistical analysis

All statistics were performed in IBM SPSS PC+ (SPSS Inc, Chicago, Illinois, USA). When experiments had only two experimental groups, they were analysed with independent or paired-samples t-tests. When experiments had three or more experimental groups, they were analysed with Analysis of Variance (ANOVA). Details of independent and dependent variables are provided in the relevant chapters. All data were checked for homogeneity of variance with Levene's test and if this was violated they were square-root, $\log_{10}$ or arc-sine transformed. If data continued to violate Levene's test, they were analysed with non-parametric tests (Kruskal-Wallis or Friedman tests followed by Mann-Whitney U or Wilcoxon tests). Data were also checked for sphericity with Mauchley's test of sphericity. If this was violated, the Greenhouse-Geisser correction was applied. In all experiments, statistical significance was set at $P<0.05$.

3 Effects of *d*-amphetamine on the performance of NK1R-/- mice in the 5-Choice Serial Reaction-Time Task

3.1 Introduction

In order to validate the NK1R-/- mouse as a model of ADHD, it was tested in the 5-CSRTT. This test measures impulsivity and attention (two diagnostic features of ADHD). NK1R-/- mice were shown to be more *inattentive* and *impulsive*, and to make more *perseverative responses* than wildtype mice (Yan et al., 2011). In this chapter, the same mice were tested in the 5-CSRTT in order to evaluate the effects of *d*-amphetamine, one of the current treatments for ADHD, on performance in this task.

3.1.1 5-Choice Serial Reaction-Time Task

3.1.1.1 Development

In humans, sustained attention and impulsivity can be assessed with the Continuous Performance Task (CPT; Beck et al., 1956). In this test, participants are asked to respond to certain stimuli and inhibit responses to others. A version of the CPT with an added spatial component was also created, in which patients have to continuously monitor the location of a light stimulus in one of five apertures (see: Wilkinson, 1963). Based on these tasks, the rodent 5-CSRTT was developed (Carli et al., 1983) in order to test attention and impulsivity in rats, and was later adapted for mice (Humby et al., 2005). By creating a rodent 5-CSRTT analogous to the tasks used to test humans, the neurochemical basis of sustained attention and impulsivity could be further understood. Attention is assessed by the animals' *accuracy* (percentage of correct responses) and also the percentage of *omissions* (no response during a trial). Impulsivity is measured by the number of *premature responses* (responses made before stimulus onset). This test also measures *perseveration*, or 'compulsivity', when more than one response into a stimulus hole is made within the same trial. Although not a diagnostic feature, perseverative responding is commonly seen in patients with ADHD (Lawrence et al., 2004).

3.1.1.2 Neurobiology

Brain structures

Despite the complex nature of the 5-CSRTT, lesions to specific brain areas do not prevent task acquisition, and in this way the neurobiology of 5-CSRTT performance can be studied. Various brain structures in the cortico-striatal network are involved in optimal performance, and this network is often implicated in the pathophysiology of ADHD (Dalley et al., 2008). Attention, impulsivity and compulsivity are thought to be underlain by different cortico-striato loops (Figure 3.1). Specifically, the dorsal part of the frontal cortex is involved in attention, with lesions to the anterior cingulate cortex reducing *accuracy* (Chudasama et al., 2003). On the other hand, the ventral part of the frontal cortex is involved in response inhibition, with lesions to the orbitofrontal cortex increasing *perseverative responses*, and lesions to the infralimbic cortex increasing *impulsivity* (Chudasama et al., 2003). Within the striatum there is also a dissociation between ventral and dorsal areas. Lesions to the core of the nucleus accumbens, which is part of the ventral striatum, decrease response inhibition (Christakou et al., 2004) as evidenced by an increase in *impulsivity* and *perseverative responses*. This mimics the effects of lesions to the ventral part of the frontal cortex. In contrast, lesions to the medial dorsal striatum cause deficits in *accuracy*, *impulsivity* and *perseveration*, while lesions to the lateral dorsal striatum prevent task performance (Rogers et al., 2001). Interestingly, despite the spatial aspect of the task, hippocampal lesions have little effect on task performance (Kirkby and Higgins, 1998).

Neurotransmitters

Dopamine

DA has long been linked to the pathophysiology of ADHD due to the fact that a deficit in DA within fronto-striatal circuits is thought to underlie the symptoms of ADHD (Levy, 1991), and also due to the ability of psychostimulants to alleviate the symptoms. For this reason, the effects of DA depletion within fronto-striatal circuits have been studied. In the 5-CSRTT, striatal DA depletion induces *omissions* and slows *response latencies* (Baunez and Robbins, 1999; Cole and Robbins, 1989). DA depletion within the dorsal striatum also induces *perseverative responding*

(Baunez and Robbins, 1999). On the other hand, DA depletion in the prefrontal cortex selectively impairs *accuracy* (Granon et al. 2000). *Premature responses* in the 5-CSRTT have also been linked to DA. Dopaminergic lesions to the nucleus accumbens reduce *premature responses* (Cole and Robbins, 1989), although more recent studies with DA agonists and antagonists suggest opposing effects whether DA is depleted in the core or the shell (Besson et al., 2010).

Figure 3.1. Fronto-striatal loops involved in optimal 5-CSRTT performance. ACC: anterior cingulate cortex; dStr: dorsal striatum; IL: infralimbic cortex; Nacc: nucleus accumbens; OFC: orbitofrontal cortex; dmStr: dorsal medial striatum. Adapted from Bari and Robbins (2011).

Noradrenaline

NA is also implicated in ADHD, and drugs that act predominantly on the noradrenergic system have shown good efficacy in the treatment of this disorder. These include guanfacine (Sallee et al., 2009), a selective alpha2-adrenoceptor agonist and atomoxetine (Kratochvil et al., 2003), a noradrenaline reuptake inhibitor. In the 5-CSRTT, NA has been linked to response *accuracy*. Cortical NA depletion decreases *accuracy* (Carli et al., 1983; Milstein et al., 2007) and NA re-uptake inhibition improves this measure (Robinson, 2012). An increase in NA within cortical areas (Navarra et al., 2008; Robinson, 2012) and the shell of the nucleus accumbens (Economidou et al., 2012) also reduce *premature responses* in the 5-CSRTT.

Serotonin

The role of 5-HT in the 5-CSRTT is unclear. Some studies report increased *premature responses* following central 5-HT depletion (Harrison et al., 1999, 1997), whilst other studies report a positive correlation between increased 5-HT and *premature responses* (Koskinen et al., 2000a, 2000b; Puumala and Sirviö, 1998). Also, the 5-HT(2C) receptor knockout mouse shows no differences in premature responses compared to wildtype mice (Fletcher et al., 2012). These discrepencies are thought to be due to the large number of 5-HT receptor subtypes: currently at least 15 have been identified. Apart from 5-HT_3 receptors, which are ligand-gated

ion channels, all the receptor subtypes are G protein-coupled receptors. Depending on the receptor subtype, they can be excitatory or inhibitory, and they perform different functions with the peripheral and central nervous system (Barnes and Sharp, 1999; Hoyer et al., 2002). Another reason for these discrepancies is the different levels of receptor subtype activation in distinct brain regions (Robinson et al., 2008). Despite these contradictory findings, studies agree that increased 5-HT release impairs response *accuracy* (see Robbins, 2002).

Acetylcholine

In line with the current treatments for ADHD, research into the neuropharmacology of the 5-CSRTT has largely focussed on monoamine systems. However, studies have also suggested a role for the cholinergic system in attentional processes. Specifically, cholinergic lesions to the nucleus basalis of Meynert, which projects to the frontal cortex, impair *accuracy* in rats in the 5-CSRTT (McGaughy et al., 2002; Muir et al., 1994). A reduction in *accuracy* was also found in mice injected systemically with scopolamine, a muscarinic receptor antagonist (Humby et al., 1999; Jones and Higgins, 1995). As mentioned above, the striatum plays an important role in 5-CSRTT performance, and cholinergic interneurons in this region exert strong control over DA (Pisani et al., 2001; Wang et al., 2006). As yet though, the effects of striatal cholinergic lesions on 5-CSRTT performance have not been studied.

3.1.2 Amphetamine

3.1.2.1 Mechanism

The current most prescribed treatments for ADHD are psychostimulants such as *d*-amphetamine (Adderall) and methylphenidate (Ritalin). Amphetamine acts primarily by increasing DA levels. This is achieved by binding to the DAT and inhibiting DA reuptake. The DAT/ amphetamine complex can also enter the cell, where amphetamine 'exchanges' with intracellular DA, causing DA to exit the cell through the sodium-mediated mechanism that clears DAT. This causes an increase in extracellular DA. At high doses, amphetamine also inhibits monoamine oxidase, an enzyme that breaks down DA (Seiden et al., 1993). Although the effects of

amphetamine are predominantly dopaminergic, it also increases extracellular 5-HT (Kuczenski and Segal, 1989) and NA (Kuczenski and Segal, 1992).

In this chapter, the dextrorotatory stereoisomer of amphetamine, dextroamphetamine (*d*-amphetamine), was used. This drug is prescribed to patients with ADHD under the name Adderall®.

3.1.2.2 Effects in the 5-CSRTT

One of the aims of the rodent version of the 5-CSRTT was to identify drugs in rodents that would have good predictive validity for treating attentional disorders. However, initial studies with amphetamine failed to show a therapeutic effect, instead showing an increase in *premature responses* following amphetamine administration in rats (Cole and Robbins, 1989, 1987; Harrison et al., 1997) and mice (Loos et al., 2010). These studies used a fixed inter-trial interval (ITI), meaning that the time between trial initiation and stimulus presentation was the same on every trial. An alternative version of the 5-CSRTT using a variable ITI has found an improvement in *premature responses* after amphetamine, although this was accompanied by an increase in *omissions* (Bizarro et al., 2004).

The effects of amphetamine on 5-CSRTT performance in other animal models of ADHD have not been tested. However, the most validated animal model of ADHD, the Spontaneously Hypertensive Rat (SHR), shows a reduction in inattention, impulsivity and activity following *d*-amphetamine in another task of visual attention (Sagvolden and Xu, 2008).

3.1.3 Aims

In order to further validate the NK1R-/- mouse as a model of ADHD, the effects of *d*-amphetamine in the 5-CSRTT were evaluated. The mice used in this experiment had previously been trained in the 5-CSRTT, as outlined in section 2.4.1.2. During training, NK1R-/- mice made significantly more *omissions* during stages 2-4 and significantly more *perseverative responses* during stages 1-5 than wildtype mice. However, there were no differences by the end of training (stage 6), apart from in the latency to collect the reward, which was longer in NK1R-/- mice (Yan et al., 2011). These mice had also been tested with a long and a variable inter-trial interval (LITI and VITI, respectively). The inter-trial interval (ITI) is the time

between trial initiation and stimulus onset. In the LITI, the ITI was increased to 7s, and in the VITI, the ITI was 2, 5, 10 or 15s. Therefore, these tests promote inattention and impulsivity. These tests revealed that NK1R-/- were more *inattentive*, *impulsive* and *compulsive* than wildtype mice (Yan et al., 2011, Figure 3.2). In the present chapter, these mice were again tested with a LITI and a VITI, but this time following administration of *d*-amphetamine. Although the hyperactivity of NK1R-/- mice was reduced with 2.5mg/kg *d*-amphetamine (Yan et al., 2010), two lower doses were used here, in line with the doses prescribed to patients with ADHD. These doses are within the range used in other studies looking at the effects of amphetamine in the 5-CSRTT (e.g. Loos et al., 2010).

Figure 3.2 Behaviour of wildtype (WT) and NK1R-/- mice when they were first tested in the 5-CSRTT with a long (LITI) and a variable (VITI) inter-trial interval (n=12). NK1R-/- mice were more inattentive (A, C, E), impulsive (F) and made more perseverative responses (B, D) than wildtype mice. *P<0.05; **P<0.01; ***P<0.001. Adapted from (Yan et al., 2011).

3.2 Methods

3.2.1 Animals

12 wildtype and 12 NK1R-/- mice (129/Sv x C57BL/6 crossed with an MF1 strain) from homozygous matings were used in this experiment. The mice were 6-8 weeks old at the start of the experiment, and weighed 25-40g (see section 2.1 for full details of breeding and housing). Throughout the experiment, food was restricted to maintain each mouse at 90% of its free-feeding weight. Water was available ad libitum and all animals were weighed daily.

The two genotypes were housed separately in cages of four, so that each cage contained four wildtype or four NK1R-/- mice. One mouse from each genotype died before the end of the experiment.

3.2.2 5-Choice Serial Reaction-Time Task

The apparatus and protocol are discussed in detail in section 2.4.1. Briefly, mice were habituated to the boxes then trained over six stages of increasing difficulty (see table Table 2.2). Once their performance at stage six was stable (total trials minus premature responses = 100; %accuracy > 75%; %omissions < 25%), treatment-naive mice were challenged with a Long Inter-Trial Interval (LITI), which increased the time between trial initiation and stimulus appearance to 7s (no injection 1, NI-1). They were then tested in the LITI 30 minutes after an intraperitoneal (i.p.) injection of *d*-amphetamine (0.3 mg/kg, A(0.3) and 1 mg/kg, A(1)), vehicle (0.9% saline) or they experienced a second un-injected test (NI-2). All mice underwent each test condition once only at weekly intervals, on a pseudo-latin square randomisation schedule. Between each weekly test, mice were trained once-daily on stage six, and were only tested if their performance was stable for 3 consecutive days prior to testing.

To further highlight deficits in attention and impulsivity, mice were then tested with a Variable Inter-Trial Interval (VITI), which set the time between trial initiation and stimulus appearance to 2, 5, 10 or 15s, on a random schedule (NI-1). Following this they received the same series of treatments, substituting the LITI for the VITI (see Figure 3.3). All tests lasted until the mice had completed 100 trials (minus premature responses), or after 45 minutes.

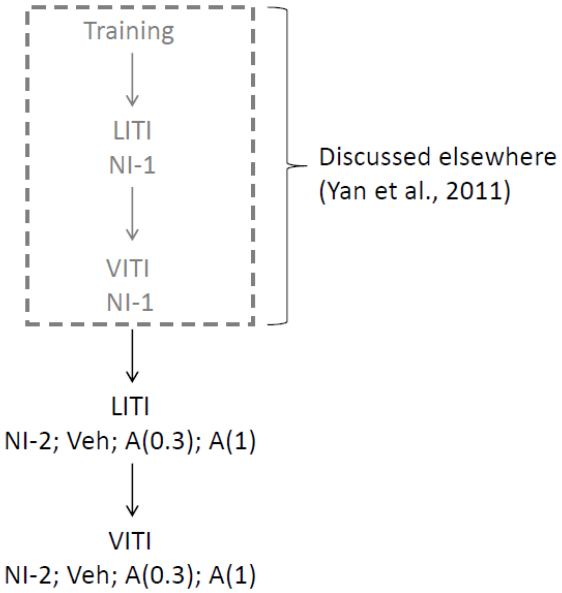

Figure 3.3 Testing sequence for wildtype and NK1R-/- in the 5-CSRTT. NI-1: no injection 1; NI-2: no injection 2; Veh: vehicle; A(0.3): *d*-amphetamine 0.3mg/kg; A(1): *d*-amphetamine 1mg/kg. LITI: long inter-trial interval. VITI: variable inter-trial interval.

3.2.3 Statisical analysis

The behaviours scored in the 5-CSRTT are described in section 2.4.1.3. They are summarised below:

- *Total number of trials*
- *%Accuracy*
- *%Omissions*
- *%Premature responses*
- *Latency to correct response*
- *Latency to collect the reward*
- *Perseverative reponses*

In addition, the total number of trials to reach baseline was calculated. Full details of the statistical analysis are given in section 2.7. The training data and first LITI and VITI tests (NI-1) have been analysed and discussed elsewhere (Yan et al., 2011, Figure 3.2). The data for the treatments and NI-2 were analysed with a 2-way repeated measures ANOVA, with '*genotype*' as the between-subjects factor and '*treatment*' as the within-subjects factor. In order to look at the effects of vehicle injection, NI-2 was first compared to vehicle, and then vehicle was compared to the treatment groups. A significant main effect of either independent variable or a

significant interaction between them led to post-hoc 1-way ANOVAs or paired sample t-tests. Data for the LITI and VITI were analysed separately. In the VITI, as well as analysing the overall data, data for each ITI (2s, 5s, 10s and 15s) was analysed separately.

3.3 Results

3.3.1 Number of trials to reach baseline

There was no genotype difference in the number of trials (t(22) = 0.279, P=0.783). Wildtype mice took an average of 32.5 trials and NK1R-/- mice took an average of 33.9 trials to complete training (Figure 3.4). As training was carried out 5 days a week, the mice were between 3-4 months when they were first tested in the 5-CSRTT.

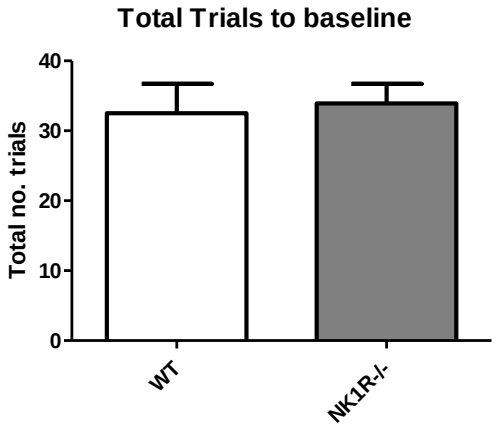

Figure 3.4 Total number of trials to reach baseline in the 5-CSRTT in wildtype (WT) and NK1R-/- mice.

3.3.2 LITI

3.3.2.1 Effects of vehicle injection

%Omissions

There was a main effect of genotype ($F(1,22) = 5.09$, $P<0.05$). At NI-2, NK1R-/- mice had significantly higher %omissions than wildtype mice ($F(1,22) = 7.61$, $P<0.05$). There was also a significant interaction ($F(1,22) = 4.83$, $P<0.05$). Whereas vehicle injection increased %omissions in wildtype mice ($t(11) = 2.84$, $P<0.05$), they remained unchanged in NK1R-/- mice (Figure 3.5A).

Perseverative responses

There was a significant interaction ($F(1,22) = 9.73$, $P<0.01$). Whereas the vehicle injection significantly *increased* perseverative responding in NK1R-/- mice ($t(11) = 2.36$, $P<0.05$), it significantly *decreased* this measure in wildtype mice ($t(11) = 2.62$,

P<0.05). This caused a genotype difference in the vehicle-injected group (F(1,22) = 6.58, *P*<0.05) (Figure 3.5C).

Latency to collect the reward

There was a significant interaction (F(1,22) = 7.15, P<0.05). The vehicle injection increased the latency to collect the reward in NK1R-/- mice only (t(11) = 3.48, P<0.01). This increase led to a genotype difference in vehicle-injected mice, with NK1R-/- mice being significantly slower to collect the reward (F(1,22) = 9.51, P<0.01) (Figure 3.5G).

%Premature responses, %accuracy, total number of trials and latency to correct response

These measures did not differ in un-injected and vehicle treated mice (Figure 3.5 and Table 3.1).

Table 3.1 Statistical values for the behaviours that did not differ in the un-injected or vehicle-injected mice tested in the 5-CSRTT with an LITI.

	Main effect of genotype	Main effect of treatment	Main interaction
%Premature responses	F(1,22) = 0.72, P=0.78	F(1,22) = 2.16, P=0.16	F(1,22) = 0.35, P=0.56
%Accuracy	F(1,22) = 2.29, P=0.14	F(1,22) = 0.82, P=0.38	F(1,22) = 0.01, P=0.94
Total number of trials	F(1,22) = 0.02, P=0.89	F(1,22) = 0.31, P=0.58	F(1,22) = 0.22, P=0.65
Latency to correct response	F(1,22) = 3.59, P=0.71	F(1,22) = 1.93, P=0.18	F(1.22) = 0.22, P=0.64

3.3.2.2 Effects of *d*-amphetamine

Perseverative responses

NK1R-/- mice perseverated significantly more in all treatment groups ($F(1,22) = 12.97$, $P<0.01$). There was also a main effect of treatment ($F(1.56, 34.13) = 5.45$, $P<0.05$). The higher dose of *d*-amphetamine reduced perseveration in both genotypes (wildtype c.f. A(0.3): $t(11) = 2.4$, $P<0.05$; NK1R-/- c.f. vehicle: $t(11) = 2.4$, $P<0.05$; NK1R-/- c.f. A(0.3): $t(11) = 2.57$, $P<0.05$) (Figure 3.5C).

Latency to correct response

There was a main effect of treatment ($F(1.35, 28.36) = 9.59$, $P<0.01$). Overall, the higher dose of *d*-amphetamine increased the latencies of both genotypes (c.f. vehicle: $t(22) = 2.67$, $P<0.05$; c.f. A(0.3): $t(22) = 3.99$, $P=0.001$) (Figure 3.5F).

Latency to collect the reward

NK1R-/- mice were consistently slower to collect the reward (main effect of genotype: $F(1,21) = 16.03$, $P=0.001$). The higher dose of *d*-amphetamine increased the latencies of both genotypes (c.f. vehicle: $t(22) = 2.80$, $P=0.01$; c.f. A(0.3): $t(22) = 2.41$, $P<0.05$) (Figure 3.5G).

%Premature responses, %accuracy, %omissions or total number of trials

There was no effect of vehicle or either dose of *d*-amphetamine on any of these measures (Table 3.2 and Figure 3.5).

Table 3.2 Statistical values for the behaviours that did not differ in the amphetamine-injected mice tested in the 5-CSRTT with an LITI.

	Main effect of genotype	Main effect of treatment	Main interaction
%Premature responses	$F(1,22) = 2.31$, $P=0.14$	$F(1.3,28.59) = 2.93$, $P=0.09$	$F(1.3,28.59) = 2.52$, $P=0.13$
%Accuracy	$F(1,21) = 0.29$, $P=0.59$	$F(2,42) = 0.63$, $P=0.54$	$F(2,42) = 0.26$, $P=0.77$
%Omissions	$F(1,22) = 0.045$, $P=0.83$	$F(1.45,31.86) = 3.642$, $P=0.051$	$F(1.45,31.86) = 1.42$, $P=0.25$
Total number of trials	$F(1,22) = 1.31$, $P=0.27$	$F(1.02,22.52) = 1.48$, $P=0.24$	$F(1.02,22.52) = 1.23$, $P=0.28$

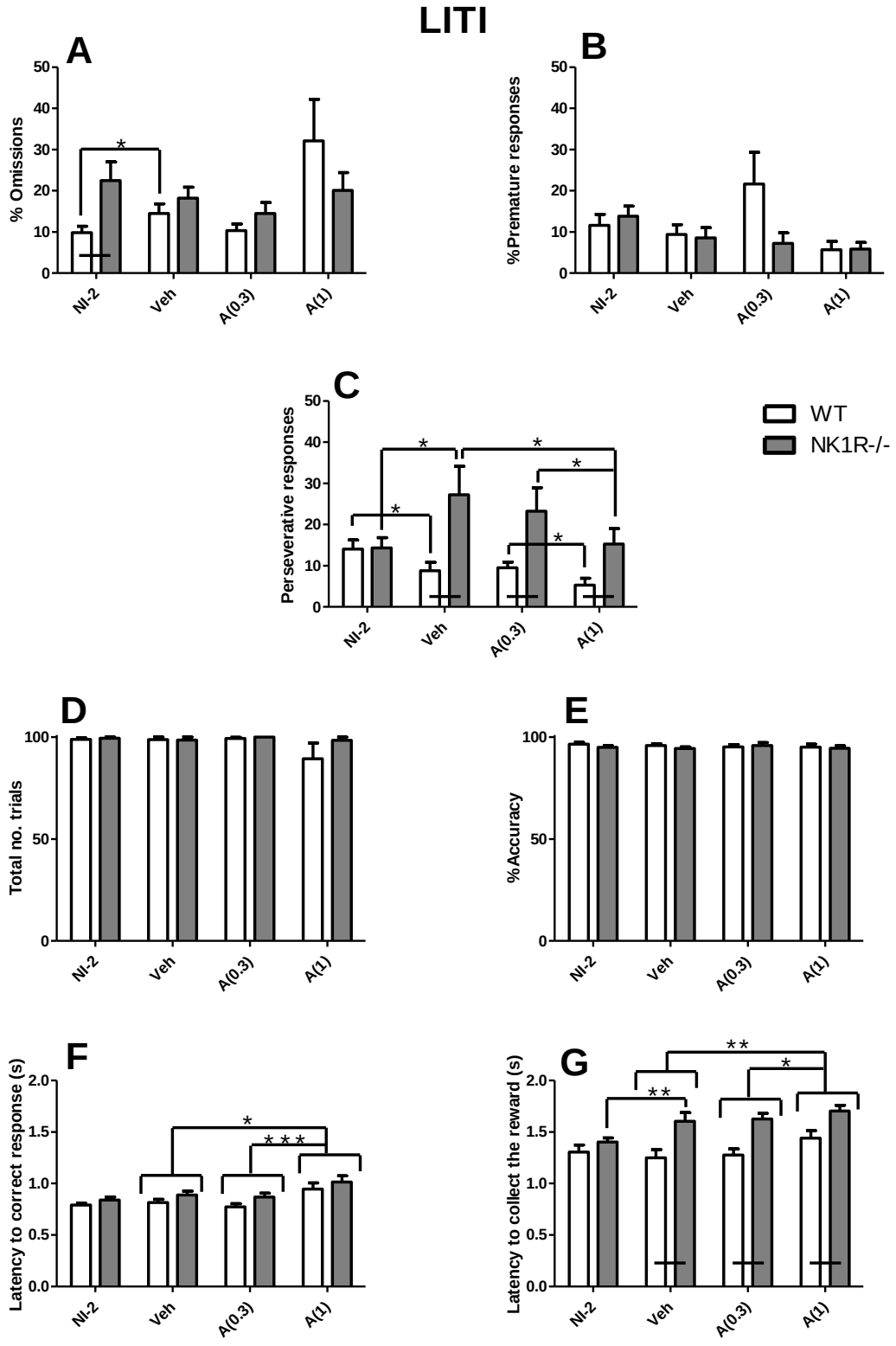

Figure 3.5 Effects of vehicle (Veh), *d*-amphetamine (0.3 and 1mg/kg; A(0.3) and A(1)) or no injection (NI-2) on performance of wildtype (WT) and NK1R-/- mice in the 5-CSRTT tested with a LITI (n=12). (A) *%Omissions,* (B) *%Premature responses*, (C) *Perseverative responses*, (D) *Total number of trials*, (E) *%Accuracy*, (F) *Latency to correct response* and (G) *Latency to collect the reward*. *P<0.05; **P<0.01; ***P<0.001. Black bars linking WT and NK1R-/- indicate a difference at P<0.05 at least.

3.3.3 VITI

Other than the effects of *d*-amphetamine on %omissions, statistical analysis of individual time bins revealed no further effects. Therefore, overall differences are presented.

3.3.3.1 Effects of vehicle injection

The vehicle injection did not modify the behaviour of either genotype, so that the genotype differences present in the un-injected groups were also present in the vehicle-injected groups. Compared to wildtype mice, NK1R-/- mice were slightly less accurate (3%), made more omissions, more perseverative responses and were slower to respond and to collect the reward. There were no genotype differences in %premature responses or total number of trials (Table 3.3).

Table 3.3 Genotype differences between un-injected and vehicle-injected wildtype and NK1R-/- mice tested in the 5-CSRTT with a VITI (n=12). Overall F values for the main effect of genotype in the repeated measures ANOVA is given, followed by the values for the post-hoc one-way ANOVA if the P value is significant.

%Accuracy	NI-2: $X^2 = 5.83$, $P<0.05$ Vehicle: $X^2 = 7.98$, $P<0.01$
%Omissions	$F(1,20) = 12.25$, $P<0.01$ NI2: $F(1,20) = 6.59$, $P<0.05$ Vehicle: $F(1,21) = 13.12$, $P<0.01$
Latency to correct response	$F(1,20) = 7.79$, $P<0.05$ NI-2: $F(1,20) = 6.31$, $P<0.05$ Vehicle: $F(1,21) = 7.12$, $P<0.05$
Latency to collect the reward	$F(1,20) = 8.55$, $P<0.01$ NI-2: $F(1,20) = 7.63$, $P<0.01$ Vehicle: $F(1,21) = 10.39$, $P<0.01$
Perseverative responses	$F(1,20) = 11.03$, $P<0.01$ NI-2: $F(1,20) = 6.50$, $P<0.05$ Vehicle: $F(1,21) = 7.96$, $P=0.01$
%Premature responses	$F(1,20) = 2.60$, $P=0.122$
Total number of trials	$F(1,20) = 0.249$, $P=0.623$

3.3.3.2 Effects of *d*-amphetamine

%Omissions

Overall there was no genotype difference in %omissions following *d*-amphetamine (Figure 3.6A). Analysis of individual time bins showed that at the 15s ITI, the higher dose of *d*-amphetamine reduced %omissions in NK1R-/- mice (X^2 = 6.62, P<0.05; c.f. vehicle: Z = 2.04, P<0.05; c.f. A(0.3): Z = 2.04, P<0.05) (Figure 3.7D).

%Premature responses

There was a main effect of treatment (F(2,42) = 3.63, P<0.05). The higher dose of *d*-amphetamine increased premature responding in NK1R-/- only (c.f. vehicle: t(11) = 2.58, P<0.05; c.f. A(0.3): t(11) = 2.94, P<0.05). This increase led to a significant genotype difference (F(1,21) = 5.32, P<0.05) (Figure 3.6B).

Perseverative responding

There was no longer a genotype difference in perseverative responding following *d*-amphetamine, but there were no significant increases or decreases in either genotype (Figure 3.6C).

%Accuracy

Both doses of *d*-amphetamine slightly reduced accuracy in wildtype mice (X^2 = 8.15, P<0.05, vehicle c.f. A(0.3): Z= 2.60, P<0.01; c.f. A(1): Z = 2.24, P<0.05) but did not affect NK1R-/- mice (Figure 3.6E).

Latency to correct response

NK1R-/- mice were slower to make a correct response across all treatment groups (F(1,21) = 8.08, P=0.01). The low dose of *d*-amphetamine slightly increased the latencies in wildtype mice (F(1.58, 33.25) = 4.11, P<0.05; vehicle c.f. A(0.3): t(10) = 3.69, P<0.01) (Figure 3.6F).

Latency to collect the reward

There was a main effect of treatment (F(1.44, 30.26) = 7.85, P<0.01). Overall, both doses of *d*-amphetamine increased the latencies (vehicle c.f. A(0.3): t(22) = 2.624,

P<0.05; c.f. A(1): t(22) = 2.312, P<0.05). This effect was seen at all ITIs (Figure 3.6G).

Total number of trials

The total number of trials was not affected by *d*-amphetamine (X^2 =0.875, P=0.65) (Figure 3.6D).

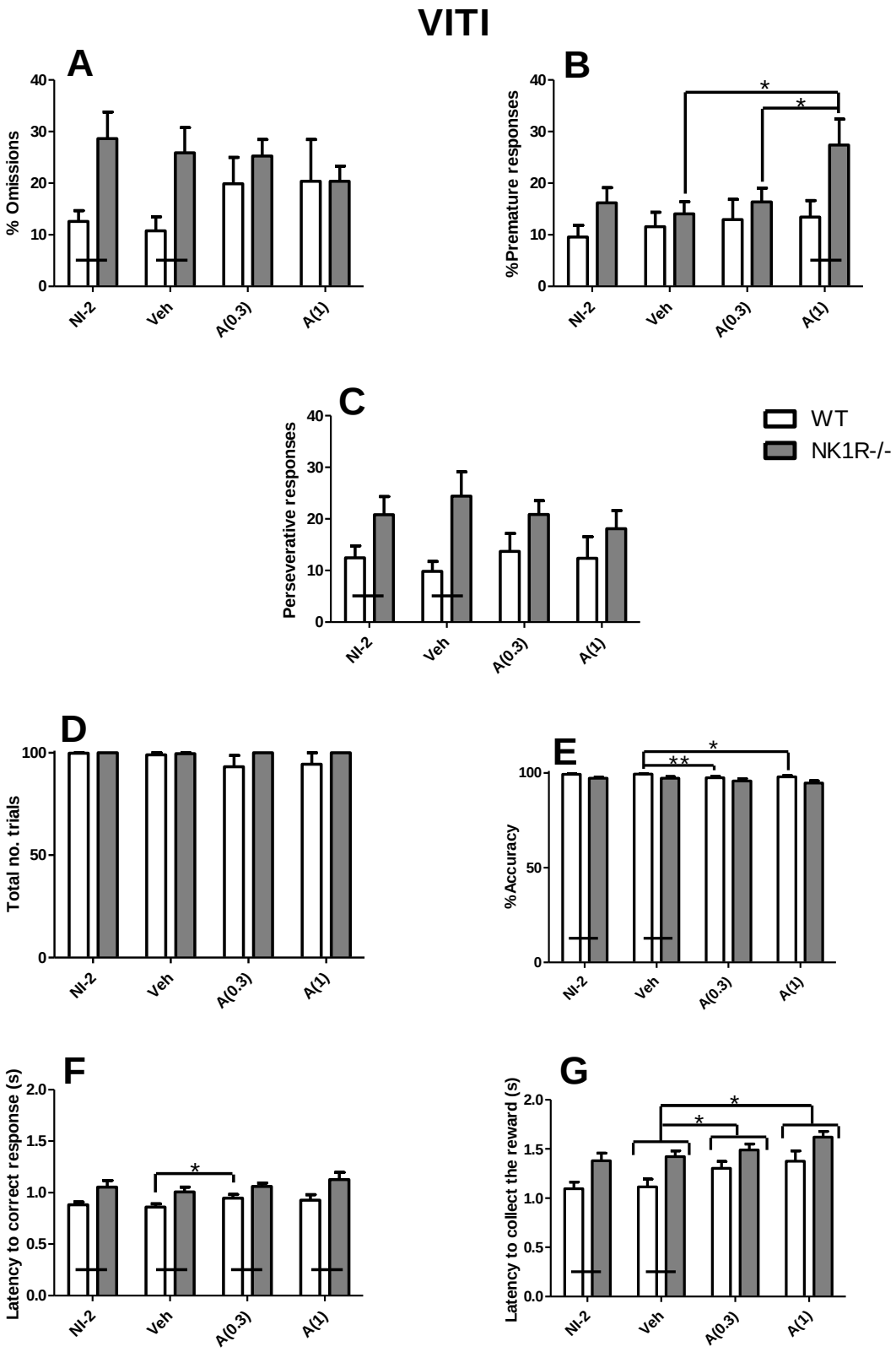

Figure 3.6 Effects of vehicle (Veh), *d*-amphetamine (0.3 and 10 mg/kg; A(0.3) and A(1)) or no injection (NI-2) on performance of wildtype (WT) and NK1R-/- mice in the 5-CSRTT tested with a VITI (n=12). (A) *%Omissions,* (B) *%Premature responses*, (C) *Perseverative responses*, (D) *Total number of trials*, (E) *%Accuracy*, (F) *Latency to correct response* and (G) *Latency to collect the reward*. *P<0.05; **P<0.01; ***P<0.001. Black bars linking WT and NK1R-/- indicate a difference at P<0.05 at least.

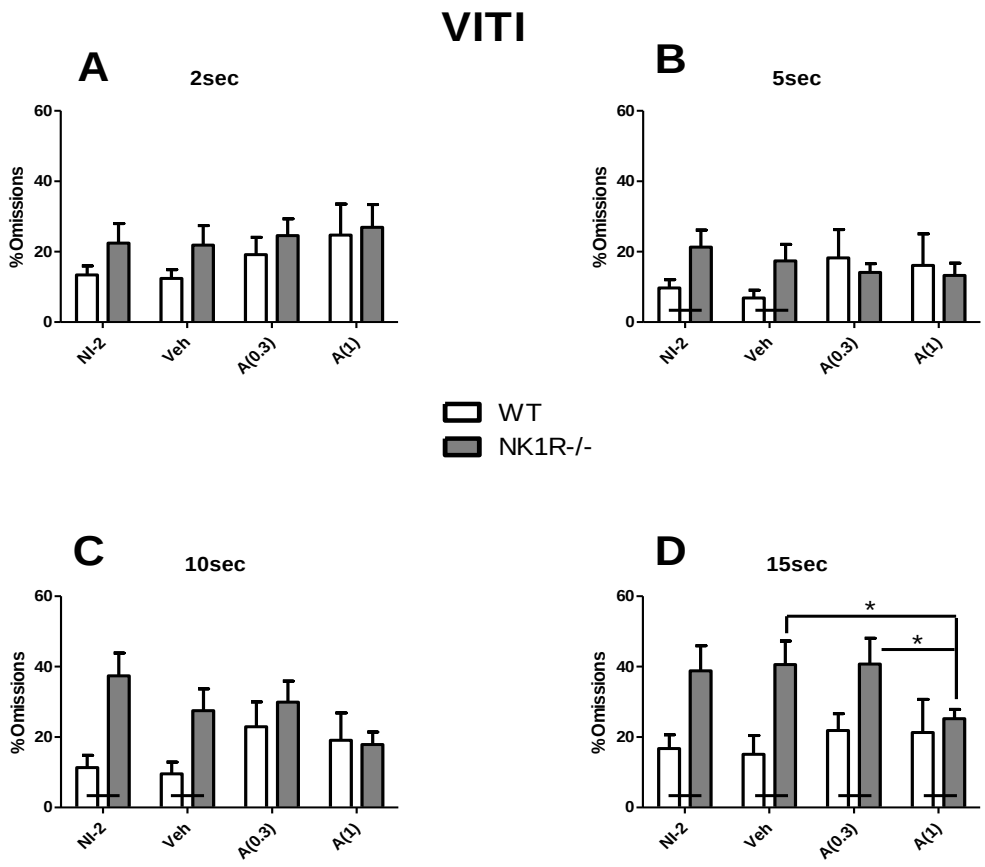

Figure 3.7 Amphetamine (1mg/kg) reduces %Omissions in NK1R-/- mice at the 15sec ITI. %Omissions of wildtype (WT) and NK1R-/- mice in the 5-CSRTT tested in the VITI at each ITI (n=12). (A) 2sec ITI, (B) 5sec ITI, (C) 10sec and (D) 15sec ITI following vehicle (Veh), *d*-amphetamine (0.3 and 1mg/kg; A(0.3) and A(1)) or no injection (NI-2). *P<0.05;**P<0.01; ***P<0.001. Black bars linking wildtype (WT) and NK1R-/- indicate a difference at P<0.05 at least.

3.4 Discussion

3.4.1 Attention

Omissions in the 5-CSRTT are an index of attention. NK1R-/- mice made more *%omissions* than wildtype mice, and this difference was no longer present after treatment with *d*-amphetamine. A significant reduction in *%omissions* following *d*-amphetamine was only evident in NK1R-/- mice in the VITI at the longest ITI (15s). Mice are trained with a 5s ITI, and between each weekly test they are kept on a baseline ITI of 5s. *%omissions* were particularly high when the ITI was increased to 15s, presumably because mice had to wait much longer than they were used to. Therefore, it was possible to pull out an effect of drug. Another index of attention in the 5-CSRTT is *%accuracy*, and this was reduced in uninjected and vehicle-injected NK1R-/- mice in the VITI. This difference was abolished following *d*-amphetamine treatment, due to a reduction in *%accuracy* in wildtype mice.

Omissions and impaired *accuracy* in the 5-CSRTT have been linked to a decrease in NA transmission (Milstein et al., 2007). Furthermore, drugs that increase NA transmission have been prescribed to patients as alternative treatments to psychostimulants. Due to the therapeutic effect of increased NA, it seems surprising that the NK1R-/- mouse, which has elevated NA in the pre-frontal cortex (Yan et al., 2009), has higher *omissions* and lower *accuracy* than wildtype mice. However, other reports have suggested that attention depends on optimal NA release (Aston-Jones and Cohen, 2005). *D*-amphetamine increases NA release, and it therefore seems surprising that it reduces omissions in NK1R-/- mice if they already have elevated levels of NA. However, *d*-amphetamine has a biphasic effect on NA, and at low doses can reduce its release (Ryan et al., 1985).

3.4.2 Perseveration

In the LITI, the vehicle injection increased *perseverative responding* in NK1R-/- mice, but reduced it in wildtype mice. In both genotypes, *perseverative responding* was reduced by the higher dose of *d*-amphetamine. The vehicle-induced increase in NK1R-/- mice was not apparent in the VITI, although this could be due to the fact that *perseverative responses* at NI-2 were already higher than in the LITI. In the

VITI, both doses of *d*-amphetamine abolished the genotype difference in *perseverative responding*.

Perseverative responding in the 5-CSRTT has been linked to a deficit in DA transmission (Baunez and Robbins, 1999), which fits with the reduction in extracellular DA found in the NK1R-/- mouse (Yan et al., 2010). Stress has previously been reported to increase DA release, which could explain why *perseveration* was reduced in wildtype mice following vehicle injection (Finlay and Zigmond, 1997). However, NK1R are required for the DA response to stress (Renoldi and Invernizzi, 2006). It follows that *perseveration* was only reduced in NK1R-/- mice following *d*-amphetamine, which mimics the dopaminergic stress response by increasing DA release. *D*-amphetamine has similarly been shown to reduce *perseveration* in rats with striatal 6-hydroxydopamine lesions (Baunez and Robbins, 1999).

3.4.3 Premature responding

%Premature responses were increased in NK1R-/- mice when they were first tested in the VITI (Yan et al., 2011, Figure 3.2). In the present chapter, when being tested for a second time without injection, this difference no longer reached statistical significance. A previous study has shown that *%premature responses* are reduced in mice tested repeatedly in the VITI (Walker et al., 2011) which could explain why this deficit is reduced on repetition of the test in NK1R-/- mice. However, it is also interesting to note that adults with ADHD are predominantly inattentive, and tend not to present with impulsive symptoms (Willcutt, 2012). Therefore, the decrease in *%premature responses* may be due to the fact that the mice are older, and the NK1R-/- mouse may be a good model for identifying more effective treatments for patients with the Predominantly Inattentive subtype of ADHD.

D-amphetamine did not reduce *%premature responses*, and even exacerbated them in NK1R-/- mice at the higher dose, as seen previously in mice treated with the same dose of amphetamine (Loos et al., 2010). Although impulsivity in the 5-CSRTT is thought to be due to disrupted 5-HT transmission, reports disagree on whether an increase (Dalley et al., 2002; Puumala and Sirviö, 1998) or decrease (Harrison et al., 1997) in 5-HT underlies impulsivity. There are at least 14 types of

5-HT receptors (Barnes and Sharp, 1999), and they have different effects on impulsivity. For example, blockade of 5-HT_{2A} receptors decreases impulsivity, while blockade of 5-HT_{2C} receptors causes an increase in this measure (Robinson et al., 2008). Therefore, the effects of 5-HT agonism or antagonism in certain brain areas could depend on the different levels of receptor subtypes. Either way, basal 5-HT in NK1R-/- mice is elevated (Yan et al., 2010) and *d*-amphetamine increases 5-HT release (Kuczenski and Segal, 1989). It follows that *d*-amphetamine would exacerbate the deficit in impulsive responding by further increasing 5-HT release.

Impulsivity in the 5-CSRTT has been described as motoric impulsivity, and other reports have similarly shown an increase in *%premature responses* following treatment with *d*-amphetamine. However, impulsivity is not a unitary construct (Evenden, 1999; Winstanley et al., 2006) and there are other rodent tests that measure different facets of impulsivity. Motoric impulsivity can be further investigated with tasks such as the go/no-go test and the stop-signal reaction-time task (Eagle and Robbins, 2003; Harrison et al., 1999). These tasks measure the ability to rapidly inhibit a pre-programmed motor response. Another test of impulsivity is delay discounting, which measures impulsive choice, rather than motoric impulsivity. As yet, the NK1R-/- mouse has not been tested in this task, although another study found that rats showing high impulsivity in the 5-CSRTT also show deficits in delay discounting (Robinson et al., 2009). It would therefore be interesting to investigate the effects of *d*-amphetamine on performance of NK1R-/- mice in other tasks of impulsivity.

3.4.4 Task performance

NK1R-/- mice were *slower to collect the reward* than wildtype mice in the LITI and VITI. This could indicate that NK1R-/- mice are less motivated by the reward. However, an increase in *latency to collect the reward* occurred in parallel with an increase in *perseverative responding* in NK1R-/- mice. Therefore, NK1R-/- mice could be slower because they were continuing to poke into the stimulus hole rather than collecting the reward. Furthermore, there were no genotype differences in the total number of trials completed, illustrating that NK1R-/- mice are as motivated to perform the task.

In all treatment groups in the VITI, NK1R-/- mice were slower to make a correct response. This difference is not apparent in the LITI, suggesting that NK1R-/- mice slow their response times when task demands are high.

3.4.5 Deficits are dependent on task parameters

Many previous reports using the 5-CSRTT either test using a long, fixed inter-trial interval (LITI) or a variable inter-trial interval (VITI). These tests measure separate response strategies (Sanchez-Roige et al., 2012). In the LITI, animals can rely on internal timing to know when the stimulus will appear, whereas in the VITI, they have to continually monitor the stimulus holes. Running both of these tests in the present experiment highlighted the importance of choosing the right task parameters. An increase in *%premature responses* is not evident in NK1R-/- mice tested in the LITI. This suggests that NK1R-/- mice have no deficits in internal timing and perform well when stimulus onset is predictable. Also, the majority of *premature responses* in the VITI were only apparent at the 10s and 15s ITIs, whereas the ITI in the LITI is only 7s. The mice were trained on a 5s ITI and it is possible that a 2s increase in ITI was not great enough to disrupt performance. The same reasoning could explain why the genotype differences in *%omissions* and *perseverative responding* are more robust in un-injected and vehicle-injected NK1R-/- mice tested in the VITI.

3.4.6 Conclusions

D-amphetamine abolished genotype differences in *%omissions*, *%accuracy* and *perseverative responding* between wildtype and NK1R-/- mice tested in the 5-CSRTT. Because there were no longer deficits in impulsivity in NK1R-/- mice on repetition of the task, it could be useful as a model of the Predominantly Inattentive subtype of ADHD. Having looked at the effects of *d*-amphetamine, the next aim was to identify alternative drugs that alleviate the deficits in NK1R-/- mice, and that could potentially be therapeutically beneficial in patients that do not currently respond to treatment.

4 Nifedipine, an L-type calcium channel blocker, reduces impulsivity but increases inattentiveness in the 5-CSRTT

4.1 Introduction

Animal models of human disorders can help the discovery of new potential treatments. This can be achieved by testing whether different compounds alleviate the animals' deficits. A report has suggested that L-type calcium channel blockers, which are currently used to treat hypertension, can alleviate ADHD-like symptoms (Krause et al., 2009). Therefore, in this chapter, the effects of nifedipine, an L-type calcium channel blocker, were evaluated in the 5-CSRTT. The results in this chapter are also discussed in Dudley et al. (2013).

4.1.1 ADHD and hypertension

One of the most widely studied rodent models of ADHD is the Spontaneously Hypertensive Rat (SHR, Okamoto and Aoki, 1963) which exhibits hyperactivity, impulsivity and attentional deficits (Russell et al., 2005). This suggests a link between ADHD and hypertension. However, hypertension is not evident in SHR until they reach adulthood, while the behavioural deficits are present before puberty (Russell et al., 2005). In a retrospective study, Krause et al. (2009) found that prior to being diagnosed, children with hypertension exhibited behavioural deficits akin to those seen in patients with ADHD, including hyperactivity and attention deficits. Interestingly, these symptoms were improved following anti-hypertensive treatment, including the L-type calcium channel blocker nifedipine. Therefore, the effects of nifedipine warrant further investigation in relation to ADHD.

4.1.2 L-type calcium channels

L-type calcium channels are voltage-gated calcium channels. Their activation is long-lasting (hence 'L'-type calcium channels) and allows calcium entry into cells. The properties of the channel are determined by four α1 subunit pores: $Ca_v1.1$-1.4. L-type calcium channels regulate various processes including muscle contraction,

synaptic plasticity and gene expression (for review see: Lipscombe et al. 2004). They are widely distributed throughout the central and peripheral nervous systems. Within the brain, they are prevalent in the striatum (Hirota and Lambert, 1997). This is of particular interest because the striatum has been implicated in the pathophysiology of ADHD, and NK1R are densely expressed in this area (Nakaya et al., 1994).

4.1.3 Nifedipine

Nifedipine is a dihydropyridine, which is a class of chemicals that block L-type calcium channels. Nifedipine has high affinity for the $Ca_v1.2$ subunit. This subunit represents the majority of L-type calcium channels found within the mouse brain, where it is particularly expressed within the striatum and cerebral cortex (Sinnegger-Brauns et al., 2009). By blocking calcium entry into smooth muscle cells, nifedipine decreases vasoconstriction and subsequently lowers blood pressure. For this reason, nifedipine is currently used to treat patients with hypertension.

4.1.4 L-type calcium channels and NK1R

Pharmacological evaluation of NK1R antagonists have revealed non-specific L-type calcium channel blocking properties (Guard et al., 1993; Rupniak et al., 1993, 2003; Schmidt et al., 1992), and a depressant effect of the NK1R antagonist RP 67580 on blood pressure has previously been reported (Laird et al., 1993). Furthermore, nifedipine and another L-type calcium channel blocker, verapamil, both induce an antinociceptive phenotype in rodents similar to that seen in wildtype mice treated with an NK1R antagonist (Miranda et al., 1992; Rupniak et al., 1993). This suggests an interaction between L-type calcium channels and NK1R. Currently, it is not known whether there are abnormalities in the expression or function of L-type calcium channels in NK1R-/- mice. However, it is possible that L-type calcium channel blockers administered to wildtype mice could mimic the behavioural deficits seen in NK1R-/- mice in the 5-CSRTT.

4.1.5 Aims

The study by Krause et al. (2009) suggests that the L-type calcium channel blocker nifedipine could alleviate the behavioural deficits seen in NK1R-/- mice tested in

the 5-CSRTT. However, pharmacological studies with NK1R antagonists suggest that L-type calcium channel antagonism could induce these behavioural deficits in wildtype mice. Therefore, in this study the effects of nifedipine on performance of wildtype and NK1R-/- mice in the 5-CSRTT were studied. In the previous chapter more behavioural deficits were evident in NK1R-/- mice under VITI testing conditions than in the LITI, presumably due to increased task difficulty. For this reason mice were only tested in the VITI in the present chapter. Also, in order to confirm that any effects on 5-CSRTT performance were not due to any sedative effects of the drug, activity of nifedipine-treated wildtype and NK1R-/- mice was measured in the LDEB.

4.2 Methods

4.2.1 Animals

The mice and housing conditions are described in section 2.4.1 and were identical to those used in chapter 3. For the LDEB experiment, mice were tested at 6-8 weeks of age.

4.2.2 5-CSRTT

The apparatus and the food deprivation protocol are decribed in section 2.4.1. Wildtype mice weighed 34.5±0.5g and NK1R-/- mice weighed 28.9±0.8g at the start of the experiment. Following habituation and training in the 5-CSRTT, wildtype and NK1R-/- mice (n=6-10) were challenged with a Variable Inter-Trial Interval (VITI) (2, 5, 10 or 15s, delivered on a random schedule). After an initial un-injected VITI test (NI-1), the mice were retested in the VITI 30 minutes after an intraperitoneal (i.p.) injection of nifedipine (3mg/kg and 10mg/kg), vehicle (20% Tween 80 in 0.9% saline), or a second un-injected test (NI-2). All mice experienced each test condition once only at weekly intervals (on a Friday), on a pseudo-latin square randomisation schedule. Between each weekly test, mice were trained once-daily on stage 6, and were only tested if their performance was stable for 3 consecutive days.

4.2.3 LDEB

The protocol is as described in (Herpfer et al., 2005). For a full description of the protocol and apparatus, see section 2.4.2. Briefly, mice (n=4-6) were placed in the dark zone for 60 minutes before being injected (i.p.) with nifedipine (3mg/kg and 10mg/kg) or vehicle (20% Tween 80 in 0.9% saline). Following a further 30 minutes in the dark zone, they were placed in the centre of the light zone and the *total number of line crosses* and *line crosses in the light zone* over 30 minutes was recorded and scored blind.

4.2.4 Statistical analysis

Full details of statistical analysis are given in section 2.7. The behaviours scored in the 5-CSRTT are identical to those in chapter 3, and described in full in section 2.4.1.3. In addition, the total number of trials to reach baseline was analysed with an independent-samples t-test and the training performance of wildtype and

NK1R-/- mice was compared with a 2-way repeated measures ANOVA, with '*stage*' as the within-subjects factor and '*genotype*' as the between-subjects variable. In the test phase, four different analyses were performed. First, uninjected wildtype and NK1R-/- mice were compared when they were first tested in the VITI (NI-1). Next, the first and second uninjected tests (NI-1 and NI-2) were compared to look at the effects of repeated testing on task performance. Then uninjected (NI-2) and vehicle-injected mice were compared to look at the effect of injection. Finally, vehicle-injected mice were compared with nifedipine-injected mice. Each comparison was analysed with a 2-way repeated measures ANOVA, with '*genotype*' as the between-subjects factor, and '*treatment*' as the within-subjects factor. A significant main effect of either independent variable or a significant interaction between them led to post-hoc 1-way ANOVA or paired sample t-tests. The LDEB data were analysed with a 2-way between subjects ANOVA, with '*genotype*' and '*treatment*' as the between-subjects factors, followed by post-hoc 1-way ANOVA and LSD tests.

4.3 Results

4.3.1 5-Choice Serial Reaction-Time Task

4.3.1.1 Number of trials to reach baseline

There was no genotype difference in the number of trials ($t(14) = 0.047$, $P=0.963$). Wildtype mice took an average of 31.5 trials and NK1R-/- mice took an average of 29 trials to complete training (Figure 4.1). As training was carried out 5 days a week, the mice were between 3-4 months when they were first tested in the 5-CSRTT.

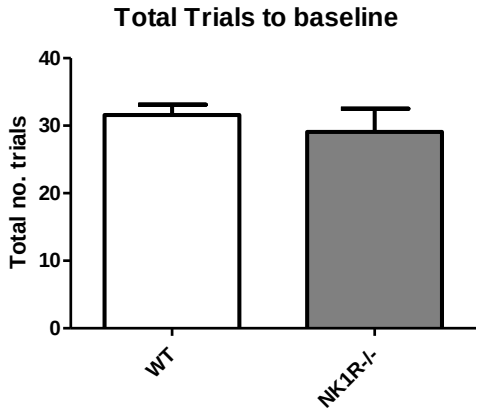

Figure 4.1 Total number of trials to reach baseline in the 5-CSRTT in wildtype (WT) and NK1R-/- mice.

4.3.1.2 Training differences

The only genotype difference during training was in %accuracy. There was a main interaction between stage and genotype ($F(5,70) = 3.98$, $P<0.01$). NK1R-/- mice were more accurate than wildtype mice at stages 3 ($F(1,21) = 13.06$, $P<0.01$), 5 ($F(1,18) = 16.64$, $P=0.001$) and 6 ($F(1,14) = 9.42$, $P<0.01$).

4.3.1.3 VITI

%Omissions

%Omissions did not differ between the two genotypes during either of the uninjected tests or following vehicle injection. Nifedipine increased %omissions, but the magnitude of this increase depended on genotype (treatment*genotype interaction: $F(2,28) = 3.98$, $P<0.05$). Whereas both doses of nifedipine increased %omissions in wildtype mice (veh c.f. nifed-3: $t(5) = 5.03$, $P<0.01$; c.f. nifed-10: $t(5)= 6.18$, $P<0.01$), only the higher dose increased this measure in NK1R-/- mice

(veh c.f. nifed-10: t(9) = 4.56, P=0.001) (Figure 4.2A). Despite this increase, wildtype mice had significantly higher %omissions than NK1R-/- mice after both doses of nifedipine (nifed-3: F(1,15) = 8.82, P=0.01; nifed-10: F(1,14) = 9.99, P<0.01).

%Premature responses

%Premature responses were increased in NK1R-/- mice at NI-1 compared to wildtype mice (t(14.46) = 2.26, P<0.05). This increase was no longer present at NI-2. %Premature responses were reduced overall by the vehicle injection (main effect of injection: $F_{1,14}$ = 7.61, P<0.05; c.f. NI-2: t(15) = 2.66, P<0.05). Nifedipine further reduced %premature responses, but this depended on genotype (treatment*genotype interaction: F(2,28) = 4.53, P<0.05). Both doses of nifedipine reduced %premature responses in wildtype mice (veh c.f. nifed-3: t(5) = 2.86, P<0.05; c.f. nifed-10: t(5) = 3.64, P<0.05). This reduction was only seen following the higher dose of nifedipine in NK1R-/- mice (veh c.f. nifed-10: t(9) = 7.62, P<0.001) (Figure 4.2B).

Perseverative responding

NK1R-/- mice made more perseverative responses than wildtype mice, especially at NI-2 (X^2 = 5.70, P<0.05). This measure was not affected by vehicle injection, and NK1R-/- mice continued to make more perseverative responses than wildtype mice following nifedipine injection (main effect of genotype: F(1,14) = 14.15, P<0.01). Nifedipine reduced perseverative responding in wildtype mice but did not affect NK1R-/- mice (treatment*genotype interaction: F(2,28) = 4.67, P<0.05; wildtype: veh c.f. nifed-3: t(5) = 2.72, P<0.05; c.f. nifed-10: t(5) = 3.94, P<0.05) (Figure 4.2C).

Total number of trials

Repetition of the test or vehicle injection did not affect the total number of trials. There was a reduction in trials completed following the higher dose of nifedipine in wildtype mice only (X^2 = 6.91, P<0.05; veh c.f. nifed-10: Z = 2.2, P<0.05) (Figure 4.2D).

%Accuracy

Repetition of the test, vehicle injection or nifedipine did not affect %accuracy in either genotype (Figure 4.2E).

Latency to correct response

The latency to correct response was slightly decreased in both genotypes on repetition of the test (main effect of repetition: $F(1,14) = 6.59$, $P<0.05$), and increased following vehicle injection (main effect of injection: $F(1.14) = 20.19$, $P=0.001$). This increase was slightly greater in wildtype mice, so that vehicle-injected NK1R-/- mice were faster to respond (main effect of genotype: $F(1,14) = 4.66$, $P<0.05$). Both doses of nifedipine increased the latency to correct response (main effect of treatment: $F(1.23,14.78) = 20.13$, $P<0.001$; veh c.f. nifed-3: $t(15) = 3.59$, $P<0.01$; c.f. nifed-10: $t(13) = 5.47$, $P<0.001$) but there were no genotype differences (Figure 4.2F).

Latency to collect the reward

Latency to collect the reward did not change on repetition of the test, but was increased following vehicle injection (main effect of treatment: $F(1,14) = 11.19$, $P<0.01$; veh c.f. NI-2: $t(15) = 3.18$, $P<0.01$). This measure was also increased following nifedipine (main effect of treatment: $F(1.25,15.02) = 17.36$, $P<0.001$; veh c.f. nifed-3: $t(15) = 3.59$, $P<0.01$; c.f. nifed-10: $t(13) = 5.47$, $P<0.001$), but did not differ in the two genotypes (Figure 4.2G).

4.3.2 Light/Dark exploration box

Nifedipine did not affect the total number of line crosses or line crosses in the light zone of either genotype (Figure 4.3). There were also no genotype differences. The statistical values are summarised in (Table 4.1).

Table 4.1 Statistical values for the total number of line crosses and line crosses in the light zone in wildtype and NK1R-/- mice tested in the LDEB.

	Main effect of genotype	Main effect of treatment	Main interaction
Total number of line crosses	F(1,32) = 0.97, P=0.33	F(2,32) = 1.92, P=0.16	F(2,32) = 0.92, P=0.41
Line crosses in the light zone	F(1,32) = 0.62, P=0.44	F(2,32) = 2.70, P=0.08	F(2,32) = 0.05, P=0.95

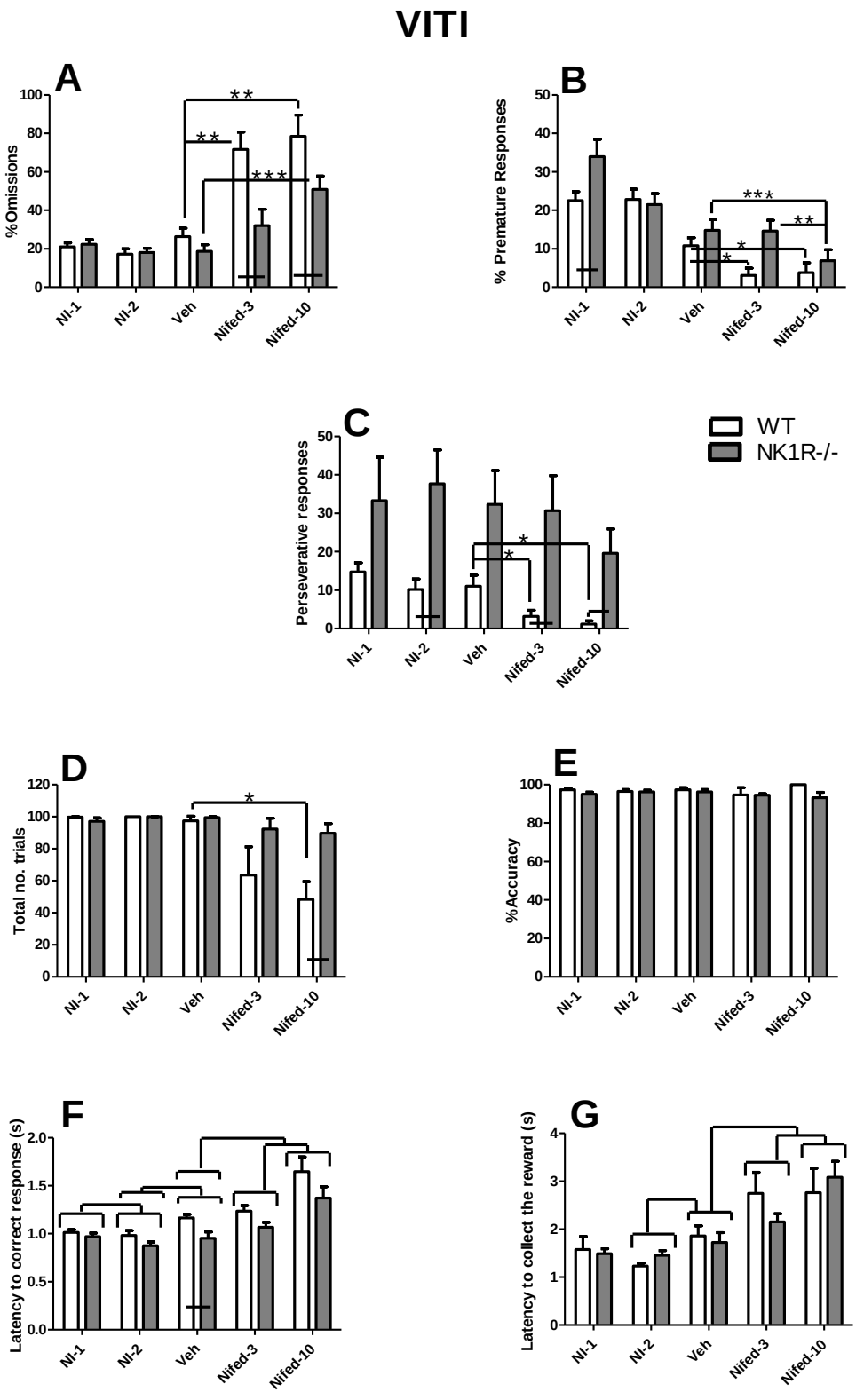

Figure 4.2 Effects of vehicle (Veh), nifedipine (3 and 10mg/kg; Nifed-3 and Nifed-10) or no injection (NI-2) on performance of wildtype (WT) and NK1R-/- mice in the 5-CSRTT tested with a VITI (n=6-10). (A) *%Omissions,* (B) *%Premature responses*, (C) *Perseverative responses*, (D) *Total number of trials*, (E) *%Accuracy*, (F) *Latency to correct response* and (G) *Latency to collect the reward*. Solid black bars linking white and black bars indicate P<0.05, at least. Black bars linking treatment groups indicate P<0.05 at least. *P<0.05; **P<0.01; ***P<0.001.

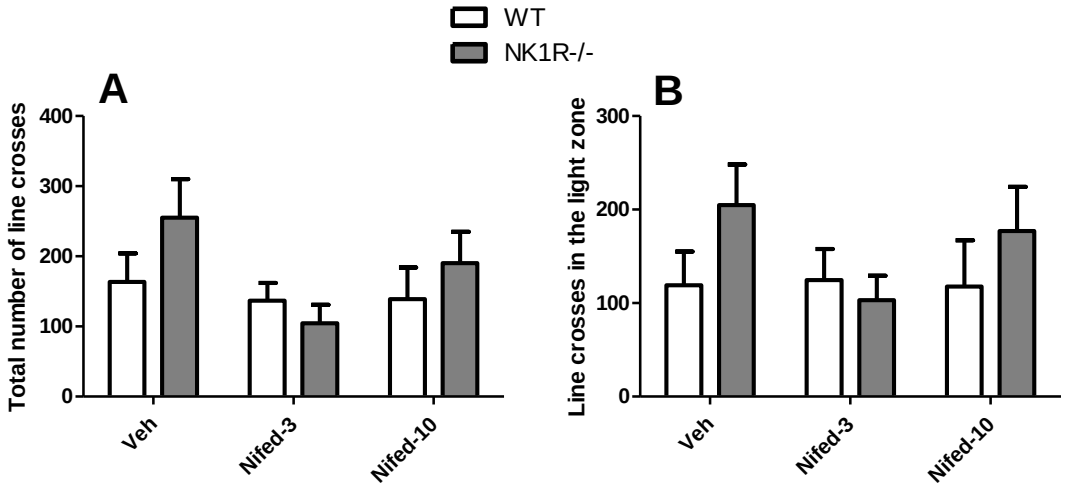

Figure 4.3 Nifedipine does not affect the activity of wildtype (WT) or NK1R-/- mice in the LDEB (n=4-6). (A) *Total number of line crosses* and (B) *number of line crosses in the light zone* made by wildtype (WT) and NK1R-/- mice in the LDEB (N=5-8). Mice received an i.p. injection of vehicle (Veh) or nifedipine (3 or 10 mg/kg; Nifed-3 or Nifed-10). There were no statistically significant differences.

4.4 Discussion

4.4.1 Effects of nifedipine on attention and impulsivity

In the 5-CSRTT, an increase in *%omissions* with no simultaneous increase in *response latencies* is an index of inattentiveness (Robbins, 2002). Whereas it was shown in chapter 3 that NK1R-/- mice have significantly higher *%omissions* than their wildtype counterparts, this difference was not replicated here. It is not clear why a deficit in attention was not present in the current study, although patients with ADHD often present with unstable symptoms (Lahey et al., 2005; Todd et al., 2008). Also, *%premature responses* (an index of impulsivity) were increased in NK1R-/- mice when first tested in the VITI, but were reduced on repetition of the test. This was also seen in the previous chapter. As discussed in section 3.4.3, a decrease in impulsivity on repetition of the 5-CSRTT has been shown in another study (Walker et al., 2011) and suggests that mice can quickly adapt to the task demands.

In order to improve the validity of the NK1R-/- mouse model of ADHD, it is important to identify conditions under which the behavioural deficits are stable. Indeed, one of the aims of this animal model is to identify novel treatments. If the behavioural deficits are not consistent in NK1R-/- mice, then it may not be possible to test whether different drugs can improve them. However, *%accuracy* in wildtype and NK1R-/- mice is very high, so perhaps making the task parameters more difficult (for example by shortening the stimulus duration) would identify more robust deficits.

The effects of nifedipine on *%omissions* and *%premature responses* differed between genotypes. While the higher dose of nifedipine (10mg/kg) increased *%omissions* and reduced *%premature responses* in both genotypes, at the lower dose (3mg/kg) only wildtype mice were affected. This highlights a link between NK1R function and L-type calcium channels. It also suggests that L-type calcium channels contribute to impulsivity and attention in the 5-CSRTT, and that their antagonism with nifedipine may be therapeutically beneficial in patients with impulse control disorders.

Surprisingly, NK1R-/- mice had higher %accuracy than wildtype mice during training. %Accuracy is an index of attention in the 5-CSRTT and this suggests that NK1R-/- are actually more attentive than wildtype mice. However, this difference was no longer present during the testing phase and unlike %omissions, %accuracy was not affected by nifedipine. This suggests that when mice missed the stimulus light, they chose not to respond, rather than guessing and making an incorrect response. This could imply that nifedipine was affecting the animals' motivation to perform the task. At the lower dose of nifedipine this did not appear to be the case because despite an increase in *%omissions* in wildtype mice, there was no significant reduction in the total number of trials completed. However, there was a reduction in trials completed at the higher dose of nifedipine, and the simultaneous decrease in *%premature responses*, increase in *%omissions* and increases in the *latencies to respond and to collect the reward* suggest that this dose impaired task performance. As seen from the LDEB data, this does not appear to be due to a sedentary effect of the drug because nifedipine did not impair the activity of wildtype or NK1R-/- mice. However, if the higher dose of nifedipine is causing a rapid reduction in blood pressure, the animals may feel sick and be unwilling to drink the milk reward.

4.4.2 Central mechanisms

The neural mechanisms underlying the decrease in impulsivity and simultaneous increase in inattentiveness by nifedipine are unclear. This bidirectional relationship between attention and impulsivity is not surprising, because redirecting attention to salient stimuli requires inhibition of ongoing actions. *%Premature responses* in the 5-CSRTT represent 'motoric impulsivity' (Winstanley et al., 2006). The suppression of unwanted motor behaviour relies on the 'indirect' striatopallidal pathway and subsequent facilitation of the 'direct' striatonigral pathway is required to initiate new actions and respond to new stimuli. These pathways form loops projecting through the cortex, basal ganglia, thalamus and striatum.

A recent study has shown that the thalamic projections in these loops converge on cholinergic interneurons in the striatum, and that these interneurons are involved in the inhibition of unwanted actions in order to redirect attention to salient

stimuli (Ding et al., 2010; Thorn and Graybiel, 2010). Thalamic activation of cholinergic interneurons causes postsynaptic activation of muscarinic M1 receptors, located on medium spiny neurons (MSNs). This suppresses L-type calcium channel currents (Wang et al., 2006), thereby reducing network excitability and inhibiting unwanted actions. Blocking L-type calcium channels with nifedipine would add to overall network inhibition, thus reducing impulsivity. It is possible that too much inhibition of the indirect 'No-Go' pathway would prevent activation of the direct 'Go' pathway, and prevent attention from being redirected. This mechanism could explain the opposing effects nifedipine has on *%omissions* and *%premature responses*. In order to test this hypothesis, it would be interesting to test an L-type $Ca_v1.2$ calcium channel knockout mouse in the 5-CSRTT.

NK1R are predominantly located on cholinergic interneurons in the striatum. Substance P binding to these receptors triggers acetylcholine release (Anderson et al., 1994; Kemel et al., 2002). In contrast, D2 receptors located on cholinergic interneurons inhibit acetylcholine release (Wang et al., 2006; Wilson, 2006). A reduction in acetylcholine release would reduce the M1 receptor suppression of L-type calcium channels in MSNs. The would lead to greater excitability within the network, and reduced behavioural inhibition. This could explain why a higher dose of nifedipine is required in NK1R-/- mice to reduce impulsivity and attention.

4.4.3 NK1R and hypertension

While the central mechanisms described above could explain the genotype-dependent responses to nifedipine, they could also be caused by a difference in blood pressure. Substance P induces smooth muscle contraction and vasodilation , and these effects are thought to be mediated by NK1R (reviewed in: Maggi, 1995; Otsuka and Yoshioka, 1993). In humans, administration of the NK1R antagonist CP99 994 blocks the vasorelaxation of pulmonary arteries (Corboz et al., 1998). It remains to be determined whether NK1R-/- mice are hypertensive. However, if this is the case, it could explain why a higher dose of nifedipine is required in NK1R-/- mice to modify behaviour in the 5-CSRTT. In order to verify this, it would be interesting to test an L-type calcium channel blocker that does not cross the blood-brain barrier.

4.4.4 Perseverative responding

Consistent with the results from the previous chapter, *perseverative responding* was greater in NK1R-/- mice than in wildtype controls. Both doses of nifedipine reduced *perseverative responding* in wildtype mice but did not affect NK1R-/- mice. In the previous chapter, *perseverative responding* was reduced following *d*-amphetamine, and this was thought to be due to an increase in DA release (see section 3.4.2). However, nifedipine does not affect DA release in the brain (El Ayadi et al., 2001; Reiriz et al., 1994), nor does it affect extracellular NA (Silverstone et al., 1992) or 5-HT (Sharp et al., 1990).

The decrease in *perseverative responding* seen in wildtype mice may result directly from blockade of L-type calcium channels, rather than through changes in monoamines. TS-2 neo mice, that have a mutation in the L-type $Ca_v1.2$ calcium channel gene, exhibit perseveration and repetitive behaviours (Bader et al., 2011). This mutation is thought to cause a failure in channel inactivation (i.e. increased channel opening), suggesting that the behavioural deficits seen in TS2-neo mice could be improved by L-type calcium channel blockers (Splawski et al., 2004). This fits with the current findings that nifedipine reduces *perseverative responding* in wildtype mice. A similar trend was seen in NK1R-/- mice, although as seen for other behaviours, this was only present at the higher dose.

4.4.5 NK1R antagonists and L-type calcium channels

Recent work in our lab tested the effects of the NK1R antagonist RP 67580 on the behaviour of wildtype and NK1R-/- mice in the 5-CSRTT (Weir, 2012). Two doses were studied, 5 and 10 mg/kg. While the lower dose increased *%premature responses* in wildtype mice, this effect was not seen at the higher dose. Also, the higher dose increased *%omissions* and the *latencies to respond and collect the reward* in wildtype and NK1R-/- mice. The finding that RP 67580 affects NK1R-/- mice suggests that it was acting at a target other than NK1R. Nifedipine induced similar deficits to those seen following the higher dose of RP 67580, suggesting that this secondary target is L-type calcium channels, as has previously been reported (Rupniak et al., 1993, 2003). This means that pharmacological studies with NK1R antagonists may not induce the deficits seen in NK1R-/- mice, even if they are caused by non-functional NK1R.

4.4.6 Activity in the LDEB

In this study, activity in the LDEB was measured in order to verify that nifedipine was not inducing gross motor deficits that could impair 5-CSRTT performance. Although there was a tendency for the *total activity* and the *activity in the light zone* to be increased in NK1R-/- mice, this did not reach statistical significance. Previous studies using the same LDEB protocol and number of animals have found an increase in activity in NK1R-/- mice (Herpfer et al., 2005; Yan et al., 2010). A lack of hyperactivity is an issue for the face validity of the NK1R-/- mouse of ADHD. Therefore, the work in chapter 5 focussed on re-establishing hyperactivity in NK1R-/- mice.

4.4.7 Conclusions

Nifedipine reduces *impulsivity* and *perseverative responding* but increases *inattentiveness* in the 5-CSRTT. These effects are greater in wildtype mice, highlighting an interaction between NK1R and L-type calcium channels. This interaction is could be linked to cholinergic interneurons in the striatum and their projections in the indirect striatopallidal pathway. Nifedipine induced a similar behavioural profile to that seen in wildtype and NK1R-/- mice given the NK1R antagonist RP 67580, indicating that the effects seen following injection of RP 67580 are related to L-type calcium channel blockade. Overall the results in this chapter suggest that L-type calcium channel blockers may be beneficial for impulse control disorders. However, the next aim was to identify treatments that would simultaneously improve *impulsivity*, *perseverative responding* and *attention*.

5 Establishing hyperactivity in NK1R-/- mice

5.1 Introduction

In order to identify drugs that improve the deficits in NK1R-/- mice, it is important to have a behavioural screen. Due to the time it takes to train and test mice in the 5-CSRTT, it is not possible to rapidly test a range of drugs in this task. It has previously been shown that NK1R-/- mice are hyperactive and that this is reduced by *d*-amphetamine (Yan et al., 2010). Tests of activity are quick and can therefore be efficient drug screens. However, activity can be measured in a variety of tests and, depending on the test, can be more or less affected by stress. In the current chapter I aim to identify testing conditions that reveal robust hyperactivity in NK1R-/- mice.

5.1.1 Hyperactivity in NK1R-/- mice

NK1R-/- mice have higher activity than wildtype mice in a locomotor activity chamber and show an increase in total line crosses in the LDEB (Herpfer et al., 2005). Also, in the LDEB, the NK1 antagonists RP 67580 and L733 060 increase activity in wildtype mice. This suggests a role for NK1R in hyperactivity. Furthermore, both the increased activity of NK1R-/- mice and the increase in activity in wildtype mice injected with NK1R antagonists are reduced by *d*-amphetamine in the LDEB (Yan et al., 2010). *D*-amphetamine is currently used to treat patients with ADHD, indicating that the LDEB could help identify drugs that are therapeutically effective in ADHD. For this reason, initial work in this chapter aimed to use the LDEB to identify novel treatments for ADHD.

5.1.2 Issues with the LDEB as a test of activity

The LDEB was initially developed as a test of anxiety, with anxiolytic drugs increasing the *number of transitions between the light and dark zone*, as well as the *time spent in the light zone* (Crawley, 1985, 1981). However, when using this test to measure activity, the varying amount of time animals spend in the light and dark zones can confound interpretation of the data. In order to overcome this limitation, (Yan et al., 2010) divided the *activity in the light zone* by the *time spent in the light zone*. Another way of controlling for this could be to analyse the overall activity

across both zones (as in Herpfer et al., 2005). However, in the protocol used, mice are habituated to the dark zone for 90 minutes prior to transferring them to the light zone. Because the animals are habituated to the dark zone, they are likely to explore it less during the 30 minute testing period. This will affect the total activity score. Therefore, if activity is the only measure of interest, it is better to use a locomotor activity chamber. As mentioned above, NK1R-/- mice are also hyperactive when tested in an activity chamber. However, the effects of drugs in this test have not yet been studied.

5.1.3 Effects of stress on activity

All rodent behavioural paradigms are susceptible to the effects of stress. When comparing the activity of NK1R-/- mice to wildtype controls it is especially important to minimise stress because NK1R-/- mice have a blunted response to anxiogenic stimuli: they attack less in the resident-intruder test (De Felipe et al., 1998), they vocalise less following maternal separation (Rupniak et al., 2000; Santarelli et al., 2001) and they show decreased anxiety in the elevated-plus maze (Santarelli et al., 2001). The stress of an injection can modify behaviour in tests of activity but as yet the activity of NK1R-/- and wildtype mice has not been measured without injection. As seen in the previous chapters, a vehicle injection in the 5-CSRTT can have genotype-dependent effects on behaviour. In this chapter the effect of vehicle injection on activity was investigated.

In order to minimise stress, animals should be well-handled and habituated to the experimental room prior to testing. However, one way of avoiding the effects of experimenter intervention is to test the activity of animals in their home cage. This can be achieved with telemetry, in which a radiotransmitter probe is implanted subcutaneously in mice and following full recovery, their activity can be monitored by placing their home cages on receiver mats. Another advantage of this technique is that activity can be monitored throughout the day and night. Previously, the activity of NK1R-/- mice has been measured in 30 minutes tests during the animals' light phase, when mice are least active.

5.1.4 Influence of breeding method

Previous studies disagree on whether activity is increased in NK1R-/- mice compared to wildtype controls. Initial investigations found no difference in activity in the open field or elevated plus-maze (De Felipe et al., 1998; Murtra et al., 2000a). However, other studies found that NK1R-/- mice showed an increase in exploratory behaviour in the open field and elevated plus-maze (Santarelli et al., 2002, 2001). This discrepancy could be due to the different background strain used. Initial investigations used mice bred on a 129/Sv x C57BL/6 background and found no differences in activity. In the studies by Santarelli et al. (2002, 2001), mice were bred on a 129SvEv line, because the authors were interested in the anxiolytic profile of NK1R-/- mice, and wildtype 129Sv mice show high levels of anxiety (Homanics et al., 1999). This highlights the influence of genetic background on the behavioural phenotype of NK1R-/- mice.

Throughout this chapter, the same activity testing conditions that identified hyperactivity in NK1R-/- mice in the study by (Herpfer et al., 2005) were used. Furthermore, the mice used here were taken from the same colony, housed in identical conditions. Despite this, hyperactivity was variable in NK1R-/- mice tested in the LDEB and could not be identified in a locomotor activity chamber. One possible reason for these differences is that the colonies have been maintained homozygously, meaning that wildtype mice are bred separately from NK1R-/- mice. If this breeding method is used, it is advised to backcross the mice to a wildtype inbred strain every few generations to avoid *de novo* genetic mutations occurring that will cause the wildtype and NK1R-/- colonies to drift (Crusio et al., 2009). An indication this may have been occurring in the colonies used in this thesis was that whereas in the study by Herpfer et al. (2005) it was not possible to distinguish the genotype based on fur colour, in this thesis younger generations of the same colony were used and all wildtype mice were white and NK1R-/- mice were brown. If coat colour is segregating with the gene of interest, it is possible that other genes are segregating too (Crusio, 2004).

Another issue with maintaining homozygous colonies is that there can be differences in early environment that affect the mouse phenotype (Crusio, 2004). Studies have shown that differences in maternal rat grooming behaviour can cause

epigenetic changes in stress responses (Meaney, 2001), and that these changes can be maintained in future generations (Francis et al., 1999). Epigenetic changes in stress responses can also occur in humans with adverse early life experiences (McGowan et al., 2009). An interaction between stress response and NK1R has previously been reported; there are differences in novelty and morphine-induced hyperlocomotion between NK1R-/- mice bred from different strains, and this has been linked to different corticosterone responses to stress (McCutcheon et al., 2008). As mentioned above, stress can affect behaviour in activity paradigms. This highlights the importance of minimising differences in early life environment by breeding wildtype and NK1R-/- mice from heterozygous parents.

5.1.5 Aims

This chapter investigates the hyperactive phenotype of NK1R-/- mice. My first aim was to identify whether vehicle-injected NK1R-/- mice in the LDEB show an increase in *total activity*, as in Herpfer et al. (2005), or an increase *in activity in the light per unit of time*, as in (Yan et al., 2010). To check whether any differences in activity were due to injection stress, I then tested uninjected activity in the LDEB and in locomotor activity chambers. Finally, in order to minimise experimental stress, I tested activity in the homecage with telemetry, initially with mice bred from homozygotes and then with littermate pairs. In the littermate pair group I also looked at whether *d*-amphetamine would reduce the activity of NK1R-/- mice while increasing it in wildtype mice, and also whether the NK1R antagonist RP 67580 increased activity in wildtype mice.

5.2 Methods

5.2.1 Mice

All mice used in this chapter were derived from the same genetic background (129/Sv x C57BL/6 crossed with an MF1 strain), but the breeding method changed (Figure 5.1). In the LDEB and activity meter experiments, all animals came from homozygous breeding pairs. In the telemetry experiment, mice from heterozygous breeding pairs were also used. Full details of breeding and housing are described in section 2.1.

5.2.2 Light/dark exploration box

The protocol was identical to that described in (Herpfer et al., 2005). Full description of the apparatus and protocol are described in section 2.4.2. Briefly, wildtype and NK1R-/- mice (8-12 weeks old) were habituated to the experimental room for 3 hours, and subsequently to the dark zone for 90 minutes. In the first experiment (n=8) they received an i.p. injection of saline (0.9%) after 60 minutes, before being replaced in the dark zone for a further 30 minutes. In the second experiment (n=4) one group received an i.p. injection of saline (0.9%) and the other was not injected. Following this, mice were transferred to the centre of the light zone and their activity was recorded for 30 minutes. The following behaviours were scored blind:

- *Total number of line crosses*
- *Time spent in the light zone*
- *Activity in the light/unit of time*
- *Number of transitions to the light zone*

5.2.3 Locomotor activity chambers

Again, the protocol was identical to that described in Herpfer et al. (2005) and is described in full in section 2.4.3. *Ambulatory activity* of wildtype and NK1R-/- mice (n=4) was scored whenever a mouse made 2 adjacent beam breaks. This was to ensure that movement caused by grooming would not count as activity.

5.2.4 Telemetry

Telemetry is a technique in which a radiotransmitter probe is implanted into an animal. Mice are placed in their homecage on a mat that contains an antenna and activity can be recorded. Full details of surgical implantation of the probe, the apparatus and the procedure are given in section 2.4.4. Mice from homozygous (n=3) or heterozygous (n=4) breeding pairs (8-12 weeks) were implanted with a TA-F10 transmitter (DataSciences, UK) and following recovery, their activity was recorded for 48h. In the homozygous breeding experiment, the dark phase was from 20.00 – 08.00, and in the heterozygous breeding experiment, the dark phase was from 19.00 – 07.00. In the littermate study only, after the initial 48h baseline recording mice received an i.p. injection every other day at 18.30h (30 minutes before the dark phase). Vehicle (10% Tween 80 in 0.9% saline), *d*-amphetamine (2.5mg/kg) and RP 67580 (5mg/kg) were injected in each mouse once only, in a Latin-square design. Mice were left to settle for 30 minutes, following which the lights went off and activity was analysed overnight. Because the effects of the injection and treatment were most prominent in the 2h post-injection period (19.00-21.00h), activity during this time period was then compared across groups. Activity recordings from this 2h time period were also taken from the 48h period in which mice were un-injected and averaged over the 2 days. This was then compared to the vehicle-injected activity in order to look at the effect of injection stress on activity.

5.2.5 Genotyping

Mice bred from heterozygous matings were ear-punched at 3 weeks old and genotyped as described in section 2.2. The genotypes of some mice from homozygous matings were tested in order to check there had been no mix-ups in the housing facility.

5.2.6 Statistical analysis

Full statistical methods are given in section 2.7. The LDEB data were analysed with a 1-way or 2-way between subjects ANOVA, with '*genotype*' and '*injection*' as between-subjects factors. The locomotor activity chamber data and the total activity recorded in the LDEB and with telemetry were analysed with an independent t-test, with '*genotype*' as the between-subjects factor. 48h activity in

the light and dark phases and overnight activity post-injection was analysed with a 2-way repeated-measures ANOVA, with '*genotype*' as the between-subjects factor and '*time of day*' as the within-subjects factor. Due to the large number of data points, data from 2h time bins were analysed separately. Uninjected telemetry data were also analysed with a 2-way repeated-measures ANOVA, with '*genotype*' as the between-subjects factor and '*time of day*' as the within-subjects factor. In order to look at the effects of injection and drug, vehicle-injected activity was first compared to uninjected activity, and then compared to drug-injected activity. Because planned comparisons were made on the effects each drug would have on activity, I also compared each treatment with vehicle alone.

In all experiments using ANOVA, a significant main effect of either independent variable or a significant interaction between them led to post-hoc 1-way ANOVA followed by LSD tests or paired sample t-tests.

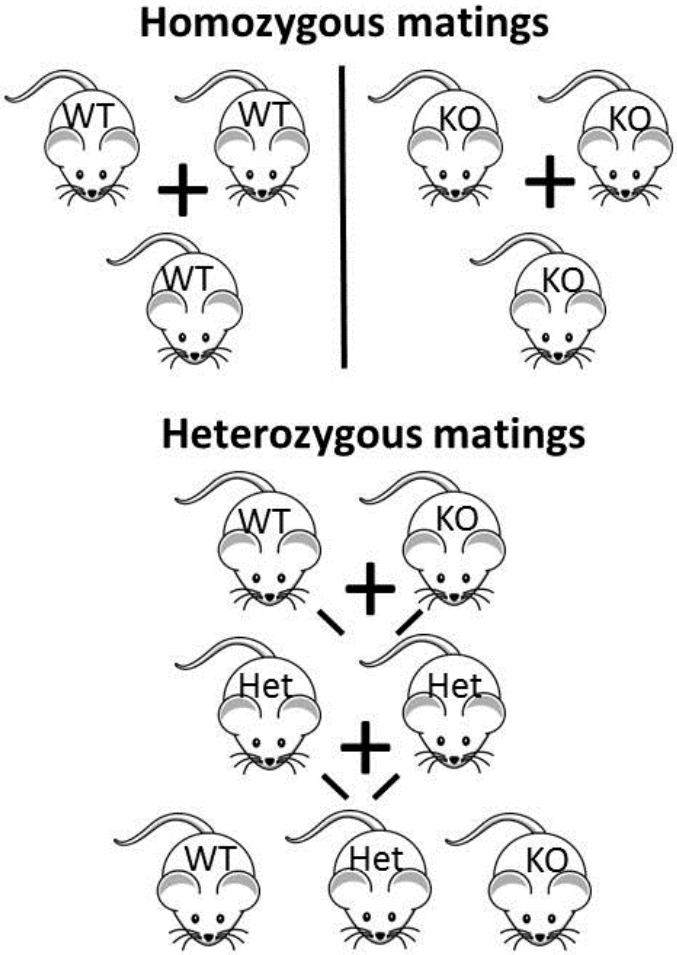

Figure 5.1 Illustration of wildtype (WT) and NK1R-/- (KO) breeding methods. Homozygous matings were obtained by breeding homozygous wildtype and NK1R-/- mice separately. Heterozygous (Het) matings were obtained by breeding homozygous wildtype and NK1R-/- with each other, then breeding the heterozygous offspring with each other.

5.3 Results

5.3.1 Vehicle-injected LDEB in offspring from homozygous matings

Activity in the light per unit of time was increased in NK1R-/- mice but this was not statistically significant (Figure 5.2A). Total activity was decreased in NK1R-/- mice compared to wildtype mice: $t(10.23) = 2.84$, $P<0.05$ (Figure 5.2B). The number of transitions to the light zone was also reduced in NK1R-/- mice compared to wildtype mice ($t(14) = 2.41$, $P<0.05$) (Figure 5.2C). NK1R-/- mice tended to spend less time in the light zone than wildtype mice, although this did not reach significance ($P=0.071$) (Figure 5.2D).

5.3.2 Un-injected LDEB in offspring from homozygous matings

Total activity was reduced in wildtype mice following vehicle injection ($F(1,12) = 6.94$, $P<0.05$; Tukey: $P<0.05$) (Figure 5.2F). Activity in the light per unit of time was not affected by vehicle injection (Figure 5.2E). In the un-injected group, NK1R-/- mice had a lower activity count and made fewer transitions to the light zone than wildtype mice (Figure 5.2G). However, the overall 2-way ANOVA was not significant so further post-hoc analyses were not performed (main effect of genotype: $P=0.072$). There was no difference in the time spent in the light zone (Figure 5.2H).

5.3.3 Activity meter in offspring from homozygous matings

There was no genotype difference in ambulatory activity in the activity meter ($t(6) = 0.329$, $P=0.754$) (Figure 5.3).

5.3.4 Telemetry

Homozygous colony

Analysis of circadian rhythms showed that NK1R-/- mice were significantly more active than wildtype mice during the first 2h of the dark phase on both days (Day 1: $F(1,4) = 8.45$, $P<0.05$; Day 2: $F(1,3) = 10.59$, $P<0.05$) (Figure 5.5). Total activity over 48h was significantly increased in NK1R-/- mice bred from homozygous matings ($t(10) = 2.62$, $P<0.05$). Activity in the dark phase was overall greater than in the light phase ($Z =2,98$, $P<0.01$). Furthermore, NK1R-/- mice were significantly more active in the dark phase ($Z=2.40$, $P<0.05$) (Figure 5.4A).

Heterozygous colony

NK1R-/- mice were significantly more active during the first 4h of the dark phase, and between 1.00 and 3.00, but only on the second night (19.00-21.00: $F(1,6) = 9.89$, $P<0.05$; 21.00-23.00: $F(1,6) = 12.42$, $P<0.05$; 1.00-3.00: $F(1,6) = 6.12$, $P<0.05$) (Figure 5.5). Total activity over 48h was not significantly different. However, there was a main effect of time ($F(1,14) = 271.27$, $P<0.001$). Mice were overall significantly more active in the dark phase ($t(15) = 12.66$, $P<0.001$). Furthermore, there was an interaction between time and genotype ($F(1,14) = 11.37$, $P<0.01$). While there was no genotype difference in the light phase, NK1R-/- mice were significantly more active in the dark phase ($F(1,14) = 8.05$, $P<0.05$) (Figure 5.4B).

Vehicle-injected NK1R-/- mice were significantly more active during the first 2h after injection ($F(1,6) = 11.21$, $P<0.05$) and between 1.00 and 3.00 ($F(1,6) = 11.43$, $P<0.05$). NK1R-/- mice were also significantly more active than wildtype mice between 23.00 and 1.00 after an injection of RP 67580 ($F(1,5) = 11.57$, $P<0.05$). There were no genotype differences following injection of *d*-amphetamine (Figure 5.6). In order to look at the effect of injection on activity, the first two hours of post-injection activity were compared to the first two hours of non-injected activity. Vehicle injection significantly reduced activity of wildtype mice ($F(1,6) = 17.24$, $P<0.01$; $t(3) = 5.54$, $P<0.05$) but not NK1R-/- mice. Furthermore, in the vehicle-injected group, NK1R-/- mice were more active than wildtype mice ($F(1,6) = 17.24$, $P<0.01$; $F(1.6) = 11.66$, $P<0.05$) (Figure 5.4C).

Overall, there was no effect of treatment on activity. However, because one mouse died before receiving RP 67580, leaving only 3 NK1R-/- mice in that group, the vehicle and *d*-amphetamine data were also analysed separately. *D*-amphetamine abolished the genotype difference in vehicle-injected wildtype and NK1R-/- mice. This was due to an increase in activity in wildtype mice (main interaction: $F(1,6) = 6.9$, $P<0.05$; $t(3) = 5.97$, $P<0.05$) (Figure 5.4C).

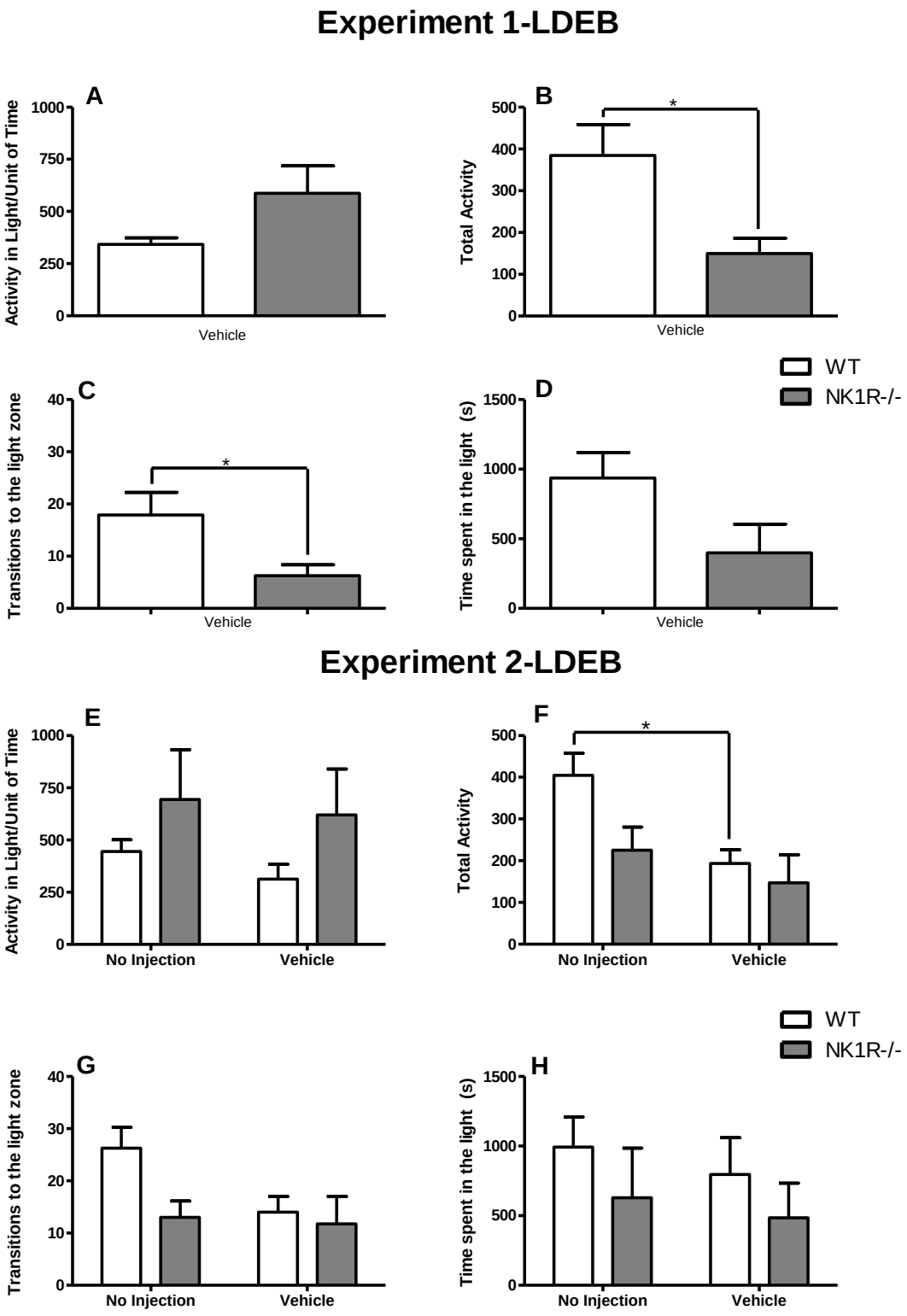

Figure 5.2 NK1R-/- mice from homozygous matings show less exploratory behaviour than wildtype (WT) mice in the LDEB. Experiment 1 (n=8): (A) *Activity in the light/unit of time*, (B) *Total Activity*, (C) *Total transitions to the light zone* and (D) *Time spent in the light zone* of wildtype (WT) and NK1R-/- mice following vehicle injection. Experiment 2 (n=4): (E) *Activity in the light/unit of time*, (F) *Total Activity*, (G) *Total transitions to the light zone* and (H) *Time spent in the light zone* of wildtype and NK1R-/- mice that were un-injected or had a vehicle injection. *P<0.05.

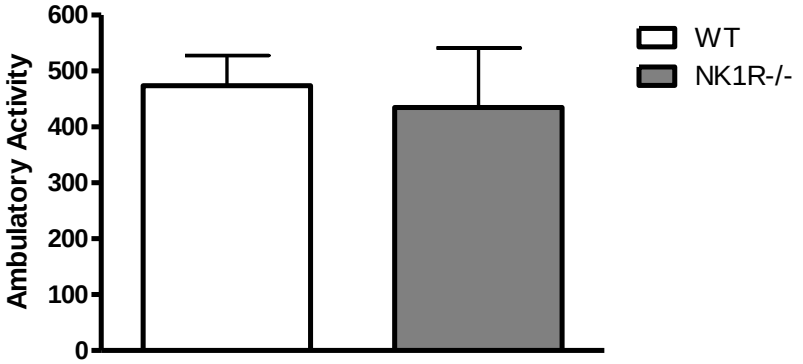

Figure 5.3 No difference in ambulatory activity measured over 10 minutes in the open field between un-injected wildtype (WT) and NK1R-/- mice from homozygous matings (n=4).

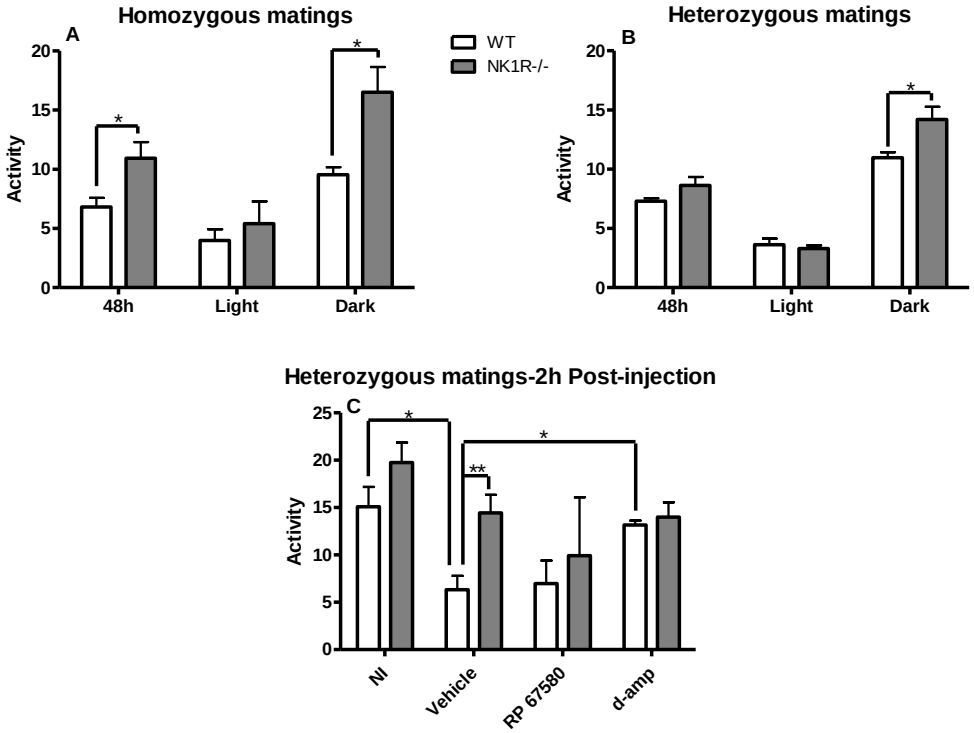

Figure 5.4 NK1R-/-mice are more active than wildtype (WT) mice in the dark phase when measured with a telemetry paradigm. NK1R-/- mice from (A) Homozygous matings (n=3) and (B) Heterozygous matings (n=4) were more active during the dark phase. (C) The effect of no injection (NI), vehicle injection, RP 67580 and *d*-amphetamine (d-amp) were compared 2h after the dark phase in mice from heterozygous matings (n=3-4). Vehicle injection reduced activity in wildtype mice, while *d*-amphetamine increased it. There were no effects on NK1R-/- mice. *P<0.05; **P<0.01.

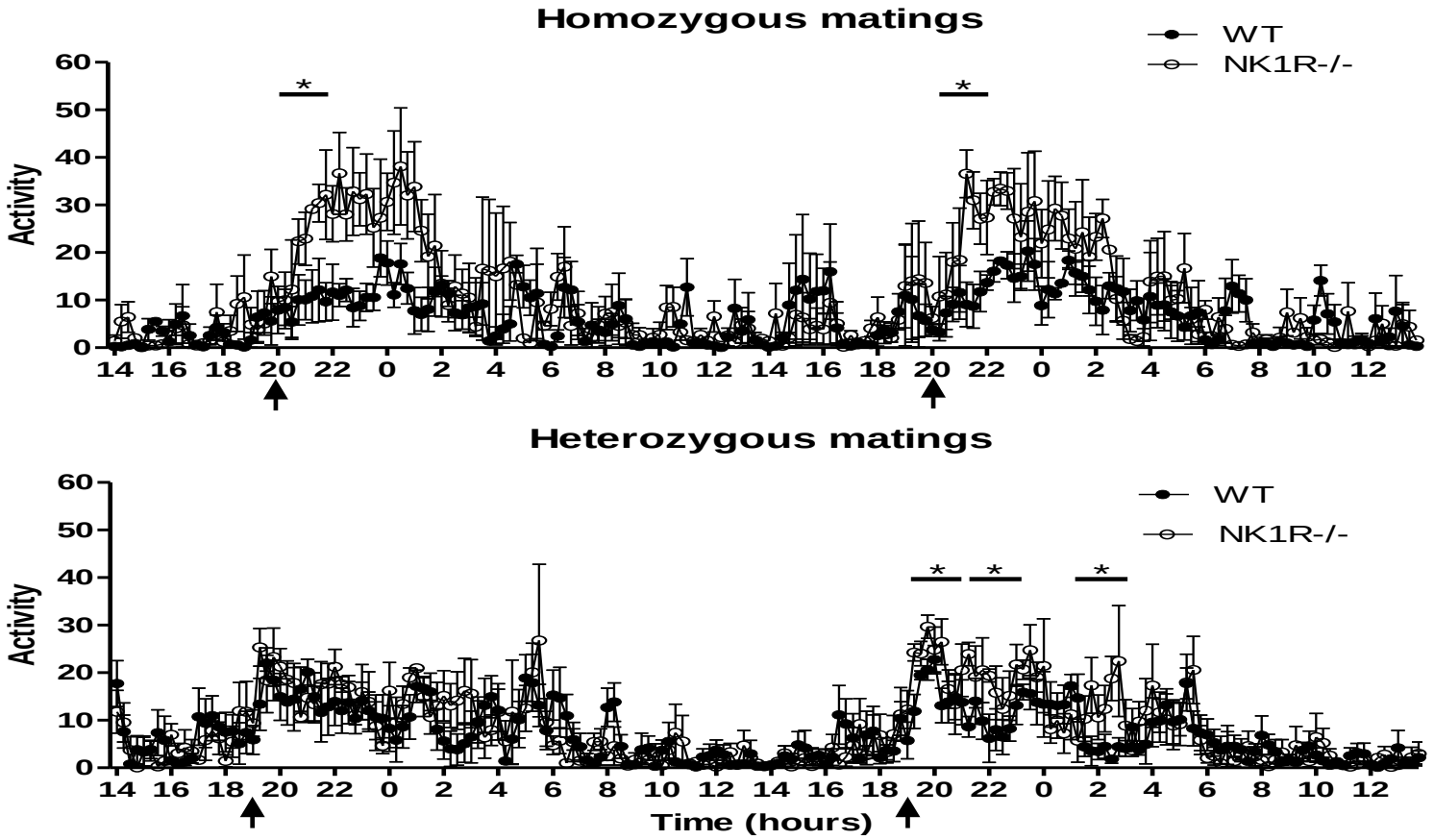

Figure 5.5 Activity of wildtype (WT) and NK1R-/- mice from homozygous and heterozygous matings over 48h. NK1R-/- mice from homozygous matings were significantly more active at the beginning of each dark phase, while NK1R-/- mice from heterozygous matings only showed an increase in activity on the second night. Black arrows indicate the start of the 12h dark phase. Black bars indicate a main effect of genotype over the 2h period (n=3-4). *P<0.05.

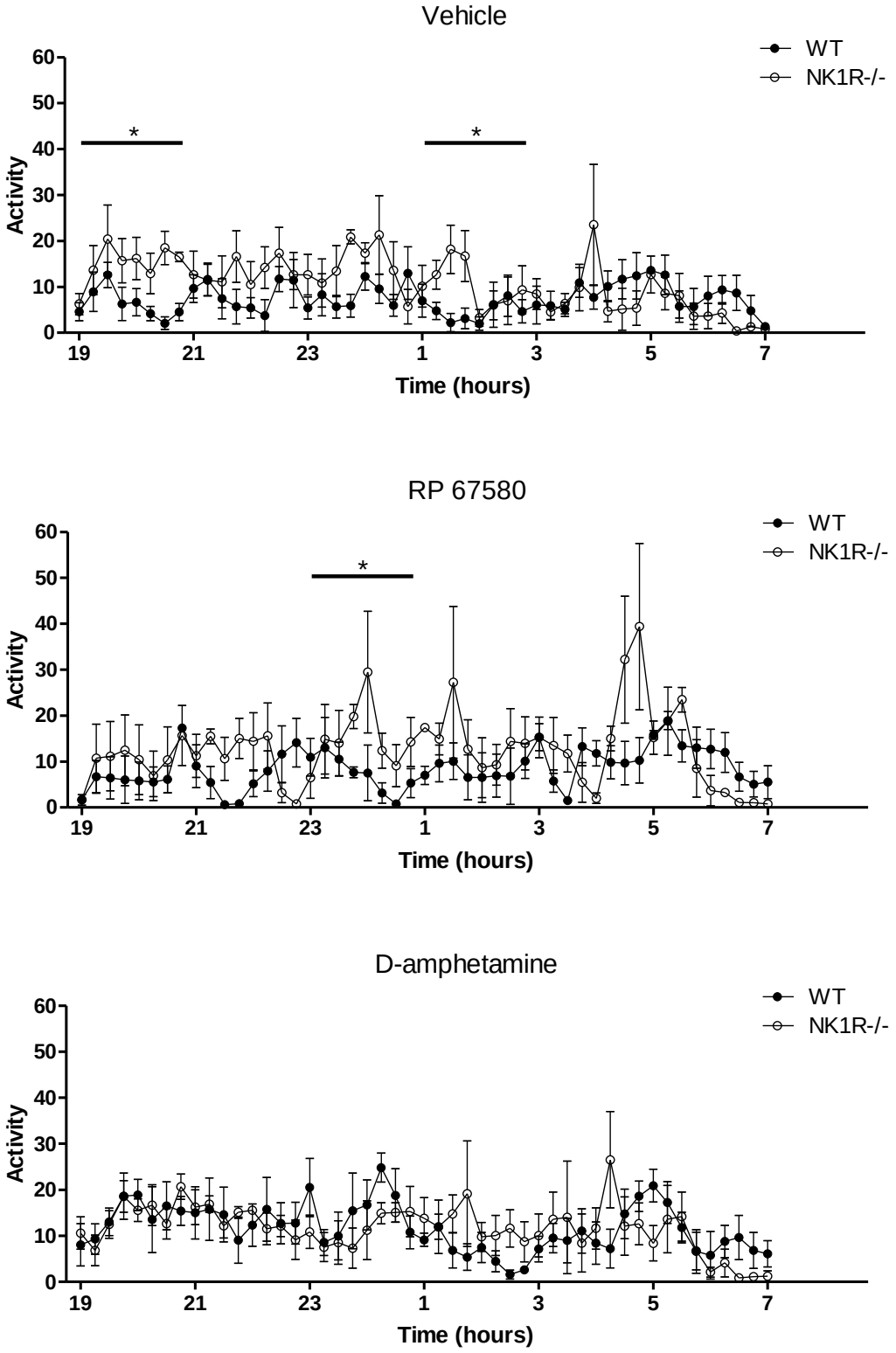

Figure 5.6 Activity of wildtype (WT) and NK1R-/- mice from heterozygous matings during the dark phase following an i.p injection of vehicle (n=4), RP 67580 (n=3) and *d*-amphetamine (n=4). Black bars indicate a main effect of genotype over the 2h period. *P<0.05.

5.4 Discussion

The aim of this chapter was to identify testing conditions that would reveal robust hyperactivity in NK1R-/- mice. This was achieved when mice were tested in a telemetry paradigm, but the difference in activity was particularly pronounced in the dark phase. NK1R-/- mice from homozygous and heterozygous parents were hyperactive, suggesting that this behavioural deficit is linked to disrupted NK1R function.

5.4.1 Activity measured in the LDEB and activity meter

In the LDEB, the activity of NK1R-/- mice tended to increase when activity was corrected for the time each mouse spent in the light zone (*activity in the light/unit of time*). This replicates the previous finding by Yan et al. (2010). However, this measure was highly variable in NK1R-/- mice and did not reach significance despite testing 8 animals in each group. This measure of activity is difficult to interpret because while it indicates that mice are moving fast, it does not mean that they have covered more distance. For example, mice could run out of the light zone within the first few seconds of the test and score highly on this measure. Herpfer et al. (2005) found an increase in *total activity* in NK1R-/- mice in the LDEB. However, here the *total activity* was decreased in NK1R-/- mice compared to wildtype mice, whether they received a vehicle injection or not. In fact, the vehicle injection only affected wildtype mice, reducing their *total activity*. This fits with previous reports that NK1R-/- mice have a blunted response to stress (De Felipe et al., 1998; Rupniak et al., 2000; Santarelli et al., 2002, 2001).

In contrast, NK1R-/- mice made fewer *transitions to the light zone* and tended to spend less *time in the light zone*. The LDEB was developed as a test of anxiety (Crawley, 1985, 1981). Anxiolytic drugs increase the *number of transitions between the light and dark zones*, and increase the *time spent in the light zone*. This would suggest that NK1R-/- mice have an enhanced, rather than blunted, response to stress. In the present experiments, the light zone was dimly light (20 lux) in order to make the zone novel but not aversive, as in Herpfer et al. (2005). However, anxiolytic drugs have not been tested under these conditions. This is necessary for the correct interpretation of NK1R-/- mouse behaviour in this paradigm. Furthermore, the use of two zones makes the interpretation of activity data

difficult. For this reason, uninjected wildtype and NK1R-/- mice were also tested in locomotor activity chambers. However, there was no difference in *ambulatory activity* between the two genotypes. This could suggest that the difference found between NK1R-/- and wildtype mice in locomotor activity chambers by Herpfer et al. (2005) was induced by vehicle injection. However, as discussed below, both uninjected and vehicle-injected NK1R-/- mice were hyperactive when measured with telemetry, indicating that an injection is not necessary to induce hyperactivity in NK1R-/- mice.

5.4.2 Telemetry

The LDEB and activity meter data presented here and in previous studies (Herpfer et al., 2005; Yan et al., 2010) was collected in the afternoon, during the animals' light phase. Testing was done at this time because 'locomotor activity of wildtype mice is low and stable' (Yan et al., 2009). However, mice are much more active in their dark phase (overnight), and it is possible that a difference in activity would be more evident and robust in this phase. This was confirmed when mice were tested with telemetry, where there was a large increase in activity in NK1R-/- mice tested in the dark phase. This increase was present in the offspring from homozygous and heterozygous breeding pairs. However, hyperactivity seems to be more pronounced in NK1R-/- mice bred from homozygous matings, because as well as a significant increase in the dark phase, the total activity was increased. Furthermore, analysis of activity over 48h showed that NK1R-/- mice from littermate pairs were only significantly more active on the second night. This could suggest that a lack of NK1R function is related to hyperactivity, but that there is something else (genetic, environmental or an interaction between the two) exacerbating the activity deficit in mice bred from homozygotes. However, in order to confirm that hyperactivity is greater in one colony, they need to be tested simultaneously.

In line with the findings from the LDEB that wildtype mice are more sensitive to injection stress, a vehicle injection reduced the activity of wildtype but not NK1R-/- mice tested with telemetry. This caused a greater difference in activity levels between the genotypes, and provides support to the previous suggestion that there was no increase in activity in NK1R-/- mice tested in the locomotor

activity chambers because the mice were not injected. Telemetry can measure more subtle changes in activity, and differences in activity in locomotor chambers may only become apparent following the stress of an injection.

Based on the finding that NK1R-/- mice are hyperactive, it was predicted that the NK1 antagonist RP 67580 would induce hyperactivity in wildtype mice. However, here this was not the case. Clearly, acute antagonism cannot replicate the effects of a life-long lack of functional NK1R. A previous study in the LDEB has reported an increase in *activity in the light zone per unit of time* in wildtype mice injected with RP 67580 from the same colony used here (Yan et al., 2010). However, another study with Swiss albino mice showed a decrease in *total line crosses in the light zone* after RP 67580 (Zernig et al., 1993). Another unexpected finding was that activity tended to decrease in NK1R-/- following RP 67580. Unfortunately, due to the death of one NK1R-/- mouse prior to being injected with RP 67580, this group had high variability. Therefore, it is not possible to determine whether this drug had any effects on NK1R-/- mice. However, based on the findings from chapter 4, if RP 67580 is affecting NK1R-/- mice, it is likely due to its L-type calcium channel blocking effects. In order to confirm the effects of RP 67580 on activity, the current telemetry study needs to be repeated in more mice.

D-amphetamine increased activity in wildtype mice but did not affect NK1R-/- mice. An increase in activity in wildtype mice following *d*-amphetamine has previously been reported in the LDEB (Yan et al., 2010). However, in the same study, activity of NK1R-/- mice was reduced by *d*-amphetamine. The reason for this discrepancy is not clear. As mentioned above, an issue with using the LDEB can be that mice spend variable amounts of time exploring the light zone, and have been habituated to the dark zone so tend not to explore it during the test period. In the study by Yan et al. (2010), the activity was presented as '*activity in the light per unit of time*'. Therefore, it is possible that *d*-amphetamine affected the time animals spent in the light zone rather than their total activity, and that there was no difference in total activity between vehicle and *d*-amphetamine-injected NK1R-/- mice, as in this study. Either way, a lack of behavioural response to *d*-amphetamine fits with the finding that there is a lack of DA response to *d*-amphetamine in the pre-frontal cortex and striatum of NK1R-/- mice (Yan et al., 2010). Also, NK1R-/-

mice show no conditioned place preference to *d*-amphetamine, indicating that the rewarding properties of *d*-amphetamine are absent in NK1R-/- mice (Murtra et al., 2000b).

5.5 Conclusions

A lack of functional NK1R causes hyperactivity in mice. Due to differential stress responses in wildtype and NK1R-/- mice, it is important to identify testing conditions that minimise stress. When tested in the home cage with telemetry, hyperactivity is present in NK1R-/- mice whether they are bred from homozygous or heterozygous parents, confirming that this deficit is due to a lack of functional NK1R rather than an interaction between early environment and behaviour. The next chapter looked at whether other behavioural and molecular deficits identified in NK1R-/- mice from homozygous breeding pairs are also present in NK1R-/- mice from heterozygous matings.

6 Increase in impulsivity and decrease in striatal cholinergic function in NK1R-/- mice bred from homozygotes

6.1 Introduction

In the previous chapter, it was shown that NK1R-/- mice bred from homozygous and heterozygous matings are hyperactive. This deficit is therefore not dependent on differences in early environment, and is likely caused by a lack of functional NK1R. In order to confirm that this is also the case for the deficits in *impulsivity*, *attention* and *perseveration*, both colonies were compared in the 5-CSRTT. Furthermore, preliminary data have suggested that NK1R-/- mice bred from homozygotes have decreased choline acetyltransferase (ChAT)-positive cells in the striatum (Slone-Murphy, 2011). This chapter aimed to further characterise this reduction in cholinergic function, and determine whether it is also present in mice bred from heterozygote breeding pairs.

6.1.1 Medium spiny neurons

Over 95% of striatal neurons are medium spiny neurons (MSNs). MSNs are the striatal output projection neurons and are inhibitory, using GABA as their main neurotransmitter. MSN output can be divided into two different pathways: the direct pathway, projecting to the internal part of the globus pallidus and the substantia nigra pars reticular, and the indirect pathway, projecting to the external part of the globus pallidus and the subthalamic nucleus (Bolam et al., 2000). The direct pathway disinhibits the basal ganglia, thereby facilitating movement, while the indirect pathway plays an opposite role. The striatum is densely innervated by DA, and DA receptor expression in this region is the highest in the brain (Missale et al., 1998). D1 DA receptors are located on striatonigral MSNs in the direct pathway, while D2 DA receptors are located on striatopallidal MSNs in the indirect pathway (Gerfen, 1992). Therefore, DA activation of these receptors can release or suppress motor movements.

6.1.2 Cholinergic interneurons

As well as expressing high levels of DA, the striatum is densely innervated by cholinergic interneurons, which are the only source of striatal acetylcholine. Despite only constituting 2% of striatal neurons, they have large, dense axonal arbors which, like dopaminergic arbors, are denser in the striatum than in any other brain region (Zhou et al., 2003). These interneurons are tonically active neurons (TANs), meaning that they fire spontaneously. Their firing can also be modulated by DA. D1 and D2 receptors are located on striatal cholinergic interneurons (Le Moine et al., 1991, 1990). Activation of D1 receptors facilitates acetylcholine release (Acquas and Di Chiara, 1999), while activation of D2 receptors inhibits its release (Bertorelli and Consolo, 1990; DeBoer et al., 1996).

Acetylcholine can also exert control over DA. Acetylcholine in the striatum acts on two different types of receptor: muscarinic acetylcholine receptors (mAChR) and nicotinic acetylcholine receptors (nAChR). MSNs express M1 and M4 mAChR subtypes (Ince et al., 1997; Levey et al., 1991), with M1 receptors mainly colocalised with D2-expressing MSNs and M4 mainly colocalised with D1-expressing MSNs. This suggests that acetylcholine can modulate the direct and indirect pathways (Zhou et al., 2003). The other type of ACh receptor, nAChRs, are located on dopaminergic axon terminals, and their activation increases tonic release of DA, while reducing phasic release (Rice and Cragg, 2004). Furthermore, activation of nAChRs is modulated by mACh autoreceptors located on cholinergic interneurons. Activation of these autoreceptors reduces acetylcholine release, thus modifying nAChR activity (Threlfell et al., 2010). DA and acetylcholine therefore have a strong reciprocal relationship within the striatum, allowing great control over striatum-mediated behaviours.

6.1.3 Cholinergic hypothesis of ADHD

Much of the research into the pathophysiology of ADHD continues to focus on the dopaminergic hypothesis (Genro et al., 2010), which states that a deficit in DA within fronto-striatal circuits causes symptoms of ADHD (Levy, 1991). Indeed, the cortex and striatum are rich in DA, and these structures are thought to play an important part in the pathophysiology of ADHD. Also, psychostimulants acting on the DA system are the most prescribed treatments for ADHD, and the majority of

animal models of ADHD have some degree of DA dysfunction (Van der Kooij and Glennon, 2007). However, the evidence described above that DA and acetylcholine interact within the striatum suggests acetylcholine could also play an important role in ADHD.

Cholinergic dysfunction has long been linked to deficits in learning and memory (Everitt and Robbins, 1997). For example, it is thought to underlie the cognitive deficits seen in Alzheimer's disease. Indeed, a decrease in cholinergic markers positively correlates with the extent of cognitive damage (Perry et al., 1978). Rodent and human studies have also indicated that acetylcholine plays an important role in the control of attention (Klinkenberg et al., 2011). A link between ADHD and acetylcholine was first suggested due to the fact that smoking is highly comorbid with ADHD. As mentioned above, activation of nAChRs increases DA release, and this is normally regulated by cholinergic interneurons (Threlfell et al., 2010) . The high incidence of smoking in patients with ADHD indicates that there may be a dysregulation of acetylcholine binding to nAChRs that patients are trying to self-medicate. In line with this, it has been shown that nicotine improves symptoms of ADHD in smokers and non-smokers (Levin et al., 1996), as do other nicotinic agonists (Wilens et al., 1999). Donepezil, an acetylcholinesterase inhibitor, also improves symptoms of ADHD (Wilens et al., 2000) and a link between choline transporter polymorphisms and ADHD has been found (English et al., 2009). This evidence suggests that cholinergic dysfunction may underlie the pathophysiology of ADHD.

6.1.4 Role of acetylcholine in the 5-CSRTT

IgG-saporin lesions to the nucleus basalis magnocellularis (a cholinergic structure in the basal forebrain) reduce acetylcholine efflux in the medial prefrontal cortex, and this reduction positively correlates with decreased *accuracy* in the 5-CSRTT (McGaughy et al., 2002). Another study found that cholinergic lesions to the basal forebrain decrease response *accuracy*, while increasing *premature* and *perseverative responses* (Muir et al., 1994). Both mAChRs and nAChRs have been shown to modulate attentional functional in the 5-CSRTT. The muscarinic receptor antagonist scopolamine causes a decrease in *accuracy* in the 5-CSRTT in mice (Humby et al., 1999) and rats (Stolerman et al., 2000). Spontaneously Hypertensive

Rats (SHRs), one of the most widely studied models of ADHD, have a reduced number of nAChRs (Gattu et al., 1997a, 1997b; Terry et al., 2000), and show deficits in *accuracy* in the 5-CSRTT. These deficits are improved by increasing acetylcholine release, suggesting that disrupted cholinergic transmission underlies deficits in selective attention (De Bruin et al., 2003). Furthermore, nicotine improves *accuracy* in the 5-CSRTT in normal rats (Stolerman et al., 2000), and reverses a deficit in *accuracy* following lesions to the basal forebrain (Muir et al., 1995).

However, while nicotine improves attentional deficits, studies have shown that it worsens impulsivity (Blondel et al., 2000; Grottick and Higgins, 2000; Mirza and Stolerman, 1998). An increase in *premature responses* can be improved by the nicotinic antagonist mecamylamine, although attention is simultaneously worsened (Grottick and Higgins, 2000). This suggests that attention and impulsivity are differentially regulated by acetylcholine. In contrast, the mAChR antagonist scopolamine increases *premature responses* when attentional demands are high (Jones and Higgins, 1995). Furthermore, using a touch screen version of the 5-CSRTT, it has recently been shown that M1 receptor knockout mice have deficits in impulsivity and perseverative responses but no attentional problems (Bartko et al., 2011). This indicates an important role for muscarinic receptors in impulse control.

6.1.5 Cholinergic interneurons and NK1R

In the rat (Gerfen, 1991) and human brain (Aubry et al., 1994), all cholinergic interneurons express NK1R, and substance P binding to these NK1R triggers acetylcholine release (Anderson et al., 1994; Kemel et al., 2002). Therefore, cholinergic activity in the striatum of NK1R-/- mice warrants further investigation. One way of measuring acetylcholine activity is to label choline acetyltransferase (ChAT) positive cells. ChAT is only found in cholinergic neurons and is involved in the synthesis of acetylcholine. Preliminary data revealed that NK1R-/- mice bred from homozygotes have fewer ChAT-positive cells in the dorsal striatum than wildtype mice (Slone-Murphy, 2011). This suggests that NK1R-/- mice have reduced cholinergic function. However, because initial data were only collected from mice bred from homozygotes, it is not clear whether the loss of ChAT activity

is due to a lack of functional NK1R or to early environmental effects on development. A lack of maternal care has been shown to induce apoptosis in rodents in other brain areas, such as the cerebral cortex and hippocampus, even if pups are only separated from their mother for 24h (Zhang et al., 2002). Although it is not currently known if NK1R-/- mice show reduced maternal care, it is possible that the care provided by parents with behavioural deficits would not be as effective, and cause the loss of ChAT-positive cells. Therefore, in this chapter, ChAT expression of mice from homozygous and heterozygous matings was measured. Also, it is not clear whether this loss of ChAT-positive cells increases with age. An age-related decline in cholinergic function has been found in patients with Alzheimer's disease, and is thought to correlate with their cognitive symptoms (Bartus et al., 1982). In order to determine whether the loss of ChAT-positive cells increases with age, 8-12 week and 9 month old mice were compared.

6.1.6 Dorsal and ventral striatum

The striatum has traditionally been divided into two main regions: the dorsal, comprising the caudate nucleus and the putamen and the ventral, comprising the nucleus accumbens and olfactory tubercle. The ventral striatum is involved in conditioned reinforcement to rewarding stimuli, while the dorsal striatum is involved in action-planning and habitual behaviour (Everitt and Robbins, 2005). However, a more global view of the striatum has also been adopted, suggesting that the striatum encodes and drives responses to salient events (Iversen and Iversen, 2007; Voorn et al., 2004). DA and acetylcholine within the striatum play an important role in these behaviours. Dopaminergic neurons fire at high frequency when encoding information about the prediction and receipt of reward-related and salient stimuli (Matsumoto and Hikosaka, 2009; Schultz, 2002). In contrast, cholinergic interneurons pause in response to salient stimuli (Aosaki et al., 1994; Morris et al., 2004).

Behaviour in the 5-CSRTT is modulated by different regions of the striatum. The dorsal striatum plays an important role in 5-CSRTT performance, with lesions to this area inducing large deficits in performance (Rogers et al., 2001). However, the extent of the effects depends on the location of the lesion. It has been proposed that the dorsal striatum can be divided into two sub-regions: the medial area,

which is involved in goal-directed behaviour (Yin et al., 2005), and the lateral area, which mediates habitual behaviours (Yin et al., 2004). Dorsolateral lesions greatly impaired 5-CSRTT performance, with rats failing to respond to over 90% of the visual stimuli. Because these lesions were made post-training, and there was no disruption of locomotor or consummatory behaviour, it has been proposed that performance deficits were due to a disruption of stimulus-response habits (Robbins, 2002; Rogers et al., 2001). In contrast, rats were able to perform the task after dorsomedial lesions, although they exhibited deficits in *accuracy*, *premature responding*, *perseveration* and a slowing of *response latencies* (Rogers et al., 2001). Likewise, sub-regions in the ventral striatum are differentially involved in 5-CSRTT performance: while DA antagonism in the shell of the nucleus accumbens increases *premature responses*, these are decreased by DA antagonism in the core region (Besson et al., 2010).

6.1.7 Aims

The aim of this chapter was to determine whether there was a decrease in ChAT expression in NK1R-/- mice bred from homozygous and heterozygous breeding pairs. It also aimed to see whether 5-CSRTT deficits are present to the same extent in NK1R-/- mice bred from both colonies, and whether a decrease in ChAT expression is greater in these mice, due to their older age. The 5-CSRTT data should be regarded as preliminary because of the small group sizes. However this research is continuing and in due course the present results will be included as part of a larger study (Grimme, Dudley, Pillidge, Hunt and Stanford, research in progress).

Initial worked confirmed that cholinergic interneurons express NK1R in the mouse striatum. Sub-regions of the dorsal and ventral striatum were analysed separately due to their different involvement in 5-CSRTT performance (Robbins, 2002). In order to control for the possibility that a decrease in ChAT expression was a consequence of general cell loss, the brains were also stained with parvalbumin because parvalbumin-positive cells in the striatum do not express NK1R (Kaneko et al., 1993). The areas of the dorsal striatal sections counted were also measured, to check whether cell loss was due to a smaller striatal area. Finally, overall expression of ChAT in the dorsal striatum was analysed with western blots.

6.2 Methods

6.2.1 Mice

As in the previous chapter, all mice were derived from the same background strain (129/Sv x C57BL/6 crossed with an MF1 strain), but half came from homozygous matings and the other half from heterozygous matings (see figure Figure 5.1). Full details of breeding and housing are described in section 2.1.

6.2.2 Genotyping

All mice bred from heterozygous matings were ear-punched and genotyped at 3 weeks of age, as described in section 2.2. The genotypes of the mice in homozygous litters were also checked, although this was only done in some offspring to ensure there had been no mix-up in the housing facility (which was unlikely due to the segregation of the coat colours in each genotype).

6.2.3 5-CSRTT

The apparatus and full training regime are described in detail in section 2.4.1. 6 wildtype and 6 NK1R-/- mice from homozygous and heterozygous breeding pairs were trained and then tested with a long and a variable inter-trial interval (LITI, 7s and VITI, 2, 5, 10 and 15s), once only. The order of these tests was randomized across groups and performed once weekly, on a Friday, if baseline performance was stable for 3 days prior to testing. 1 wildtype and 1 NK1R-/- mouse from heterozygous matings did not reach training criteria and were excluded from the experiment.

6.2.4 Body weights

In order to check whether any differences in 5-CSRTT performance between the two colonies could be due to a difference in body weight, the weights of a separate group of 6-10 week old wildtype and NK1R-/- mice from homozygous and heterozygous breeding pairs were compared (n=6).

6.2.5 Immunohistochemistry

In order to confirm co-localisation of ChAT and NK1R, a double fluorescent stain was carried out in a wildtype mouse. The dorsal striata of 5 wildtype and 5 NK1R-/- mice aged 8-12 weeks from homozygote breeding pairs were stained with

ChAT and parvalbumin in order to replicate the previous finding that there is a decrease in ChAT in the dorsal striatum of NK1R-/- mice (Slone-Murphy, 2011). Next, the dorsal striata of 5 wildtype and 5 NK1R-/- from heterozygote breeding pairs aged 8-12 weeks were stained with ChAT and parvalbumin. After completing the LITI and VITI, as described in section 6.2.3, all mice were used in an experiment looking at the effects of guanfacine on performance of wildtype and NK1R-/- mice in the 5-CSRTT. They had therefore all received 3 i.p. injections of guanfacine (0.1, 0.3 and 1 mg/kg), as well as a vehicle injection of 0.9% saline. On completion of this experiment, when they were 9 months old, all mice were killed and their brains were stained for ChAT and parvalbumin.

Full details of the immunohistochemistry protocol are given in section 2.5. This is briefly summarised below.

Tissue preparation

Mice were killed by transcardial perfusion (see section 2.3.1). Brain sections were cut coronally at 40μm and every third section was stained (120 μm apart). This maximised the number of sections available for counting, while ensuring that cholinergic interneurons were not double-counted.

3,3'-Diaminobenzidine (DAB) immunohistochemistry

Sections were placed in a blocking solution for 1h with hydrogen peroxide to eliminate any endogenous peroxidase activity. They were then incubated overnight at room temperature with the primary antibody (Table 6.1). The next day, sections were washed in 0.1M PB and placed in the secondary antibody for 2h (Table 6.1), before being washed again. In order to improve detection, Avidin-Biotin Complex (ABC, Vectastain kit, Vector labs) was made and added to the sections for 1h. Finally, sections were washed again, staining was visualised and sections were mounted.

Fluorescent immunohistochemistry

Sections were placed in a blocking solution for 1h and incubated in the primary antibody (Table 6.1) for 3 nights at 4 degrees. Following this, sections were incubated for 90 minutes in the secondary antibody (Table 6.1). Then, sections

were incubated with the ABC solution (Vectastain kit, Vector labs) for 30 minutes. Biotinylated tyramide solution (PerkinElmer, MA, USA) was added to the sections for 7 minutes, before being placed in the dark in FITC-conjugated avidin (Vector, Burlingame, USA) for 2 hours. Sections were washed in 0.1M PB between each step. For double-labelled fluorescence, sections were subsequently placed in the second primary antibody (Table 6.2) overnight at room temperature then incubated in the secondary antibody for 2 hours (Table 6.2). Finally, sections were washed and mounted.

6.2.6 Cell counting

Sections were counted on a Nikon Eclipse E800 microscope. Dorsal striatal sections were taken between 0.4 and 0.1 mm rostral of bregma, and nucleus accumbens sections were taken between 1.5 and 1.2 mm rostral of bregma. For dorsal counts, the contour of each striatal area was drawn with a camera lucida, and the dorsal striatum was divided into 4 areas (ventrolateral; ventromedial; dorsolateral; dorsomedial). This was done by drawing 2 lines roughly parallel to the tops and sides of each striatal drawing (Figure 6.1A). For nucleus accumbens counts, the contour was also drawn with a camera lucida, and subsequently the core and shell were outlined with the help of a mouse brain atlas (Franklin and Paxinos, 1997) (Figure 6.1B). At least 2 sections were counted per animal, bilaterally, meaning that there were at least 4 counts. These counts were averaged for each animal, and the means were used for statistical analysis.

6.2.7 Area measurements

In order to compare the striatal areas of wildtype and NK1R-/- mice from both colonies, a picture of each counted section was taken with a Nikon E4500 camera attached to a Nikon Eclipse E800 microscope. Using ImageJ, a scale bar was set and the circumference of each striatum was drawn, from which an area could be automatically calculated.

6.2.8 Western blots

In order to measure overall ChAT expression in the dorsal striatum, western blots were performed on tissue from 8-12 week old wildtype and NK1R-/- mice from homozygous and heterozygous matings (n=5). Tissue preparation, protein

extraction and immunoblotting were carried out as described in section 2.6. Data were normalised with GAPDH (Santa Cruz, USA). Details of ChAT and GAPDH antibodies are provided in Table 6.3. The molecular weight of ChAT was 70/74 kDA and for GAPDH it was 37 kDA.

6.2.9 Statistical analysis

Full details of statistical analysis are given in section 2.7. 5-CSRTT data for the number of trials to reach training baseline, the LITI and the VITI were analysed separately, with a 2-way between-subjects ANOVA. The between subjects factors were '*genotype*' and '*colony*'. The behaviours scored were identical to those in chapters 3 and 4 and are described in full in section 2.4.1.3. In addition, the training performance of wildtype and NK1R-/- mice was compared with a 2-way repeated measures ANOVA, with '*stage*' as the within-subjects factor and '*group*' (wildtype homozygous, NK1R-/- homozygous, wildtype heterozygous, NK1R-/- heterozygous) as the between-subjects variable. The VITI data from each time bin (2s, 5s, 10s, 15s) was also analysed separately. However, the pattern of results did not differ from the overall results, so the data are not presented. Body weight data was compared with a 2-way between subject ANOVA, with '*genotype*' and '*colony*' as the between subjects factors. Cell counts from each colony and age group were analysed with a 2-way within-subjects ANOVA, with '*genotype*' as the between-subjects factor and region (either *ventromedial / ventrolateral / dorsomedial / dorsolateral* or *core / shell*) as the within-subjects factor. A main effect or interaction between the factors lead to post-hoc one-way ANOVAs with LSD post-hoc tests. Total striatal cell counts and area measurements were analysed with an independent-samples t-test, with '*genotype*' as the between-subjects factor. Western blots were analysed separately for each colony, in order to normalise the NK1R-/- mouse data to their respective wildtype controls. A ratio of the band density of ChAT to GAPDH was calculated and expressed as a percentage of mean wildtype control. These percentages were analysed with an independent samples t-test.

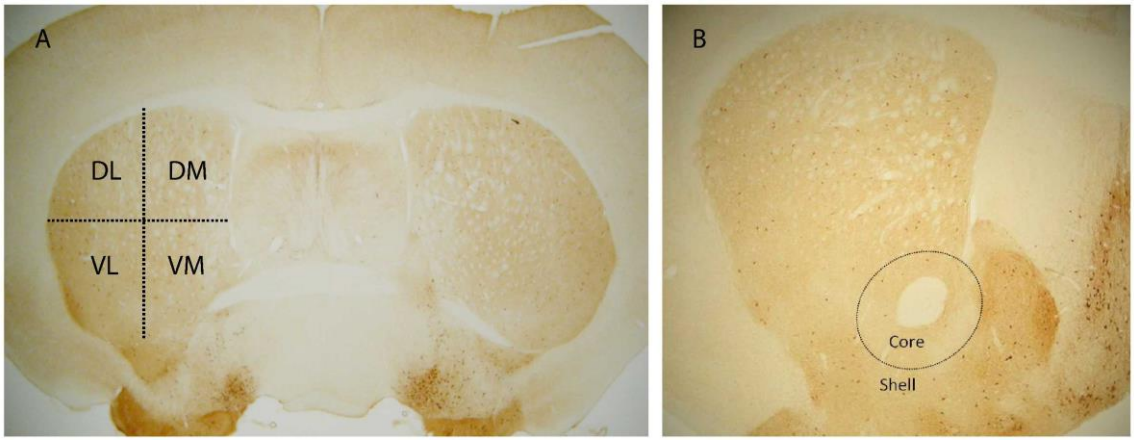

Figure 6.1 Division of the striatum for counting. Representation of the subregions of the (A) dorsal and (B) ventral striatum that were quantified. DL: Dorsolateral; DM: Dorsomedial; VL: Ventrolateral; VM: Ventromedial.

Table 6.1 Antibodies for single antigen immunohistochemistry. ChAT: Choline acetyltransferase; PV: Parvalbumin; NK1R: Neurokinin-1 receptor. Secondary antibodies were diluted 1:400. DAB: 3,3'-Diaminobenzidine; FITC: fluorescein isothiocyanate.

Antigen	Primary Antibody		Secondary Antibody	
	Antibody	Dilution	Antibody	Visualisation
ChAT	Goat α-ChAT (Millipore)	1:100	Biotinylated rabbit α-goat	DAB
PV	Rabbit α-PV (Synaptic Systems)	1:20 000	Biotinylated goat α-rabbit	DAB
NK1R	Rabbit α-NK1R (Millipore)	1:10 000	Biotinylated goat α-rabbit	FITC

Table 6.2 Antibodies for double antigen labeling. NK1R: Neurokinin-1; ChAT: Choline acetyltransferase; TSA: Tyramide Signal Amplification. The biotinylated secondary antibody was diluted 1:400 and the Alexa Fluor secondary antibody was diluted 1:500.

Antigen	TSA		Direct	
	Primary antibody	Secondary antibody	Primary antibody	Secondary antibody
NK1R + ChAT	Rabbit α-NK1R 1:10 000 (Millipore)	Biotinylated Horse α-rabbit	Goat α-ChAT 1:100 (Millipore)	Alexa Fluor 594 Chicken α-goat

Table 6.3 Antibodies for immunoblotting. ChAT: choline acetyltransferase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase. HRP: horseradish peroxidase.

Antigen	Primary Antibody		Secondary Antibody	
	Antibody	Dilution	Antibody	Dilution
ChAT	Goat α-ChAT (Millipore)	1:500	HRP-conjgated rabbit α-goat	1:1 000
GAPDH	Mouse α-GAPDH (Santa Cruz)	1:1 000	HRP-conjugated goat α-mouse	1:1 000

6.3 Results

6.3.1 5-CSRTT

6.3.1.1 Number of trials to reach baseline

There was no genotype difference ($F(1,18) = 0.978$, $P=0.336$), no colony difference ($F(1,18) = 1.104$, $P=0.307$) and no interaction ($F(1,18) = 1.344$, $P=0.262$). In the homozygous colony, wildtype mice took an average of 53 trials and NK1R-/- mice took an average of 37.8 trials to complete training. In the heterozygous colony, wildtype mice took an average of 37.4 trials and NK1R-/- mice took an average of 38.6 trials (Figure 6.2). As training was carried out 5 days a week, the mice were between 3-5 months when they were first tested in the 5-CSRTT.

Figure 6.2 Total number of trials to reach baseline in the 5-CSRTT in wildtype (WT) and NK1R-/- mice from homozygous and heterozygous breeding pairs.

6.3.1.2 Training

There was a main effect of group for perseverative responses ($F(3,17) = 5.50$, $P<0.01$). There were differences in stages 1 and 3, as summarised in (Table 6.4). There were no other differences in training performance.

Table 6.4 Differences in perseverative responses between wildtype and NK1R-/- mice from homozygous and heterozygous breeding pairs during 5-CSRTT training. F values represent the One-way ANOVA for the corresponding stage. P values represent significance in post-hoc LSD tests. WT: wildtype; hom: offspring from homozygous parents; het: offspring from heterozygous parents.

Stage 1	NK1R-/-(hom)>WT(hom), P<0.05
F(3,20) = 4.09, P<0.05	NK1R-/-(het)>WT(het), P<0.05
Stage 3	NK1R-/-(hom)>WT(hom), P<0.05
F(3,20) = 1.17, P<0.05	

6.3.1.3 LITI

There was a main effect of colony for the latency to correct response (F(1,17) = 5.52, P<0.05). Overall, mice bred from heterozygotes were quicker to respond than mice bred from homozygotes (F(1,19) = 6.01, P<0.05) (Figure 6.3). There were no other differences between genotype or colony (Table 6.5).

Table 6.5 Statistical values for the behaviours that did not differ between genotype or colony for wildtype and NK1R-/- mice tested in the 5-CSRTT with a LITI.

	Effect of genotype	Effect of colony	Interaction
%Accuracy	F(1,17) = 0.02, P=0.90	F(1,17) = 0.61, P=0.45	F(1,17) = 1.53, P=0.23
Latency to collect the reward	F(1,17) = 0.13, P=0.72	F(1,17) = 0.33, P=0.58	F(1,17) = 1.09, P=0.31
%Omissions	F(1,17) = 0.01, P=0.94	F(1,17) = 1.22, P=0.28	F(1,17) = 0.71, P=0.41
Perseverative responses	F(1,17) = 0.71, P=0.41	F(1,17) = 0.05, P=0.83	F(1,17) = 0.39, P=0.54
%Premature responses	F(1,17) = 0.01, P=0.93	F(1,17) = 2.53, P=0.13	F(1,17) = 1.64, P=0.22
Total number of trials	F(1,17) = 0.01, P=0.95	F(1,17) = 1.73, P=0.21	F(1,17) = 0.01, P=0.95

6.3.1.4 VITI

%Premature responses

There was a main interaction between genotype and colony (F(1,18) = 22.81, P<0.001). Post-hoc LSD tests revealed that NK1R-/- mice bred from homozygotes were significantly *more* impulsive than wildtype mice (P=0.001). In contrast,

NK1R-/- mice from heterozygotes were significantly *less* impulsive than wildtype mice (P=0.01). Finally, wildtype mice from homozygotes were significantly *less* impulsive than wildtypes from heterozygotes (P<0.001). The difference between NK1R-/- from each colony just missed criterion for significance (P=0.051) (Figure 6.4A).

Perseverative responding

There was a main effect of genotype ($F(1,18) = 4.72$, $P<0.05$), but no effect of colony or interaction (Figure 6.4C). Therefore, the 2 colonies were merged. NK1R-/- mice from both colonies made consistently more perseverative responses than wildtype mice ($F(1,20) = 4.90$, $P<0.05$) (Figure 6.4D).

%Accuracy

There was an interaction between genotype and colony $F(1,18) = 5.06$, $P<0.05$. However, post-hoc analyses highlighted no further differences (Figure 6.4B).

Latency to correct response, latency to collect the reward, %Omissions and total number of trials

There were no statistical differences for any of these behaviours (Table 6.6)

Table 6.6 Statistical values for the behaviours that did not differ between genotype or colony for wildtype and NK1R-/- mice tested in the 5-CSRTT with a VITI.

	Effect of genotype	Effect of colony	Interaction
Latency to correct response	$F(1,18) = 0.59$, P=0.45	$F(1,18) = 0.42$, P=0.52	$F(1,18) = 0.15$, P=0.70
Latency to collect the reward	$F(1,18) = 2.07$, P=0.17	$F(1,18) = 2.44$, P=0.14	$F(1,18) = 0.24$, P=0.63
%Omissions	$F(1,18) = 0.22$, P=0.12	$F(1,18) = 0.77$, P=0.39	$F(1,18) = 0.15$, P=0.70
Total number of trials	$F(1,18) = 1.98$, P=0.18	$F(1,18) = 0.001$, P=0.98	$F(1,18) = 0.001$, P=0.98

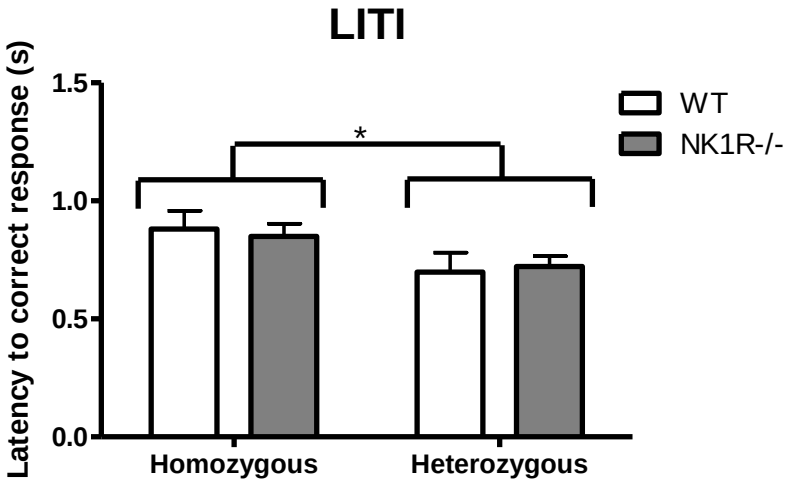

Figure 6.3 Latency to correct response of wildtype (WT) and NK1R-/- mice tested in the 5-CSRTT with a LITI (n=5-6). Overall, mice bred from homozygotes were slower to make a response than mice bred from heterozygotes. *P<0.05

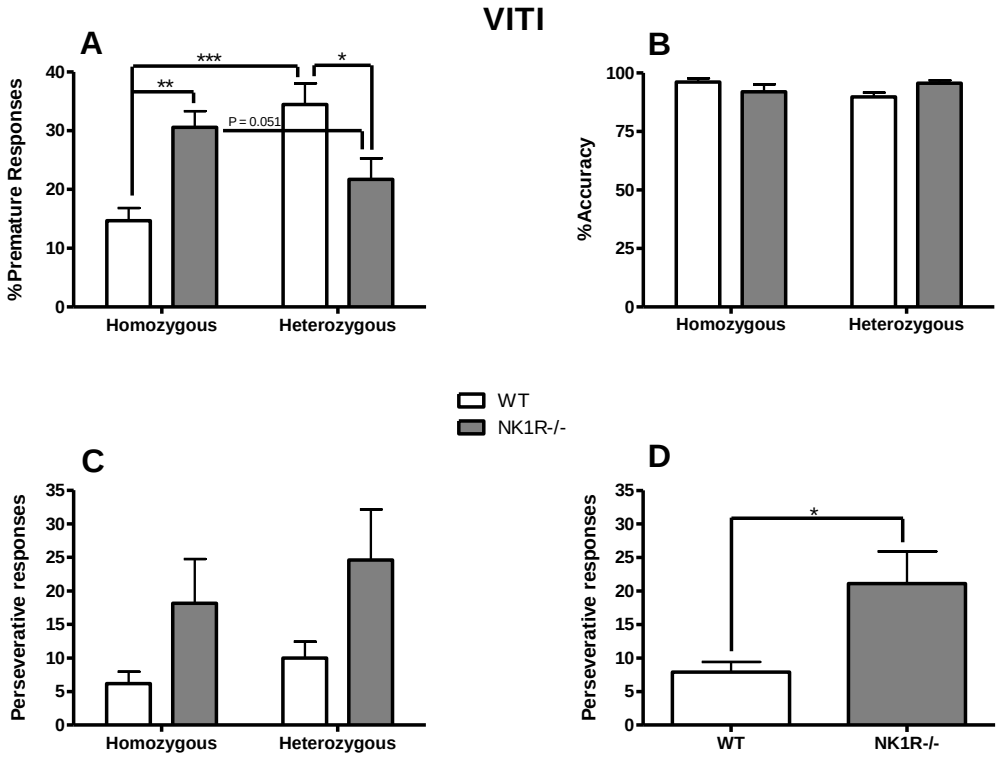

Figure 6.4 Behaviour of wildtype (WT) and NK1R-/- mice tested in the 5-CSRTT with a VITI. (A) *%Premature responses*, (B) *%Accuracy*, (C) *Perseverative responses* of wildtype and NK1R-/- mice from homozygous and heterozygous matings in the VITI. (D) *Perseverative responses* of wildtype and NK1R-/- mice regardless of colony. n=5-6 per group. *P<0.05; **P<0.01; ***P<0.001.

6.3.2 Body weights

In mice from homozygous breeding pairs, wildtype mice weighed 35.6±1.5g and NK1R-/- mice weighed 30.5±0.7g. In mice from heterozygous breeding pairs, wildtype mice weighed 34.1±2.8g and NK1R-/- mice weighed 33.8±1.9g. Although NK1R-/- mice from homozygous breeding pairs weighed less than the other groups of mice, there were no overall significant main effects (Effect of genotype: $F(1,18)=2.33$, $P=0.14$; Effect of colony: $F(1,18) = 0.28$, $P=0.60$; Interaction: $F(1,18) = 1.87$, $P=0.19$) (Figure 6.5).

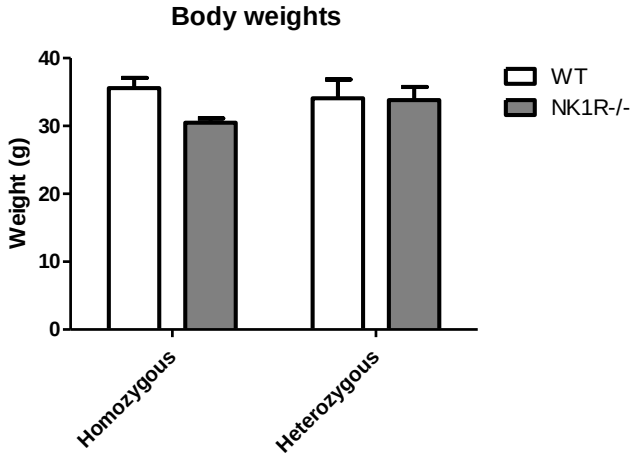

Figure 6.5 Body weights of 6-10 week old wildtype (WT) and NK1R-/- mice from homozygous and heterozygous breeding pairs (n=6).

6.3.3 Immunohistochemistry

An immunohistochemical stain of wildtype and NK1R-/- mice from both colonies confirmed the loss of NK1R in NK1R-/- mice (Figure 6.6). A double fluorescent stain in the striatum of a wildtype mouse confirmed the co-localisation of ChAT and NK1R (Figure 6.7).

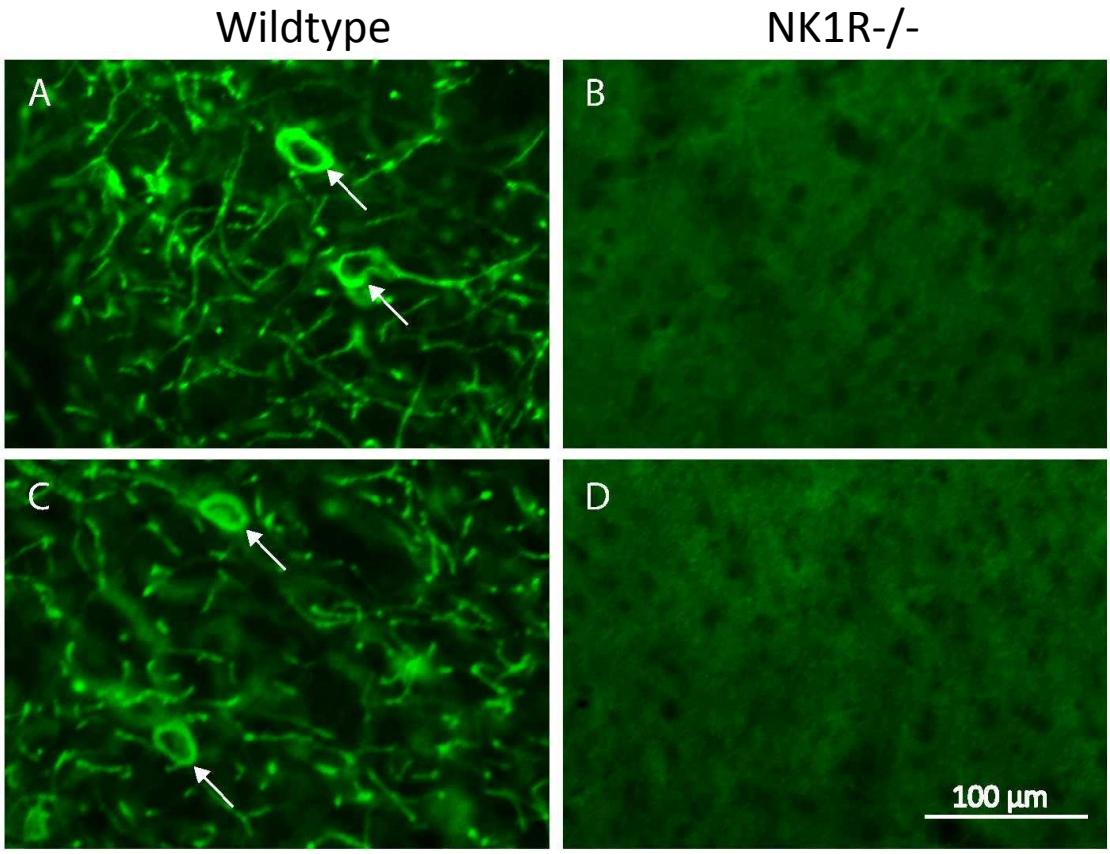

Figure 6.6 NK1R immunoreactivity in wildtype and NK1R-/- mice. Fluorescent staining of NK1R in wildtype (A) and NK1R-/- (B) mice bred from homozygotes and wildtype (C) and NK1R-/- (D) mice bred from heterozygotes. Arrows indicate NK1R-positive cells.

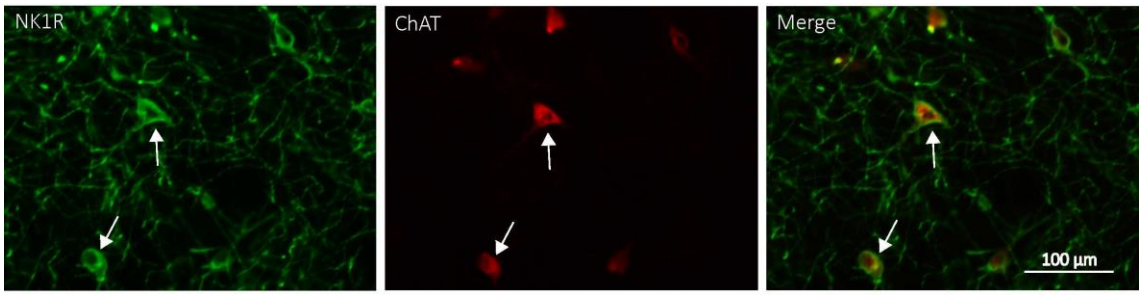

Figure 6.7 Co-localisation of ChAT and NK1R. Fluorescent staining of NK1R and ChAT in the dorsal striatum of a wildtype mouse. Arrows indicate examples of NK1R and ChAT positive cells.

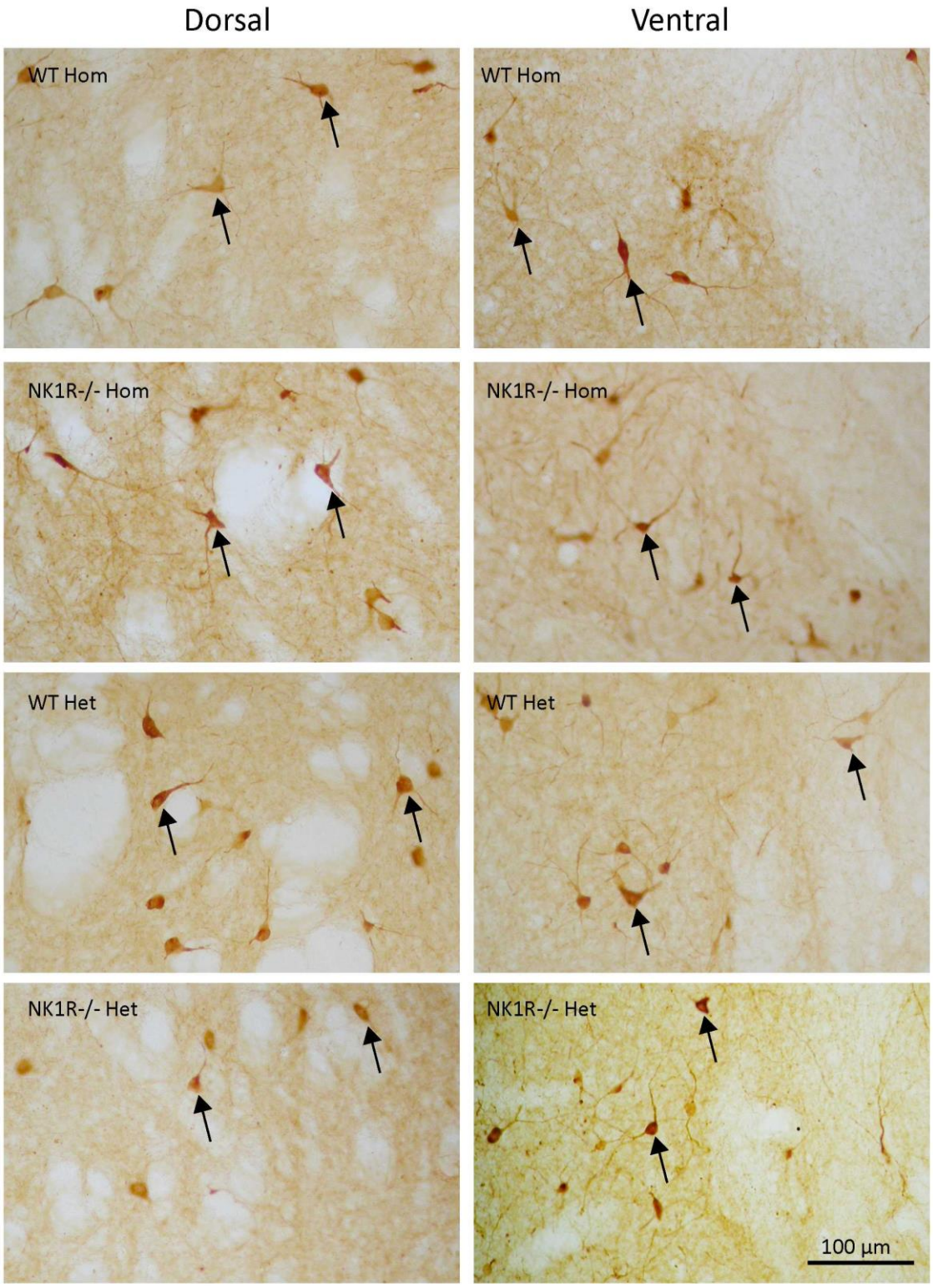

Figure 6.8 ChAT immunoreactivity in 8-12 week old wildtype (WT) and NK1R-/- mice. Representative sections of the dorsal and ventral striatum of wildtype and NK1R-/- mice bred from homozygotes (WT Hom; NK1R-/- Hom) and from heterozygotes (WT Het; NK1R-/- Het). Arrows indicate examples of ChAT-positive cells that were labelled with 3,3'-Diaminobenzidine.

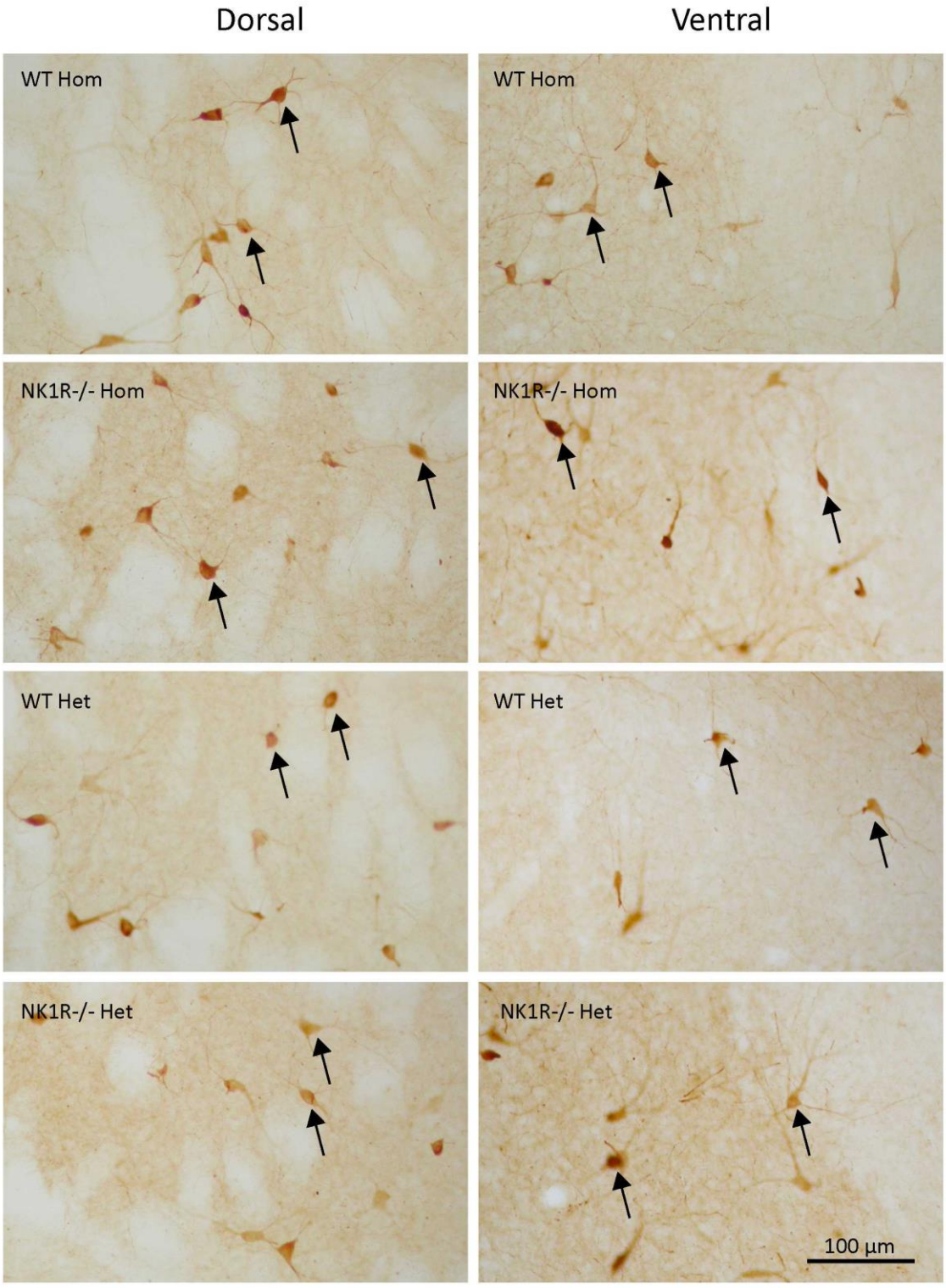

Figure 6.9 ChAT immunoreactivity in 9 month old wildtype (WT) and NK1R-/- mice. Representative sections of the dorsal and ventral striatum of wildtype and NK1R-/- mice bred from homozygotes (WT Hom; NK1R-/- Hom) and from heterozygotes (WT Het; NK1R-/- Het). Arrows indicate examples of ChAT-positive cells that were labelled with 3,3'-Diaminobenzidine.

6.3.4 ChAT immunohistochemistry

Overall differences in cell count between striatal sub-regions that were independent of genotype are summarised in Table 6.7. The mean cell count for each group is shown for 8-12 week old mice (Table 6.8) and 9 month old mice (Table 6.9).

Table 6.7 Significant differences in the number of ChAT positive cells in each striatal sub-region of mice from homozygous and heterozygous matings, regardless of genotype (n=10). DStr: dorsal striatum; VStr: ventral striatum; DM: dorsomedial; VM: ventromedial; DL: dorsolateral; VL: ventrolateral.

	Homozygous		Heterozygous	
	DStr	VStr	DStr	VStr
8-12 weeks	DM>VM; DM>VL DL>VM	Shell>Core	DM>VM DL>VM	Shell>Core
9 months	DM>VM; DM>VL DL>VM	Shell>Core	DM>VM; DM>VL DL>VM; DL>VM	Shell>Core

ChAT expression in the dorsal striatum of 8-12 week old wildtype and NK1R-/- mice (Figure 6.8)

The difference in total number of ChAT-positive cells just missed criterion for significance (P=0.059) (Figure 6.10A). The 2-way ANOVA showed a main effect of area ($F(3,24) = 17.28$, $P<0.001$). Further analysis within each area revealed that NK1R-/- mice had significantly fewer ChAT-positive cells in the dorsomedial striatum compared to NK1R-/- mice ($F(1,8) = 7.14$, $P<0.05$). The dorsolateral striatum just missed criterion for significance (P=0.066) (Figure 6.12A).

In wildtype and NK1R-/- mice from heterozygous matings there was no overall difference in total number of ChAT-positive cells (Figure 6.10B). The 2-way ANOVA revealed a main effect of area ($F(3,24) = 6.19$, $P<0.01$). However, there were no differences between the two genotypes (Figure 6.12B).

ChAT expression in the ventral striatum of 8-12 week old wildtype and NK1R-/- mice (Figure 6.8)

There was no difference in total ChAT-expression in the nucleus accumbens between wildtype and NK1R-/- mice from the homozygous or heterozygous colonies (Figure 6.10C, D). The 2-way ANOVA revealed a main effect of area for the homozygous ($F(1,8) = 88.69$, $P<0.001$) and the heterozygous colonies ($F(1,7) = 187.82$, $P<0.001$) However, there were no differences between the two genotypes (Figure 6.12C, D).

ChAT expression in the dorsal striatum of 9 month old wildtype and NK1R-/- mice (Figure 6.9)

There were significantly fewer ChAT-positive cells in NK1R-/- mice than wildtype mice bred from homozygotes ($t(8) = 3.22$, $P<0.05$) (Figure 6.11A). The 2-way ANOVA revealed a main effect of area ($F(1.6,12.4) = 14.41$, $P=0.001$) and genotype ($F(1,8) = 10.34$, $P<0.05$). Further analysis within each area revealed that there were significantly fewer ChAT-positive cells in NK1R-/- mice in the ventrolateral ($F(1,8) = 13.95$, $P<0.01$) and dorsolateral ($F(1,8) = 5.34$, $P=0.050$) striatum compared to wildtype mice. Although ChAT-expression was lower in NK1R-/- mice in the dorsomedial striatum, this did not reach significance ($P=0.093$) (Figure 6.13A).

In the heterozygous colony there was no overall difference in total number of ChAT-positive cells (Figure 6.11B). The 2-way ANOVA showed a main effect of area ($F(3,27) = 33.83$, $P<0.001$). However, there were no genotype differences (Figure 6.13B).

ChAT expression in the ventral striatum of 9 month old wildtype and NK1R-/- mice (Figure 6.9)

Overall there was greater expression of ChAT in wildtype than NK1R-/- mice in the homozygous colony ($t(8) = 2.83$, $P<0.05$) (Figure 6.11C). In the 2-way ANOVA there was a main effect of area ($F(1,8) = 134.39$, $P<0.001$) and a main effect of genotype ($F(1,8) = 7.99$, $P<0.05$). Analysis of individual areas revealed a significant decrease in ChAT-positive cells in NK1R-/- mice in the core ($F(1,8) = 12.03$, $P<0.01$) and in the shell ($F(1,8) = 5.37$, $P<0.05$) (Figure 6.13C).

There was no overall difference in the total number of ChAT-positive cells in the heterozygous breeding colony (Figure 6.11D). There was a main effect of area in the 2-way ANOVA ($F(1,7) = 136.9$, $P<0.001$), but there were no genotype differences (Figure 6.13D).

Table 6.8 Number of ChAT-positive cells in 8-12 week old wildtype and NK1R-/- mice from homozygous and heterozygous matings (n=5). VM: Ventromedial; VL: Ventrolateral; DM: Dorsomedial; DL: Dorsolateral. Data indicate mean ± standard error. These data are also represented in Figure 6.10.

		Colony							
		Homozygous				Heterozygous			
Count	Genotype	VM	VL	DM	DL	VM	VL	DM	DL
ChAT, Dorsal	Wildtype	30.95 ±3.59	34.48 ±6.34	55.58 ±4.07	47.83 ±7.39	30.20 ±5.23	35.75 ±2.84	42.95 ±7.50	43.90 ±4.96
	NK1R-/-	24.19 ±1.96	29.10 ±1.50	37.92 ±5.20	31.96 ±1.02	31.07 ±5.28	39.78 ±2.05	39.17 ±7.74	42.72 ±4.64
		Core		Shell		Core		Shell	
ChAT, Ventral	Wildtype	19.60 ±1.91		63.20 ±5.75		17.13 ±0.43		68.75 ±5.55	
	NK1R-/-	17.80 ±3.31		65.86 ±2.64		20.54 ±0.60		65.86 ±2.64	

Table 6.9 Number of ChAT-positive cells in 9 month old wildtype and NK1R-/- mice from homozygous and heterozygous matings (n=5). VM: Ventromedial; VL: Ventrolateral; DM: Dorsomedial; DL: Dorsolateral. n=5 per group. Data indicate mean ± standard error. These data are also represented in Figure 6.11.

		Colony							
		Homozygous				Heterozygous			
Count	Genotype	VM	VL	DM	DL	VM	VL	DM	DL
ChAT, Dorsal	Wildtype	31.10 ±3.19	39.80 ±3.01	53.80 ±5.49	49.50 ±6.57	29.00 ±0.95	35.25 ±2.58	56.35 ±2.31	46.45 ±5.01
	NK1R-/-	26.15 ±2.27	26.80 ±1.75	41.95 ±2.88	33.95 ±1.44	28.65 ±1.17	32.30 ±2.18	46.65 ±2.45	42.85 ±1.52
		Core		Shell		Core		Shell	
ChAT, Ventral	Wildtype	19.60 ±1.54		69.75 ±6.59		11.63 ±3.25		62.63 ±8.27	
	NK1R-/-	11.85 ±1.62		50.30 ±5.20		12.90 ±1.69		60.30 ±6.11	

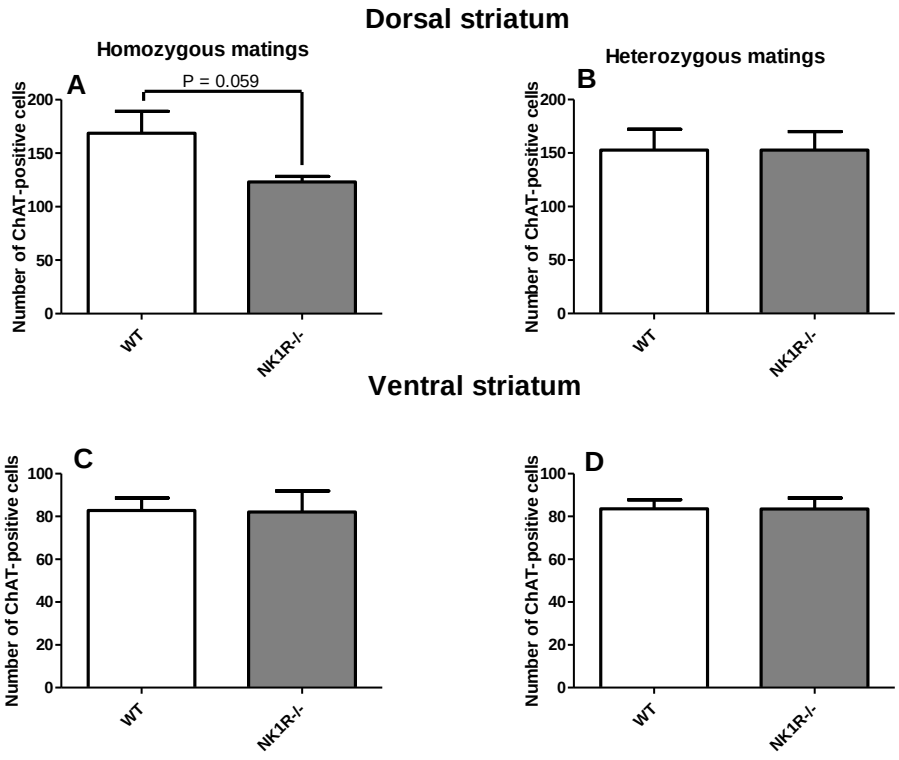

Figure 6.10 Total number of ChAT-positive cells in the striatum of 8-12 week old wildtype (WT) and NK1R-/- mice (n=5). Total counts in the dorsal and ventral striatum of (A) and (C) homozygous matings, and (B) and (D) heterozygous matings.

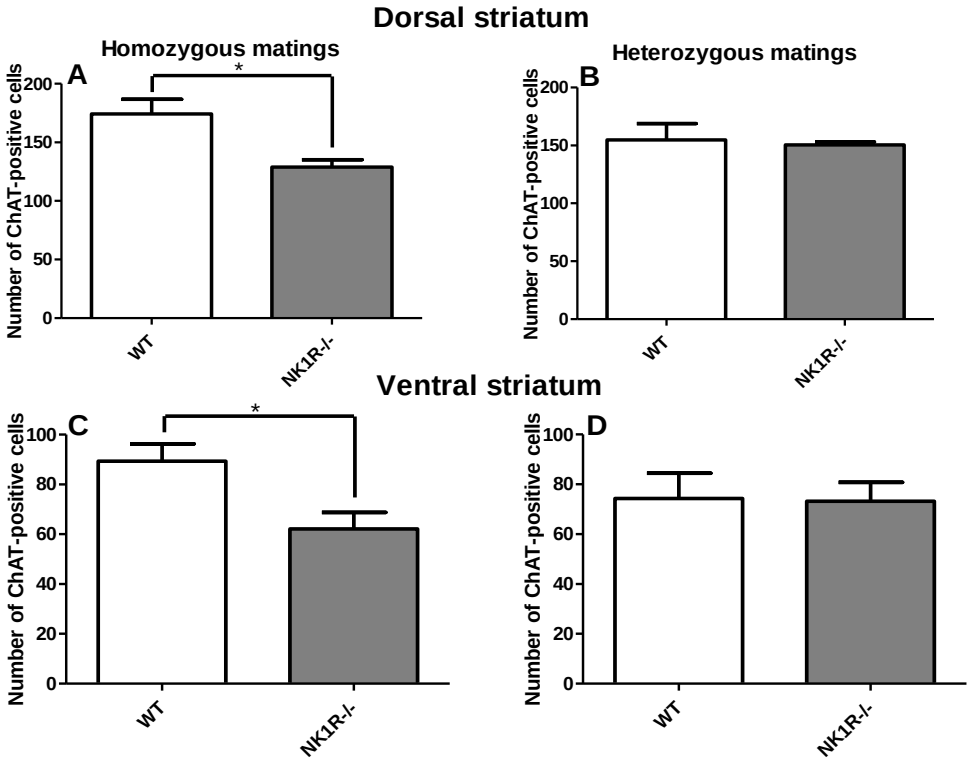

Figure 6.11 Total number of ChAT-positive cells in the striatum of 9 month old wildtype and NK1R-/- mice (n=5). Total counts in the dorsal and ventral striatum of (A) and (C) homozygous matings, and (B) and (D) heterozygous matings. *P<0.05.

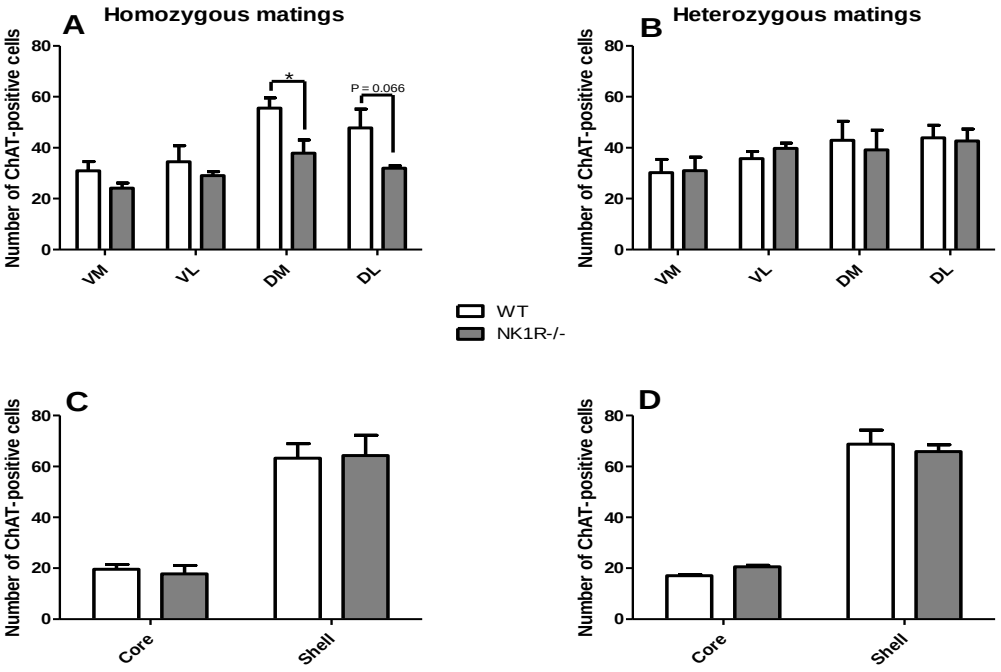

Figure 6.12 Number of ChAT-positive cells in the striatum of 8-12 week old wildtype (WT) and NK1R-/- mice (n=5). Total counts in the dorsal striatum of (A) mice bred from homzygotes and (B) mice bred from heterozygotes, and total counts in the ventral striatum of (C) mice bred from homozygotes and (D) mice bred from heterozygotes. VM: Ventromedial; VL: Ventrolateral; DM: Dorsomedial; DL: Dorsolateral. *P<0.05.

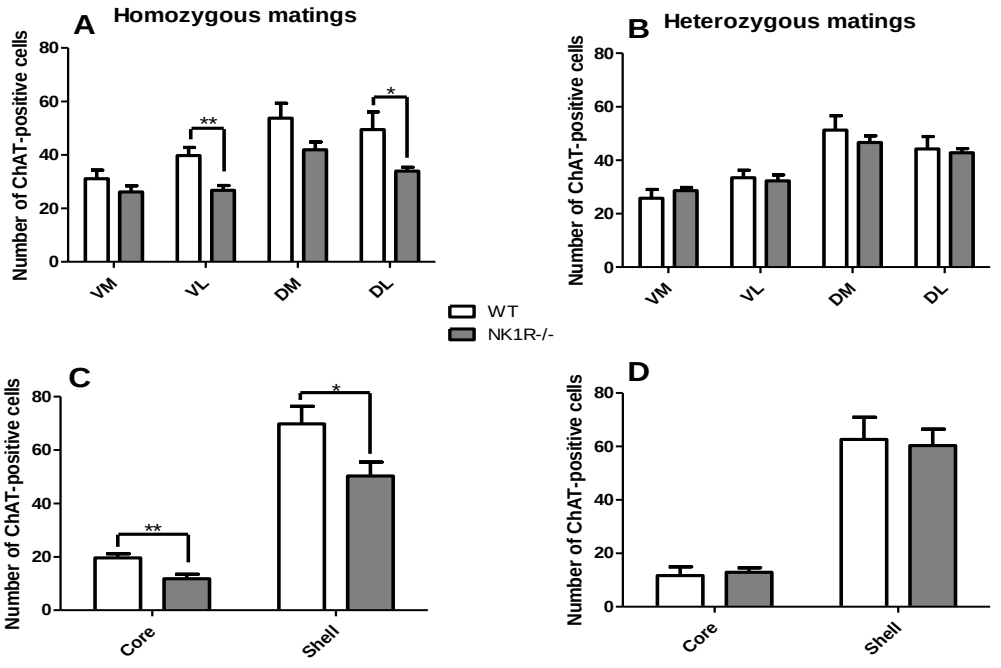

Figure 6.13 Number of ChAT-positive cells in the striatum of 9 month old wildtype (WT) and NK1R-/- mice (n=5). Total counts in the dorsal striatum of (A) mice bred from homzygotes and (B) mice bred from heterozygotes, and total counts in the ventral striatum of (C) mice bred from homozygotes and (D) mice bred from heterozygotes. VM: Ventromedial; VL: Ventrolateral; DM: Dorsomedial; DL: Dorsolateral. *P<0.05; **P<0.01.

6.3.5 Parvalbumin immunohistochemistry

Mean cell counts for 8-12 week old mice and 9 month old mice are summarised in Table 6.10 and Table 6.11, respectively. An example of parvalbumin-positive cells is shown in Figure 6.14. There were no genotype differences in either colony at either age.

6.3.6 Area measurements

In both colonies the area of the striatum was bigger in younger mice (homozygous matings: $X^2 = 6.64$, $P=0.01$; $Z = 2.58$, $P<0.01$; heterozygous matings: $F(1,17) = 39.82$, $P<0.001$; $t(18) = 6.46$, $P<0.001$). There were no genotype differences (Figure 6.15).

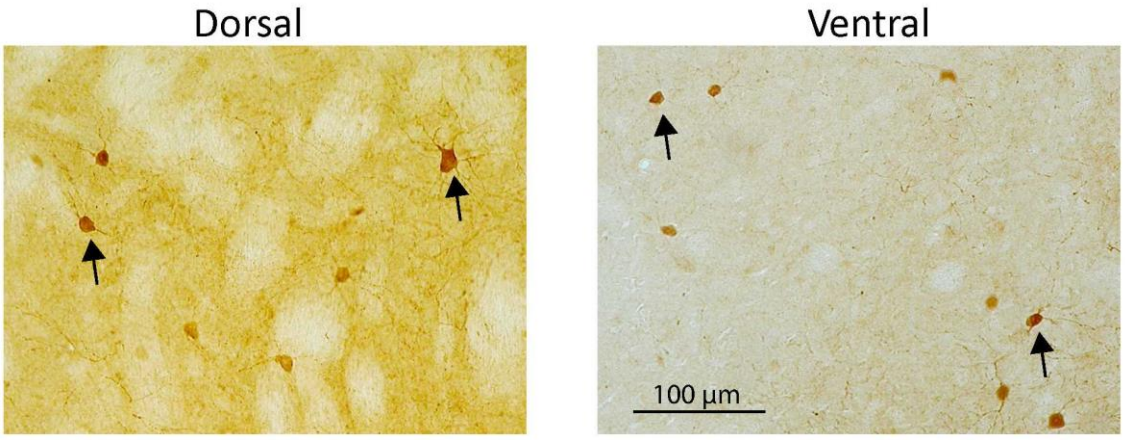

Figure 6.14 Parvalbumin immunoreactivity in a wildtype mouse. Representative sections of the dorsal and ventral striatum of a wildtype mouse bred from homozygotes. Arrows indicate examples of Parvalbumin-positive cells that were labelled with 3,3'-Diaminobenzidine.

Table 6.10 Number of Parvalbumin-positive cells in 8-12 week old wildtype and NK1R-/- mice from homozygous and heterozygous matings (n=5). VM: Ventromedial; VL: Ventrolateral; DM: Dorsomedial; DL: Dorsolateral. Data indicate mean ± standard error.

		Colony							
		Homozygous				Heterozygous			
Count	Genotype	VM	VL	DM	DL	VM	VL	DM	DL
Parv, Dorsal	Wildtype	10.5 ±0.14	34.67 ±1.24	36.75 ±6.13	46.33 ±9.37	19.50 ±3.02	29.19 ±2.67	52.13 ±7.46	39.13 ±1.88
	NK1R-/-	13.42 ±3.58	31.75 ±4.13	36.75 ±5.77	38.33 ±5.66	26.13 ±3.18	32.50 ±3.96	56.69 ±16.57	43.56 ±9.30
		Core		Shell		Core		Shell	
Parv, Ventral	Wildtype	3.33 ±0.73		15.83 ±2.67		4.92 ±0.36		35.42 ±3.43	
	NK1R-/-	2.5 ±0.58		15.67 ±1.67		8.67 ±1.88		44.25 ±4.69	

Table 6.11 Number of parvalbumin-positive cells in 9 month old wildtype and NK1R-/- mice from homozygous and heterozygous matings (n=5). VM: Ventromedial; VL: Ventrolateral; DM: Dorsomedial; DL: Dorsolateral. Data indicate mean ± standard error.

		Colony							
		Homozygous				Heterozygous			
Count	Genotype	VM	VL	DM	DL	VM	VL	DM	DL
Parv, Dorsal	Wildtype	25.23 ±3.90	30.54 ±5.15	46.92 ±3.94	34.39 ±1.52	19.81 ±1.55	23.06 ±2.38	38.75 ±2.36	27.25 ±0.44
	NK1R-/-	31.00 ±1.45	34.50 ±1.99	43.94 ±1.77	32.56 ±3.54	21.92 ±3.32	21.69 ±0.43	39.48 ±4.83	26.71 ±3.61
		Core		Shell		Core		Shell	
Parv, Ventral	Wildtype	5.90 ±1.12		24.90 ±3.83		4.40 ±1.37		23.10 ±4.12	
	NK1R-/-	7.05 ±0.78		27.48 ±2.65		3.10 ±1.03		24.60 ±3.25	

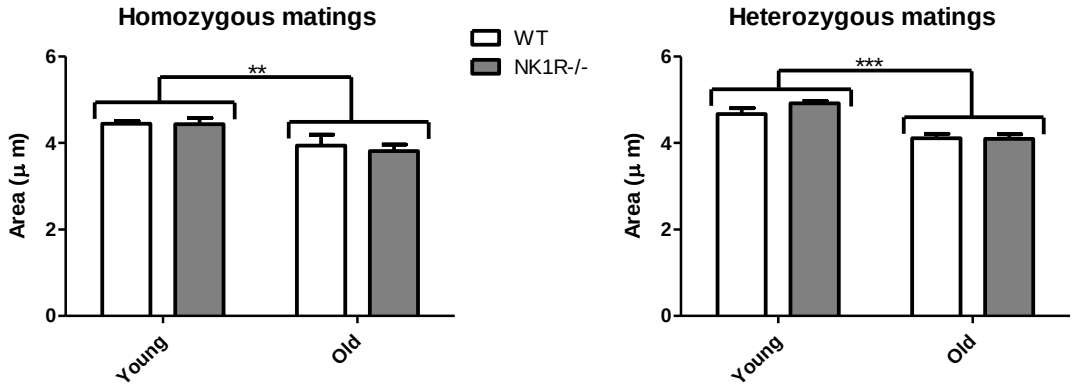

Figure 6.15 Striatal areas of wildtype (WT) and NK1R-/- mice from homozygous and heterozygous matings (n=5). Area of the dorsal striatum in 8-12 week (young) and 9 month (old) mice. **P<0.01; ***P<0.001.

6.3.7 ChAT western blots

The mean ChAT expression appeared to be reduced in NK1R-/- mice from homozygous matings compared to wildtype mice. However, due to the large variance there was no significant difference (P=0.42). There was also no genotype difference in the mice from heterozygous matings (Figure 6.16).

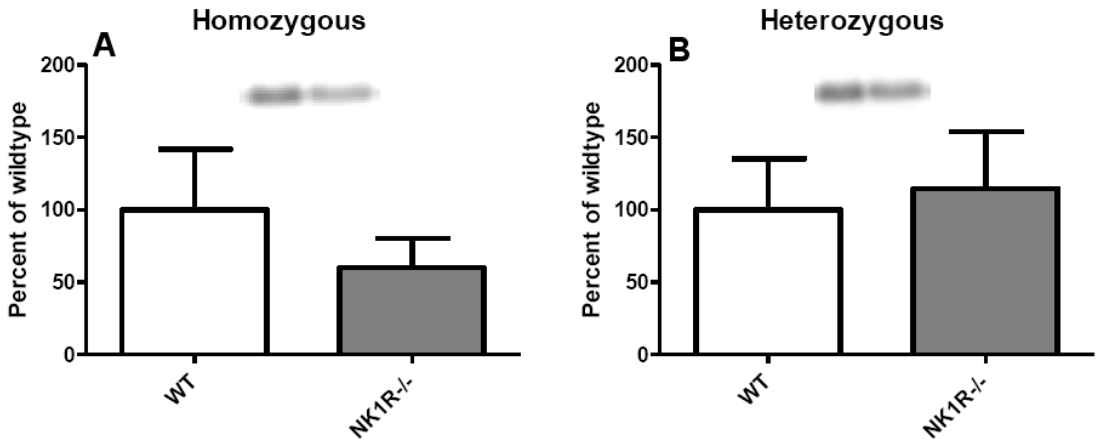

Figure 6.16 ChAT expression in the dorsal striatum of 8-12 week old wildtype (WT) and NK1R-/- mice (n=5). The density of each band was normalized to GAPDH and the ratios were expressed as a percentage of control. (A) Mice from homozygous matings and (B) mice from heterozygous matings.

6.4 Discussion

NK1R-/- mice bred from homozygotes have a reduction in ChAT-positive cells within the striatum. This deficit is not present in NK1R-/- mice bred from heterozygous matings. Because NK1R-/- mice from both colonies are bred on the same background strain, this suggests that there could be an important effect of early environment on cholinergic cell development. Alternatively, other genetic mutations could have occurred in NK1R-/- mice bred homozygously that are passed down through generations and cause the loss of ChAT-positive cells. Furthermore, the deficits in impulsivity seen in NK1R-/- mice from homozygous litters were not present in those bred heterozygously.

6.4.1 Differences in impulsivity in the 5-CSRTT

As seen previously (Yan et al., 2011), NK1R-/- mice bred from homozygous parents are significantly more impulsive than their wildtype counterparts. In contrast, NK1R-/- mice bred from heterozygote parents are not impulsive, and are even significantly less impulsive than their wildtype littermate controls. This difference was not caused by a difference in body weight between the two colonies. Although *premature responding* in mice tested in the 5-CSRTT tends to be low, especially compared to rats (Humby et al., 1999), other studies have found high *premature responses* in wildtype mice when they are first tested with a LITI (Peña-Oliver et al., 2012). In the present experiment it is surprising that there is a large difference in premature responses in the VITI between the wildtype mice from each colony since no differences were seen during training. However, it is possible that the task demands during training were not great enough to pull out differences. Regardless, the disparity in the behaviour of wildtype mice from different breeding methods highlights how the interpretation of data can differ greatly depending on the control animals used.

The difference in impulsive responding between the two colonies suggests that the behavioural deficits seen previously in NK1R-/- mice tested in the 5-CSRTT are not only caused by a lack of functional NK1R. As discussed in chapter 5, random mutations can occur in mice that are passed down to future generations (Crusio et al., 2009). If wildtype and NK1R-/- mice are bred separately, their genetic background could gradually segregate. Crossing wildtype and NK1R-/- mice and

creating heterozygous mating pairs prevents this drift, and increases the likelihood that differences between the genotypes are linked to a lack of functional NK1R.

The behavioural differences seen between the two colonies could also arise from a different early environment. Early life maternal deprivation can induce lifelong behavioural deficits, including deficits in attentional set-shifting (Lovic and Fleming, 2004) and motor impulsivity tested on a DRL schedule (differential reinforcement of low response rate, Lovic et al., 2011). It is therefore possible that the deficits seen in the 5-CSRTT in NK1R-/- mice bred from homozygotes is a consequence of poor maternal care from NK1R-/- mothers. However, as discussed below, comparison of NK1R-/- mice from both colonies revealed differences in ChAT-positive cells in the striatum, suggesting that the differences seen in the 5-CSRTT may be linked to an interaction between early environment and brain development.

6.4.2 Cholinergic deficit in the striatum of NK1R-/- mice bred from homozygotes

In this chapter, a deficit in ChAT-positive cells was identified in the striatum of homozygously-bred NK1R-/- mice. It was also shown that in the mouse striatum, NK1R are expressed on cholinergic interneurons, as has been reported in the rat (Gerfen, 1991). The deficit in ChAT expression was not due to a general cell loss in the striatum because there was no difference in parvalbumin-positive cells. The striatum was smaller in older mice from both colonies compared to young mice, and a reduction in striatal area has previously been found in aging humans (Gunning-Dixon et al., 1998). However, the cell loss was not due to a reduction in the area of the striatum, as this did not differ between wildtype and NK1R-/- mice.

As well as being located on cholinergic interneurons in the striatum, NK1R are expressed on a subtype of interneurons expressing somatostatin (SS), neuropeptide Y (NPY) and nitric oxide synthase (NOS) (Kaneko et al., 1993). It is possible that SS, NPY and NOS expression would also be reduced in NK1R-/- mice. However, preliminary data found no decrease in NPY expression in the striatum of young NK1R-/- mice bred from homozygotes (D'angelo and Weir, unpublished data).

The loss of ChAT-positive cells within the dorsal striatum was present to the same extent in 8-12 week old and 9 month old NK1R-/- mice, suggesting there is no further cell death throughout the animals' lives. However, this does not confirm that there was never any cell death, as it could have occurred at a younger age. In order to confirm this, cell death needs to be checked at many different time points. This could be done with an immunohistochemical stain for caspase-3, a protein that plays an essential role in cell apoptosis (Porter and Jänicke, 1999). However, identifying the exact time point when this would occur in NK1R-/- mice is difficult. Another possibility is that the cholinergic interneurons within the striatum of NK1R-/- mice have shrunk. This has previously been reported in a patient with Alzheimer's disease (Pearson et al., 1983), and in rats with lesions to the basal nucleus of Meynart (Sofroniew et al., 1983). Regardless of whether ChAT-positive cells have died or shrunk, it may be possible to restore ChAT function by administering nerve growth factor (NGF, Mobley et al., 1985). Indeed, patients with Alzheimer's disease, that have a loss of cholinergic cells and ChAT activity, also have reduced NGF expression (Mufson et al., 2008). Similarly, ChAT function could be restored with brain-derived neurotrophic factor (BDNF), as it has been shown that BDNF is vital for the survival of striatal cholinergic neurons in mice (Ward and Hagg, 2000). Since ChAT is involved in the synthesis of acetylcholine, it is also possible that the cholinergic interneurons of NK1R-/- mice are synthesising lower levels of ChAT. Western blots were performed to see whether this is the case. However, despite a tendency for there to be a reduced amount of ChAT protein in NK1R-/- mice, this did not reach significance due to the large variance between samples.

Whereas there was no decrease in ChAT expression within the ventral striatum of young NK1R-/- mice, there were significantly fewer ChAT-positive cells in the ventral striatum of 9 month old NK1R-/- mice. This suggests that there is a gradual decrease in ChAT function in the ventral striatum throughout the animals' lives, whereas this is present from an early age in the dorsal striatum. Previously, a loss of cholinergic cells in the striatum has been shown to increase with age (Altavista et al., 1988). Interestingly, the extent of this loss positively correlates with the extent of spatial learning deficits in the Morris water maze (Fischer et al., 1989).

This fits with the present finding that NK1R-/- mice bred from homozygotes show behavioural deficits in the 5-CSRTT and have a decrease in ChAT-positive cells in both the dorsal and ventral striatum. It is important to note that the mice were between 3 and 5 months old when they were tested in the 5-CSRTT in this chapter, and as they were subsequently used in another experiment, they were 9 months old when they were perfused. It would be interesting to see whether there is a decrease in cholinergic activity in the ventral striatum of NK1R-/- mice at 3-5 months. In addition, the drug treatment experienced by the mice prior to perfusion may have affected cholinergic transmission. However, guanfacine is an alpha-receptor agonist that inhibits NA, not acetylcholine, and levels of NA are very low in the dorsal striatum (Glowinski and Iversen, 1966). Also, mice only received 3 acute injections of guanfacine, making it unlikely that this caused a long-term deficit in cholinergic transmission. Furthermore, this loss was specific to NK1R-/- mice bred from homozygotes. If the treatment was causing a depletion of cholinergic activity, it would be present in both colonies. Regardless, in order to control for the effects of the 5-CSRTT, cell counts in 9 month old naïve mice are needed.

As well as a lack of difference in cholinergic activity in the ventral striatum of littermate pairs, there was no loss of ChAT-positive cells in the dorsal striatum of NK1R-/- mice from heterozygote parents. This suggests that a deficit in cholinergic transmission is not a direct consequence of non-functional NK1R, but could instead be linked to the early environment. If NK1R-/- mothers are providing poor maternal care, this could induce apoptosis in their offspring (Zhang et al., 2002). As yet, no studies have looked at whether maternal care is altered in NK1R-/- mothers. However, NK1R-/- mice have a blunted response to anxiogenic stimuli (De Felipe et al., 1998; Rupniak et al., 2000; Santarelli et al., 2001) and mice treated with an anxiolytic show reduced maternal care (D'Amato et al., 1998). Also, high-responding rats, that show reduced anxiety, exhibit a reduction in maternal care (Clinton et al., 2010, 2007). Further studies are required to look at maternal behaviours in NK1R-/- mice. It would also be interesting to look at how cross-fostering NK1R-/- pups to wildtype mothers, and vice-versa, affects cholinergic development. Alternatively, the loss of cholinergic transmission could be caused by

a mutation that has occurred in the NK1R-/- mouse colony and passed down through generations (Crusio et al., 2009).

6.4.3 Striatal sub-region

In young NK1R-/- mice, the deficit in ChAT expression was found in the dorsomedial striatum, whilst in older mice the deficit was found in the dorsolateral and ventrolateral striatum. The dorsomedial striatum is involved in goal-directed behaviours, and as actions become habitual, they rely on dorsolateral mechanisms (Belin et al., 2009; Everitt and Robbins, 2005). When the mice are tested with a VITI or LITI in the 5-CSRTT, they have been trained on the task for several months, and it is therefore likely that in wildtype mice the actions have become habitual. It is therefore not surprising that behavioural deficits in NK1R-/- mice correlate with cell loss in the dorsolateral region of the striatum, because this could cause a disruption of habit learning. However, in old NK1R-/- mice there tended to be fewer ChAT-positive cells in the dorsomedial striatum, and overall counts in the dorsal striatum were significantly reduced. Therefore, a general reduction in ChAT-positive cells in the dorsal striatum regardless of sub-region may underlie deficits in the 5-CSRTT.

6.4.4 Cholinergic deficits and the 5-CSRTT

A loss of ChAT-positive cells was found in the ventral and dorsal striatum of NK1R-/- mice bred from homozygotes. These mice showed increased impulsivity in the 5-CSRTT. Cholinergic transmission has previously been linked to attention in the 5-CSRTT, with lesions to the basal forebrain and systemic cholinergic agents affecting *accuracy* (for review see (Robbins, 2002). However, basal forebrain lesions affect cholinergic transmission within the cortex, and as yet the effects of selective cholinergic lesions to the striatum have not been studied in the 5-CSRTT. Cholinergic interneurons in the dorsal and ventral striatum are innervated by the thalamus, not the cortex (Lapper and Bolam, 1992; Meredith and Wouterlood, 1990). As discussed in chapter 4, basal ganglia loops projecting through structures including the striatum and the thalamus are involved in motor inhibition, and thus could play an important role in impulsivity. Indeed, an increase in *premature responding* following *d*-amphetamine is thought to be mediated by the nucleus accumbens (Cole and Robbins, 1989), and lesions to the core of the nucleus

accumbens increase *premature responding* and *perseverative responding* without affecting *accuracy* (Christakou et al., 2004). Futhermore, ablation of cholinergic interneurons in the ventral striatum increases conditioning to cocaine (Hikida et al., 2001), indicating increased impulsivity. In light of the results in the current chapter, it would be interesting to look at the effects of selective dorsal and ventral striatal cholinergic lesions on 5-CSRTT performance.

6.4.5 Other behaviours in the 5-CSRTT

Perseverative responding was increased in NK1R-/- mice in the VITI regardless of colony. This increase was also seen in stages 1 and 3 of training, suggesting that NK1R-/- mice learn to inhibit this behaviour during training and it only re-emerges when task demands were high. Overall, this agrees with previous evidence that *perseverative responding* in the 5-CSRTT is linked to a lack of functional NK1R (Dudley et al., 2013; Yan et al., 2011). It also suggests that *perseverative responding* is independent of alterations in the striatal cholinergic system. There was a slight increase in the *latency to make a correct response* in mice bred from homozygotes when they were tested in the LITI. However, this was not affected by genotype. There was a main interaction between genotype and colony for *%accuracy* in the VITI. However, further post-hoc tests did not reach significance. Due to the number of 5-CSRTT boxes, it was only possible to test six wildtype mice and six NK1R-/- mice from each colony. Also, one wildtype and one NK1R-/- mouse bred from heterozygous matings did not complete training. In order to see whether any further differences in attention can be identified, this experiment needs to be repeated. These experiments are in progress and the present data will be combined with 5-CSRTT data from another cohort of mice derived from homozygous and heterozygous matings (A. Grimme, work in progress). However, significant differences in impulsivity and perseverative responses were identified here, suggesting that these deficits are more robust.

6.4.6 Conclusions

NK1R-/- mice bred from homozygote matings show a deficit in impulsivity in the 5-CSRTT and a loss of ChAT-positive cells in the dorsal and ventral striatum. In contrast, these deficits are not present in NK1R-/- mice bred from heterozygote matings. Although the behavioural results are preliminary they do suggest a

relationship between cholinergic activity in the striatum and impulsivity. The results also highlight an important interaction between early environment, brain development and behaviour.

7 General discussion

ADHD affects between 5.9 and 7.1% of the population worldwide (Willcutt, 2012), yet the aetiology remains poorly understood. There are also concerns over the safety and efficacy of the use of psychostimulants such as *d*-amphetamine and methylphenidate for the treatment of ADHD. Animal models play a crucial role in our understanding and treatment of human disorders. Although various animal models of ADHD have been proposed, none completely satisfies all the criteria (face, construct and predictive validity) for a good animal model. The validation of animal models of neuropsychiatric disorders is complicated due to the fact that the symptoms presented tend to be human-specific. Also, the diagnosis of the vast majority of these disorders is based on the patient's description of symptoms, rather than an objective diagnostic test (Nestler and Hyman, 2010). Therefore species-specific tests have been developed that aim to mimic individual aspects of the disorder.

Locomotor activity paradigms are commonly used to test for ADHD in rodents. These can be used in conjunction with the current treatments for ADHD, to determine whether they alleviate hyperactivity. However, hyperactivity alone does not define ADHD, and it is also seen in animal models of other disorders, including models of autism (Dichter et al., 2012) and addiction (Fernando and Robbins, 2011). It is therefore important to test other symptoms of the disorder. The 5-CSRTT measures sustained visual attention and motoric impulsivity. It also provides a measure of perseveration, which is commonly seen in patients with ADHD, although it is not a diagnostic criterion. The 5-CSRTT was developed from the human CPT test, which patients with ADHD perform poorly on. Therefore, deficits in this paradigm in rodents may provide important insights into the deficits seen in humans.

The NK1R-/- mouse has recently been proposed as a new animal model of ADHD. It is hyperactive and this is reduced by the psychostimulants *d*-amphetamine and methylphenidate. It also has abnormal DA, 5-HT and NA transmission within fronto-striatal circuits and polymorphisms of the human equivalent of the NK1R

gene (TACR1) have been found in patients with ADHD (Yan et al., 2010) . Finally, NK1R-/- mice have deficits in attention, impulsivity and perseverative responding in the 5-CSRTT (Yan et al., 2011). The work in this thesis aimed to further validate the NK1R-/- mouse model of ADHD. Due to the emerging evidence that a deficit in cholinergic transmission may underlie ADHD, the striatal cholinergic neuroanatomy of the NK1R-/- mouse was also investigated.

7.1 Summary of findings

7.1.1 Effects of *d*-amphetamine on performance of NK1R-/- mice in the 5-CSRTT

D-amphetamine is one of the most widely prescribed treatments for ADHD and it alleviates the hyperactivity seen in NK1R-/- mice tested in the LDEB (Yan et al., 2010). To further validate the NK1R-/- mouse model of ADHD, the effects of *d*-amphetamine on attention, impulsivity and perseveration were tested in the 5-CSRTT (Yan et al., 2011). NK1R-/- mice were more inattentive than wildtype mice, as measured by an increase in *%omissions* and a decrease in *%accuracy*. This difference was abolished by *d*-amphetamine. However, *%accuracy* was not improved in NK1R-/- mice by *d*-amphetamine, but instead was decreased in wildtype mice. Furthermore, a significant improvement in *%omissions* in NK1R-/- mice was only seen when task demands were high (when the ITI was increased to 15s on a random schedule). *D*-amphetamine also prevented a vehicle-induced increase in perseveration in NK1R-/- mice. *D*-amphetamine failed to reduce *%premature responses* in NK1R-/- mice. However, a therapeutic effect of *d*-amphetamine on impulsivity may not have been detectable because NK1R-/- no longer showed an increase in *%premature responses* on repetition of the test. This suggests that the mice adapt quickly to the task parameters and a deficit in impulsivity may reappear if task demands are heightened by increasing the ITI.

These results suggest that *d*-amphetamine has some therapeutic effect on attention and perseverative responding in NK1R-/- mice. However, the effects varied depending on the test parameters used. It could be that there was no robust therapeutic effect of *d*-amphetamine because a lack of functional NK1R prevents the normal response to *d*-amphetamine. Indeed, although patients with ADHD may

have polymorphisms of the NK1R gene, the receptor will still maintain a certain level of functionality. A lack of normal response to *d*-amphetamine is supported by a microdialysis study showing that wildtype mice injected with an NK1R antagonist and NK1R-/- mice do not show an increase in striatal extracellular DA following administration of *d*-amphetamine (Yan et al., 2010).

7.1.2 Identifying new treatments for ADHD

In chapter 4, nifedipine was tested in the 5-CSRTT as a potential alternative treatment for ADHD. A study has shown that the L-type calcium channel blocker nifedipine can alleviate ADHD-like symptoms in children with renal hypertension (Krause et al., 2009). Other studies have shown that NK1R antagonists have L-type calcium channel blocking effects (Guard et al., 1993; Rupniak et al., 1993, 2003). These findings suggest an interaction between NK1R and L-type calcium channels.

Nifedipine had similar effects on the performance of wildtype and NK1R-/- mice in the 5-CSRTT. It reduced *%premature responses*, increased *%omissions* and increased the *latencies to respond and collect the reward*. In the striatum, L-type calcium channel currents are suppressed by the activation of DA D2 receptors (Hernandez-Lopez et al., 2000) and M1 muscarinic receptors (Wang et al., 2006) located on MSNs. This reduces overall network excitability. D2-expressing MSNs are part of the indirect pathway, which is involved in action inhibition. Therefore, direct blockade of L-type calcium channels would theoretically increase network inhibition and reduce impulsivity. An increase in network inhibition would also inhibit the direct pathway, preventing new actions from being initiated and increasing *%omissions*.

Although similar effects were seen in both genotypes, a higher dose was required to see the decrease in *%premature responses* and increase in *%omissions* in NK1R-/- mice. Substance P binding to NK1R located on cholinergic interneurons in the striatum stimulates acetylcholine release which binds to M1 receptors located on MSNs. As mentioned above, this reduces L-type calcium channel opening (Wang et al., 2006). Therefore, in the absence of NK1R, it is possible that there is overall less network inhibition due to a reduction in acetylcholine release, and subsequently a higher dose of nifedipine is required to see the inhibitory effects.

As seen in the first 5-CSRTT experiment, an increase in impulsivity in NK1R-/- mice was not seen on repetition of the test and, in contrast to the first experiment, deficits in attention were no longer present. Therefore, it was not possible to determine whether nifedipine has a therapeutic effect on these deficits in NK1R-/- mice. Overall this experiment highlighted an interaction between NK1R and L-type calcium channels, and suggested that blockade of these channels may be beneficial in impulse control disorders. However, the lack of therapeutic effect on perseverative responding, the increased inattention in both genotypes and the non-specific increases in latencies to perform the task suggest that nifedipine may not be useful for the treatment of ADHD.

Behavioural screens are commonly used to identify new drug targets. Ideally, these tests are quick to run, so that a range of drugs can be rapidly screened. Because it takes many months to train and test rodents in the 5-CSRTT, locomotor activity paradigms are more suitable drug screens. However, these paradigms are often confounded by stress, and NK1R-/- mice have altered stress responses (De Felipe et al., 1998; McCutcheon et al., 2008; Rupniak et al., 2000; Santarelli et al., 2001). Also, previous work measuring the activity of NK1R-/- mice used a LDEB paradigm. This was initially developed as a screen for anxiolytic drugs (Crawley, 1985, 1981), and is therefore susceptible to stress. Moreover, animals were tested in the light phase when they are least active. Possibly due to these confounds, the hyperactivity of NK1R-/- mice was highly variable, and not significantly different from wildtype mice. The work in chapter 5 aimed to identify conditions under which hyperactivity in NK1R-/- mice was robust. This was achieved by measuring their activity in the home cage during the dark phase with telemetry. Telemetry is therefore a good screen for hyperactivity in NK1R-/- mice, and may help identify new treatments for ADHD.

7.1.3 Behavioural and molecular comparison of mice from homozygote and heterozygote matings

The mice tested in the 5-CSRTT in chapters 3 and 4 had been bred from homozygotes for many generations. The deficits seen in NK1R-/- mice tend to be attributed to a lack of functional NK1R. However, the deficits could be caused by other *de novo* genetic mutations occurring independently in the wildtype and

NK1R-/- colonies that are passed down to future generations (Crusio et al., 2009). Also, maternal care could be different in mothers lacking functional NK1R, and early environment can have long-term effect on animals' phenotypes (Crusio, 2004). In order to overcome these limitations, mice were also bred from heterozygous matings. They were then tested with telemetry and compared in the 5-CSRTT with mice bred from homozygotes. NK1R-/- mice from heterozygous matings continued to show hyperactivity although this was less pronounced than in NK1R-/- mice from homozygous matings. This suggests that a lack of functional NK1R is linked to hyperactivity, but that something else is exacerbating this deficit in mice from homozygous matings. As discussed below, this could be due to a loss of striatal cholinergic function. NK1R-/- mice from both colonies also showed an increase in perseverative responses. However, the deficit in impulsivity was only present in NK1R-/- mice from homozygote breeding pairs. No deficits in attention were present in either colony.

Further work looked at whether *d*-amphetamine could reduce the hyperactivity of NK1R-/- mice bred from heterozygotes, and whether the NK1R antagonist RP 67580 induced hyperactivity in their wildtype counterparts. In line with the lack of clear therapeutic effect of *d*-amphetamine in the 5-CSRTT, *d*-amphetamine failed to reduce the hyperactivity of NK1R-/- mice, but increased activity in wildtype mice, thereby abolishing the genotype difference. There was no clear effect of RP 67580 on activity, although group sizes were small so this experiment needs to be replicated.

Increasing evidence suggests that abnormalities in cholinergic transmission may be involved in the pathophysiology of ADHD. Studies have shown that acetylcholine receptor agonists, such as nicotine and the acetylcholinesterase inhibitor donezepil can improve symptoms of ADHD (Levin et al., 1996; Wilens et al., 2000). Also, all cholinergic interneurons in the striatum express NK1R (Aubry et al., 1994; Gerfen, 1991). Therefore, chapter 6 compared the number of ChAT-positive striatal cells in 8-12 week old wildtype and NK1R-/- mice from each colony, as well as in 9 month old mice that had completed the 5-CSRTT experiment. The results showed that in NK1R-/- mice bred from homozygote matings there was a loss of ChAT-positive cells in the dorsal and ventral striatum, although in the

latter region this was only seen in 9 month old mice. These experiments also revealed a decrease in overall striatal area in all mice as they age, regardless of genotype.

7.2 The NK1R-/- mouse as a model of ADHD

Extending previous findings, NK1R-/- mice are hyperactive, and this is the case whether they are bred from homozygous or heterozygous parents. However, previous studies found hyperactivity after a vehicle injection. When NK1R-/- mice were uninjected, hyperactivity was only consistently identified when they were tested in their home cage, with telemetry. It has previously been reported that hyperactivity in animal models of ADHD should be observed in a familiar environment, as this is when symptoms of ADHD are observed in patients (Russell et al., 2005). The findings presented here suggest that the hyperactivity of NK1R-/- mice measured with telemetry could mimic the hyperactivity observed in patients with ADHD. On the other hand, the lack of effect of *d*-amphetamine on the hyperactivity of NK1R-/- mice tested with telemetry could be an issue for the predictive validity of the model.

The deficits in attention previously observed in NK1R-/- mice tested in the 5-CSRTT (Yan et al., 2011) were only present in the first experiment in this thesis. It is not clear why these deficits disappeared, but perhaps increasing the difficulty of the task will reinstate attentional deficits. Another possibility is that the NK1R-/- mouse is a model of the Predominantly Hyperactive/Impulsive clinical subtype of ADHD. However, impulsivity was only present in mice from homozygote matings. Also, in all the 5-CSRTT experiments presented in this thesis, impulsivity was only evident when animals were first tested post-training. Reinstatement of impulsivity may depend on increasing the task difficulty. Alternatively, testing mice in the dark phase may increase impulsivity. Indeed, testing in the dark phase revealed greater hyperactivity in NK1R-/- mice, and this could also be the case for impulsivity. However, NK1R-/- mice have yet to be tested with paradigms that measure more cognitive forms of impulsivity, such as delay discounting. This is discussed in more detail in section 7.4.1. One deficit that was consistently seen in NK1R-/- mice from both colonies was an increase in perseverative responding. This is not a diagnostic symptom of ADHD, but is often found in patients (Lawrence et al., 2004).

In summary, the phenotype of the NK1R-/- mouse investigated in this thesis suggests that it could be used for investigating hyperactive and perseverative deficits. Furthermore, NK1R-/- mice from homozygote matings could provide insights into the mechanisms involved in impulsivity. However, further studies are needed to investigate impulsivity under different experimental parameters and in different paradigms. Additionally, the predictive validity of the NK1R-/- mouse model needs further confirmation.

7.3 Cholinergic hypothesis of ADHD

The dominant theory of ADHD suggests that it is caused by abnormal DA transmission within fronto-striatal circuits (Levy, 1991), and it has also been suggested that symptoms of ADHD are caused by a dysregulation of catecholamines (Pliszka et al., 1996). In line with this, the majority of animal models of ADHD show some degree of DA dysfunction (Van der Kooij and Glennon, 2007) and psychostimulants, which are used to treat ADHD, increase DA transmission. However, the mechanism of action of psychostimulants remains poorly understood and the beneficial effects of *d*-amphetamine on hyperactivity, impulsivity and attention are found in healthy children as well as children with ADHD (Zahn et al., 1980). Also, imaging studies of patients with ADHD disagree on whether there is an increase, a decrease or no change in expression of the DA transporter in the striatum (Hesse et al., 2009; Krause et al., 2000; van Dyck et al., 2002).

The reduction in ChAT-positive cells in the striatum of NK1R-/- mice supports increasing evidence that a deficit in cholinergic function underlies the symptoms of ADHD (discussed in section 7.1.3). More specifically, because the loss was specific to NK1R-/- mice bred from homozygous breeding pairs, the cholinergic deficit is likely to underlie behavioural deficits that are not present in NK1R-/- mice bred from heterozygous breeding pairs. When directly compared in the 5-CSRTT, only NK1R-/- mice from homozygotes were impulsive. Therefore, a new mechanism underlying impulsivity is proposed, caused by disruption of normal cholinergic function in the dorsal and ventral striatum. This fits with a recent study showing that mice lacking muscarinic M1 receptors have deficits in impulsivity in the 5-CSRTT without showing attentional deficits (Bartko et al., 2011). In addition, M1

receptor knockout mice are hyperactive (Miyakawa et al., 2001), so the increased hyperactivity seen in NK1R-/- mice bred from homozygous matings could also be due to a decrease in cholinergic function.

The striatum is densely innervated by DA neurons and cholinergic interneurons that exert control over each other. As discussed in chapter 6, dopaminergic neurons fire at high frequency when encoding information about the prediction and receipt of reward-related and salient stimuli (Matsumoto and Hikosaka, 2009; Schultz, 2002). In contrast, cholinergic interneurons pause in response to salient stimuli (Aosaki et al., 1994; Morris et al., 2004), and this pause is mediated by activation of mACh autoreceptors located on cholinergic interneurons. A pause in firing reduces acetylcholine binding to nAChRs located on DA neurons. This increases the phasic, high frequency firing rate of DA neurons (Rice and Cragg, 2004). Striatal DA and acetylcholine therefore interact in the striatum to signal events of high saliency (Threlfell and Cragg, 2011).

Cholinergic interneurons alter their firing pattern during learned and rewarded motor tasks (Graybiel et al., 1994; Kitabatake et al., 2003). GABAergic projections from the ventral tegmental area to the nucleus accumbens cause a pause in cholinergic interneuron firing to signal events of motivational significance, and reinforce stimulus-outcome associations (Brown et al., 2012). In addition, cholinergic interneurons modulate the activity of MSNs in the direct and indirect pathways through the activation of mAChRs (Zhou et al., 2003). Thalamic input to these interneurons causes a burst of acetylcholine release in response to salient stimuli which enhances the activation of the indirect pathway. More specifically, the thalamic-mediated increase in acetylcholine release transiently activates presynaptic M2 receptors located on corticostriatal glutamatergic terminals. This inhibits the activation of direct and indirect MSNs. Subsequently, acetylcholine has a slower, long-lasting facilitatory effect on M1 receptors located on indirect pathway MSNs. The pause in cholinergic interneuron firing ensures there is no concomitant corticostriatal suppression of the indirect pathway. Activation of the indirect pathway inhibits locomotor activity (Miyakawa et al., 2001) and suppresses actions, thus allowing attention to be redirected (Ding et al., 2010; Thorn and Graybiel, 2010). Therefore, a reduction in cholinergic function would

theoretically increase impulsivity and hyperactivity due to hypoactive indirect pathway MSNs. This is illustrated in Figure 7.1. Furthermore, the activation of nAChRs through nicotine injection or smoking increases impulsive behaviour (Ohmura et al., 2012). Interestingly, nAChRs are upregulated following striatal cholinergic interneuron ablation (Kitabatake et al., 2003). Therefore, the impulsivity seen in NK1R-/- mice with a reduction in ChAT-positive cells could result from an upregulation of nAChRs. This requires further investigation.

7.4 Future directions

In this thesis, work was done to validate the NK1R-/- mouse model of ADHD. The findings presented here can help guide future clinical studies. However, further work with NK1R-/- mice is required to establish the nature and extent of the deficits, both behaviourally and molecularly. These are discussed below.

7.4.1 Behavioural studies with NK1R-/- mice

NK1R-/- mice bred from homozygote breeding pairs are impulsive when first tested in the 5-CSRTT. Impulsivity is not a unitary construct, and different behavioural paradigms are thought to represent different facets of impulsivity, relying on different biological mechanisms (Evenden, 1999). In the 5-CSRTT, impulsivity is measured by *premature responses*. This represents the animal's failure to withhold an action, which is a type of 'motoric' impulsivity (Winstanley et al., 2006). Other behavioural paradigms that measure this type of impulsivity exist, such as the go/no-go task (Harrison et al., 1999), and the stop-signal reaction-time task (Eagle and Robbins, 2003). In these tasks, rodents have to nose-poke in response to certain stimuli. However, on some trials other cues are presented indicating that the animal must not respond. These tests were specifically designed to test the ability to quickly inhibit a pre-programmed action. These tests are yet to be published in mice, but it would be interesting to test NK1R-/- mice in these paradigms to see whether they are impulsive and whether the impulsivity is more robust than when measured in the 5-CSRTT.

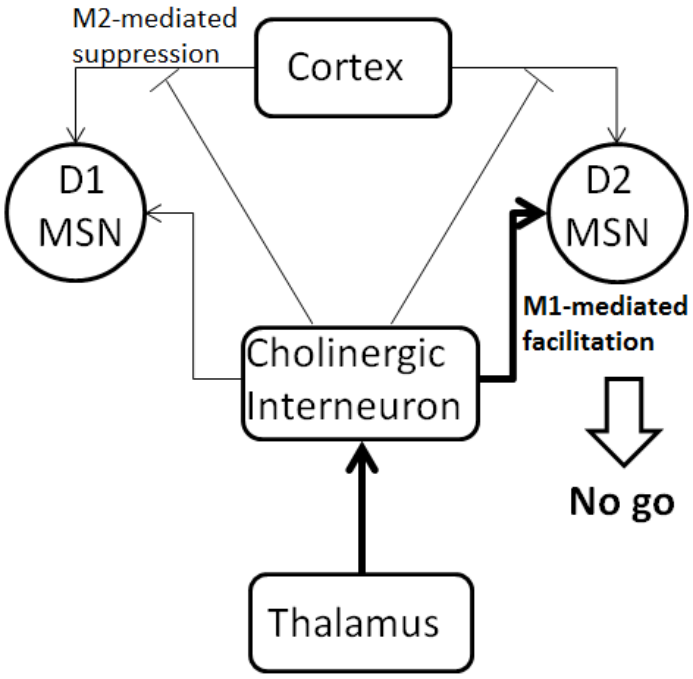

Figure 7.1 Illustration of the thalamic facilitation of the indirect pathway. A burst of thalamic activity in response to a salient stimulus triggers a burst of acetylcholine release. This activates postsynaptic M1 receptors on D2 MSNs, facilitating the 'no go' pathway, while also suppressing cortical input through the activation of presynaptic M2 receptors. MSN: medium spiny neuron. DA: dopamine.

Another type of impulsivity is choice impulsivity (Winstanley et al., 2006). This represents the avoidance of delay and can be measured with a delay discounting task. This task measures the ability to wait and receive a large reward rather than taking a smaller, more immediate one. When patients with ADHD are tested in this task, they prefer to avoid delay and take the smaller, more immediate reward (Schweitzer and Sulzer-Azaroff, 1995) and poor performance in delay discounting correlates with symptoms of ADHD (Solanto et al., 2001). It would therefore be interesting to test NK1R-/- mice in this paradigm.

Perseverative responses were higher in NK1R-/- mice in all 5-CSRTT experiments. This was true regardless of breeding method, suggesting that it is linked to a deficit in NK1R function. Although it has been described as a measure of compulsivity, it is not clear what *perseverative responding* in the 5-CSRTT represents. Recent work in our lab has looked at species –typical behavioural tests that measure perseverative digging (Weir, 2012), but results from NK1R-/- mice are contradictory and the cause of perseveration remains unknown. When mice are first habituated to the 5-CSRTT apparatus, milk is placed in the stimulus holes to encourage nose poking. It is possible that NK1R-/- mice fail to extinguish this stimulus/reward association.

This could be further tested using reversal learning paradigms such as the T-maze or attentional set shifting. In these tasks, animals learn that a reward is found in a certain location, and this location is then changed. The inability of an animal to learn the new reward location is a measure of perseverative behaviour (Izquierdo and Belcher, 2012).

7.4.2 Molecular studies

There were fewer ChAT-positive cells in the striatum of NK1R-/- mice compared to their wildtype counterparts. ChAT is an enzyme involved in the synthesis of acetylcholine. There are various ways in which this reduction needs to be further studied. Microdialysis in the striatum of NK1R-/- mice can determine whether there is reduced acetylcholine efflux compared to wildtype mice. As yet, it is not known whether there are fewer cholinergic cells in the striata of NK1R-/- mice, or whether they are simply reduced in size. This can be determined with more precise counting techniques. Another possibility is that cholinergic cells are synthesising lower levels of ChAT in NK1R-/- mice, meaning that some cells failed to be detected with the immunohistochemical technique used here. Western blots suggested that there was overall less ChAT protein in young NK1R-/- mice bred from homozygote matings. However, due to the high variability within each sample, no significant differences were identified. This could be due to the fact that striatal sub-regions were not analysed separately. However, this study should also be repeated with a larger number of samples.

If it turns out there is a reduction in the number of cholinergic interneurons, it remains to be determined when this loss happens. Cell death could occur during the first few weeks of the animals' lives. Alternatively, disruption of the NK1R gene could prevent normal embryonic development or migration of striatal cholinergic interneurons. However, disruption of the NK1R gene alone does not cause the reduction in ChAT expression, because it was not found in NK1R-/- mice bred from heterozygotes. This suggests that there could be other genetic modifications in homozygous NK1R-/- breeders that are causing the reduced ChAT expression. Alternatively, early environment may prevent normal development of the striatal cholinergic system. This possibility can be investigated by fostering wildtype mice to NK1R-/- breeding pairs.

Finally, in young mice, the loss of cholinergic function was found in the dorsal striatum, but in 9 month old mice there was also a loss in the ventral striatum. This could indicate that there is on-going cell death in the striatum throughout the animals' lives. However, the 9 month old mice were not treatment-naïve, having been trained and tested in the 5-CSRTT. Therefore, it would be interested to look at ChAT expression in the striatum of old, naïve mice. The dorsal striatum has been linked to motoric impulsivity, while the ventral striatum has been linked to reward impulsivity. The latter type of impulsivity is thought to mimic aspects of compulsive drug seeking in addicts. In this context an increase in impulsivity in NK1R-/- mice is surprising, because a previous study has found that NK1R antagonists can reduce alcohol craving in patients (George et al., 2008). This would suggest that NK1R antagonism reduces, rather than increases, impulsivity. However, the findings presented here help to reconcile this contradiction, suggesting that it is a loss of striatal cholinergic function, rather than disruption of the NK1R gene, that causes the increase in impulsivity. This should be further investigated by selectively lesioning striatal cholinergic cells in wildtype mice and testing them in the 5-CSRTT. It would also be interesting to test the effects of cholinergic agonists in NK1R-/- mice tested in the 5-CSRTT.

7.4.3 Other neurokinin receptors

Substance P preferentially binds to NK1R. However, disruption of the NK1R gene may not mean there is reduced substance P binding, since it also shows some binding affinity to NK2 and NK3 receptors (Maggi and Schwartz, 1997). It is possible that there has been an upregulation of these receptors to compensate for the loss of NK1R, although initial studies with NK1R-/- mice showed that there was no substance P binding in the brain (De Felipe et al., 1998). Furthermore, acetylcholine release from striatal cholinergic interneurons is modulated by all three tachykinin receptor subtypes (Preston et al., 2000). The role of other neurokinin receptors in ADHD remains to be investigated.

7.4.4 Extending the findings to the clinic

The aim of developing an animal model is to gain further understanding of a clinical disorder and to identify new ways of treating it. Imaging studies have aimed to identify biomarkers for ADHD in the brains of patients. So far, these

investigations have focused on the DA system. The findings presented here suggest that striatal cholinergic markers could identify patients with impulse control disorders. There are no radioligands for ChAT, so it cannot be measured in vivo (Bohnen and Frey, 2007). However, there are radioligands for other markers of cholinergic activity, including acetylcholinesterase, which hydrolyses acetylcholine, and vesicular acetylcholine, the acetylcholine transporter. Other radioligands can also image both nicotinic and muscarinic receptors (Bohnen and Frey, 2007). If binding studies reveal a loss of cholinergic function in patients with impulse control disorders, clinical studies should also investigate the effects of cholinergic agonists.

The deficit in perseverative responding was consistently found in NK1R-/- mice regardless of breeding method, suggesting that it is due to a lack of NK1R function. It remains to be determined how a deficit in perseverative responding translates to the clinic, and further understanding of this deficit could lead to a new therapeutic effect of NK1R agonists. The finding that disruption of the NK1R gene results in hyperactivity also suggests that NK1R agonists could alleviate hyperactive symptoms in ADHD.

7.5 Conclusions

The work presented in this thesis further investigated the NK1R-/- mouse as a model of ADHD. Hyperactivity and perseveration appear to be linked to a loss of NK1R function, suggesting that NK1R agonists may be therapeutically beneficial for these symptoms. This work also suggests that impulsivity is not caused by a loss of NK1R function, but rather by reduced striatal cholinergic function. Therefore, abnormal striatal cholinergic activity could underlie impulse control disorders. There was no consistent deficit in attention. Overall, these results suggest that the NK1R-/- mouse can be used to study specific molecular and behavioural deficits in ADHD and related disorders. This will allow further elucidation of the different biological mechanisms underlying the symptoms of ADHD, as well as other comorbid disorders.

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