The Role of the Neurokinin-1 Receptor in Behaviour and Cognition: an interaction with the Brain Renin Angiotensin System and its implications for Attention Deficit Hyperactivity Disorder

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I, Ashley James Porter, hereby confirm that the work presented in this thesis is original and my own. Where contributions have been made by others, this is stated clearly within the thesis.

Abstract

Mice lacking functional neurokinin-1 receptors (NK1R-/-) display several behavioural abnormalities that resemble Attention Deficit Hyperactivity Disorder (ADHD), including locomotor hyperactivity (which is alleviated by ADHD medication), impulsivity and inattentiveness. These findings prompted the proposal that NK1R-/- mice offer a novel murine model of ADHD. The first aim of this thesis was to investigate the influence of non-genetic (epigenetic/environmental) factors on the behaviour of NK1R-/- mice by comparing animals from two different breeding protocols. These studies revealed that certain elements of their impulsivity are influenced by non-genetic factors, but other behaviours are not. The second aim was to compare the behaviour of male and female animals, to determine whether there are sex differences in their behaviour. This study revealed that the hyperactivity of NK1R-/- mice is not evident in females, echoing typical sex-differences in ADHD patients. Following this, the behavioural effects of several drugs that target the brain renin angiotensin system (BRAS) are reported. This was prompted by preliminary evidence that the BRAS and NK1R interact in the regulation of locomotor activity. The angiotensin converting enzyme (ACE) inhibitor, captopril, abolished the hyperactivity and impulsivity of (male) NK1R-/- mice, suggesting that ACE could provide a novel therapeutic target for the treatment of ADHD. By contrast, angiotensin receptor antagonism either exacerbated or had no effect on these behaviours, suggesting that captopril's therapeutic effect is not due to a reduction in angiotensin II. Finally, the performance of NK1R-/- mice in the 5-Choice Continuous Performance Test was investigated. NK1R-/- mice displayed performance deficits only during certain phases of training, suggesting that detection of the impulsive or inattentive phenotype might require unpredictable test parameters. Overall, these studies further validate/phenotype the NK1R-/- mouse model of ADHD, and point to an interaction between the BRAS and NK1R in the regulation of ADHD-related behaviours, which warrants further investigation.

Table of Contents ***Page***

List of Figures *Page*

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables *Page*

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Appendix I

List of Abbreviations

5-CCPT	5-Choice Continuous Performance Test
5-CSRTT	5-Choice Serial Reaction-Time Task
5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1A} receptor	5-hydroxytryptamine 1A receptor
5-HT1A-/- mice	5-hydroxytryptamine 1A receptor knockout mice
AC	adenylyl cyclase
ACE	angiotensin converting enzyme
ACh	acetylcholine
ADHD	Attention Deficit Hyperactivity Disorder
Am-A	glutamyl aminopeptidase A
Am-N	alanyl aminopeptidase N
Ang(1-7)	angiotensin (1-7)
Ang(1-9)	angiotensin (1-9)
Ang(2-7)	angiotensin (2-7)
Ang(3-7)	angiotensin (3-7)
AngI	angiotensin I
AngII	angiotensin II
AngIII	angiotensin III
AngIV	angiotensin IV
ANOVA	Analysis of Variance
AT receptor	angiotensin II receptor (non-specific)
AT_1 receptor	angiotensin II type 1 receptor
AT_2 receptor	angiotensin II type 2 receptor
AT_4 receptor	angiotensin II type 4 receptor

BRAS	brain renin angiotensin system
cAMP	cyclic adenosine monophosphate
CAP / Cap	captopril
Carb-P	carboxypeptidase P
ChAT	choline acetyltransferase
CNS	central nervous system
CPT	continuous performance test
D_1 receptor	dopamine D1 receptor
D_2 receptor	dopamine D2 receptor
DA	dopamine
DAG	diacylglycerol
DAT	dopamine transporter
DAT-/- mouse	dopamine transporter knockout mouse
dNTP	deoxyribonucleotide triphosphate
DOPA decarboxylase	aromatic L-amino acid decarboxylase
DOPAC	3,4-dihydroxyphenylacetic acid
DSM	Diagnostic and Statistical Manuel of Mental Disorders
DZ	dark zone of the Light/Dark Exploration Box
EDTA	ethylenediaminetetraacetic acid
EPM	elevated plus maze
Fig.	figure
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GPCR	G-protein coupled receptor
Het	heterozygous

Hom	homozygous
i.p.	intraperitoneal
ICD	International Classification of Diseases
IGL	intergeniculate leaflet
IP_3	inositol triphosphate
ITI	intertrial interval
KO	knockout
KO-Het	NK1R-/- mice derived from heterozygous breeding pairs
KO-Hom	NK1R-/- mice derived from homozygous breeding pairs
LDEB	Light/Dark Exploration Box
LITI	Long Intertrial Interval
LOS / Los	losartan
LSD	Fisher's Least Significant Difference test
LTP	long term potentiation
LZ	light zone of the Light/Dark Exploration Box
MAP kinase	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
NA	noradrenaline
NAT	noradrenaline transporter
NI / No Inj.	no injection
NK1R	neurokinin-1 receptor
NK2R	neurokinin-2 receptor
NK3R	neurokinin-3 receptor
NK1R-/- mice	neurokinin-1 receptor gene 'knockout' mice
NKA	neurokinin A

NKB	neurokinin B
NMDA	N-methyl-D-aspartate
NOL test	novel object location test
NOR test	novel object recognition test
NKγ	neuropeptide γ
NPK	neuropeptide K
PCR	polymerase chain reaction
PD	PD 123319
PE-P	prolylendopeptidase
PFC	prefrontal cortex
PKC	protein kinase C
PLA_2	phospholipase A2
PLC	phospholipase C
PO	propyl oligopeptidase
PPT	pre-pro-tachykinin
PPT-A gene	pre-pro-tachykinin A gene
PPT-B gene	pre-pro-tachykinin B gene
RAS	renin angiotensin system
RHT	retinohypothalamic tract
RTH syndrome	Resistance to Thyroid Hormone syndrome
SCN	suprachiasmatic nucleus
SD	stimulus duration
SHR	Spontaneously Hypertensive Rat
SNAP-25	synaptosomal-associated protein 25
SP	substance P

SP(1-7)	substance P(1-7)
SSRI	selective serotonin reuptake inhibitor
TR-ß1	thyroid hormone receptor-beta 1
Veh	vehicle
VITI	Variable Intertrial Interval
VMAT-2	vesicular monoamine transporter-2
VTA	ventral tegmental area
WKY rat	Wistar Kyoto rat
WT	wildtype
WT-Het	wildtype mice derived from heterozygous breeding pairs
WT-Hom	wildtype mice derived from homozygous breeding pairs

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Chapter 1

General Introduction

1.1. Substance P and NK1R

1.1.1. Background

Substance P was first discovered by Von Euler and Gaddum (1931), who reported that extracts from equine brain and intestine tissue caused contraction of smooth muscle and hypotension. It was named by Gaddum and Schild in 1934, and it is thought that the 'P' originates from 'preparation' or 'powder' written in a laboratory notebook. Following decades of failed attempts, it was almost 40 years later that Chang and Leeman (1970) successfully isolated and purified this substance, this time in bovine hypothalamus, and identified 'substance P' as an undecapeptide with the amino acid sequence: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.

Substance P belongs to a family of neuropeptides called the tachykinins (Table 1.1; for reviews, see: Page, 2004; Pennefather et al., 2004). Substance P was the first of these to be discovered, but was later followed by the discovery of neurokinin A (NKA) and neurokinin B (NKB) (Kangawa et al., 1983; Kimura et al., 1983; Nawa et al., 1983; Minamino et al., 1984). Following this, two biologically active extended forms of NKA, termed neuropeptide K (NPK) and neuropeptide γ (NPγ), as well as a truncated form of NKA, termed NKA(3-10), were also identified (Tatemoto et al., 1985; Kage et al., 1988; MacDonald et al., 1989). More recently, a separate group of tachykinins, termed endokinins, has also been discovered (Zhang et al., 2000; Kurtz et al., 2002; Page et al., 2003). All tachykinins, by definition, share the common amino acid sequence: Phe-X-Gly-Leu-Met-NH_2, where X is either a β-branched aliphatic or an aromatic hydrophobic amino acid residue. It is this hydrophobic sequence that is central to the activation of the three known tachykinin receptors (Cascieri et al., 1992; Maggi, 1995). By contrast, their divergent hydrophilic regions are thought to underlie the differences in specificity that each of these peptides express for these receptors (Ingi et al. 1991).

Tachykinin:		**Amino Acid Sequence:**
Neurokinin	Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
	Neuropeptide K	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	Neuropeptide γ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	Neurokinin A(3-10)	Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Endokinins (human)	Endokinin A	Asp-Gly-Gly-Glu-Glu-Gln-Thr-Leu-Ser-Thr-Glu-Ala-Glu-Thr-Try-Val-Ile,Val-Ala-Leu-Glu-Glu-Gly-Ala-Gly-Pro-Ser-Ile-Gln-Leu-Gln-Leu-Gln-Glu-Val-Lys-Thr-Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	Endokinin B	Asp-Gly-Gly-Glu-Glu-Gln-Thr-Leu-Ser-Thr-Glu-Ala-Glu-Thr-Try-Glu-Gly-Ala-Gly-Pro-Ser-Ile-Gln-Leu-Gln-Leu-Gln-Glu-Val-Lys-Thr-Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	Endokinin A/B	Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	Hemokinin-1 (human)	Thr-Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	Hemokinin-1(4–11)	Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Endokinins (rat/mouse)	Hemokinin-1 (rat/mouse)	Arg-Ser-Arg-Thr-Arg-Gln-Phe-Tyr-Gly-Leu-Met-NH_2
Endokinins (rabbit)	Endokinin-1	Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Table 1.1. The tachykinin family of peptides
(adapted from: Page, 2004)

All tachykinins are encoded by three genes: pre-pro-tachykinin A (PPT-A), pre-pro-tachykinin B (PPT-B), and pre-pro-tachykinin C (PPT-C) (Figure 1.1; see: Pennefather et al., 2004). Alternative splice variants of the PPT-A gene encode for four different types of mRNA (α-PPT-A, ß-PPT-A, γ-PPT-A, and δ-PPT-A). Substance P is encoded by all four, NKA is encoded by ß-PPT-A and γ-PPT-A, NPK is encoded by ß-PPT-A and NPγ is encoded by γ-PPT-A. By contrast, the PPT-B gene encodes for NKB, only. Alternative splice variants of PPT-C result in four different types of mRNA (α-PPT-C, ß-PPT-C, γ-PPT-C and δ-PPT-C), which produce different forms of the endokinin peptides.

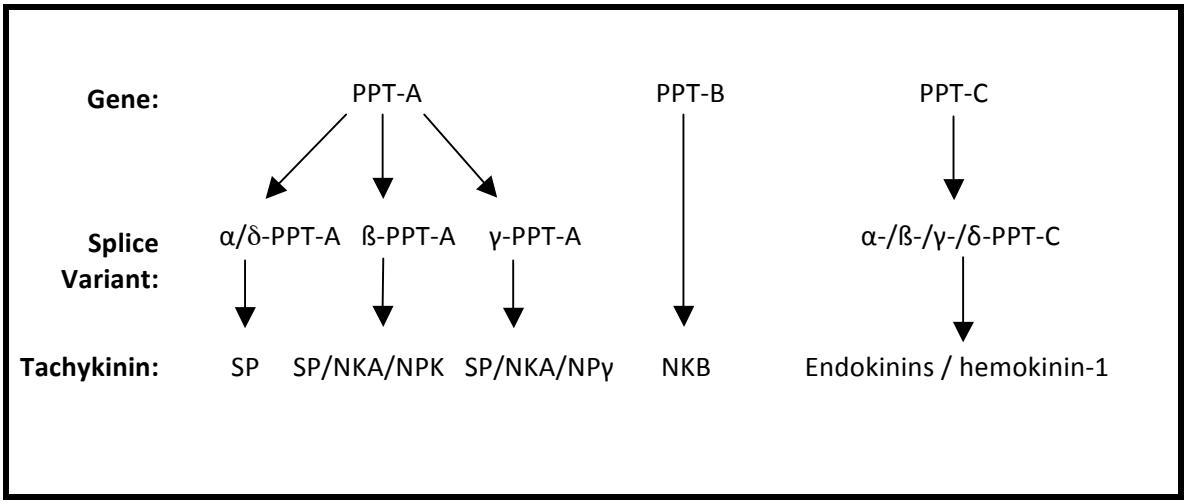

Figure 1.1. The biosynthesis of mammalian tachykinins
PPT, pre-pro-tachykinin; SP, substance P; NKA, neurokinin A;
NKB, neurokinin B; NPK, neuropeptide K; NPγ, neuropeptide γ

1.1.2. Tachykinin receptors

The tachykinins exert their physiological and behavioural effects through interaction with specific membrane-bound tachykinin receptors, which belong to the large family of 7 transmembrane G-protein coupled receptors (GPCRs). To date, three different tachykinin receptors have been

identified in mammals: neurokinin-1 (NK1R), neurokinin-2 (NK2R) and neurokinin-3 (NK3R) (Regoli et al., 1994; Maggi, 1995). Whilst each tachykinin is capable of binding to all three receptor subtypes, each neuropeptide binds with moderate selectivity to specific receptor subtypes over others: substance P binds preferentially to NK1R; neurokinin A binds preferentially to NK2R; and neurokinin B binds preferentially to NK3R (Maggi, 1995; Maggi and Schwartz, 1997) (Table 1.2).

Tachykinin Receptor	Rank order of specificity
NK1R	SP ≥ NKA > NKB
NK2R	NKA > NKB > SP
NK3R	NKB > NKA > SP

Table 1.2. Specificity of the tachykinin peptides to different receptor subtypes.

Activation of NK1R by substance P is coupled to three different second messenger systems, depending on the alpha subunit of the receptor (see: Quartara and Maggi, 1997) (Figure 1.2). $G_{\alpha s}$ activates adenylyl cyclase, which results in the production of cyclic adenosine monophosphate (cAMP). $G_{\alpha o}$ activates phospholipase A_2, which results in the release of arachidonic acid through the hydrolysis of phospholipids on the cell membrane. Arachadonic acid, in turn, is involved in the regulation of signalling enzymes such as phospholipase C (PLC) and protein kinase C (PKC). G_{q11} activates PLC, which results in the production of inositol triphosphate (IP_3) and diacylglycerol (DAG), which in turn cause an increase in intracellular calcium and the production PKC, respectively.

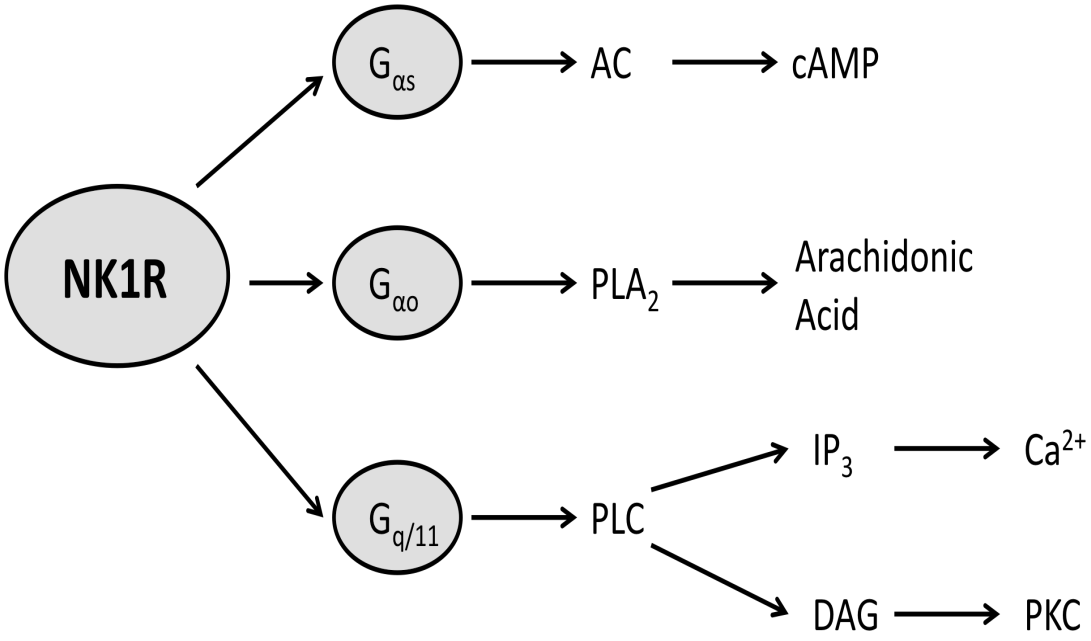

Figure 1.2. Intracellular signalling linked with NK1R
AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PLA_2, phospholipase A2; PLC, phospholipase C; IP_3, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C

1.1.3. Distribution of substance P and NK1R within the central nervous system

In mammals, substance P is the most abundant of the tachykinin peptides, and is widely distributed across the central, peripheral and enteric nervous systems (for review, see: Severini et al., 2002). The distribution of substance P was first described by Hökfelt et al. (1975), and there have since been a number of reports that have provided a comprehensive description of substance P distribution in both the central (Ljungdahl et al., 1978; Cuello and Kanazawa, 1978; Ribeiro-da-Silva and Hokfelt, 2000) and peripheral (Costa et al., 1980; Dalsgaarg et al., 1982) nervous systems. The distribution of NK1R has been determined using a number of different methods, such as autoradiography (Shults et al., 1982; Mantyh et al., 1984; Saffroy et al., 2003), in situ hybridisation (Maeno et al., 1993; Caberlotto et al., 2003), and immunohistochemistry

(Nakaya et al., 1994). The focus of the following section is the distribution of substance P and NK1R within the central nervous system (CNS).

In the CNS, substance P and NK1R are concentrated in regions of the brain responsible for governing mood, cognition and motor control. The density of substance P is particularly high, for example, in the monoaminergic nuclei of the brainstem. These include the noradrenergic locus coeruleus, the serotonergic dorsal raphé nucleus, and the dopaminergic ventral tegmental area and substantia nigra, with the density of substance P particularly high within the substantia nigra pars reticulata (Ljungdahl et al., 1978; Ribeiro-da-Silva and Hokfelt, 2000). NK1R are similarly expressed in both the locus coeruleus and the dorsal raphé nucleus: in the locus coeruleus, NK1R are found on all noradrenergic neurons (Chen et al., 2000); in the dorsal raphé nucleus, NK1R are found on both serotonergic neurons, and on local glutamatergic neurons which modulate serotonergic activity (Liu et al., 2002; Lacoste et al., 2006). Interestingly, despite high levels of substance P in the substantia nigra, few (if any) NK1R are expressed in this region (Nakaya et al., 1994). This provides an example of how there can be a mismatch between the distribution of substance P and NK1R, suggesting that volume transmission plays an important role in the function of this neuropeptide.

High densities of substance P and NK1R are also found within the striatum, a region strongly implicated in motor control, habit formation, action selection/inhibition, and reward. Striatal substance P is found within GABAergic medium spiny output neurons, which send projections to regions such as the substantia nigra and globus pallidus (Anderson and Reiner, 1990), as well as collaterals to striatal cholinergic interneurons, where NK1R expression is high (Bolam et al., 1986; Gerfen, 1991). Other regions that express substance P and NK1R include the amygdala, hypothalamus and habenula (Iversen et al., 1976; Emson et al., 1977, 1978; Nakaya et

al., 1994). Substance P is also found in moderate densities in the medial prefrontal cortex, but NK1R density is generally low to moderate in cortical areas (Mantyh et al., 1984).

1.1.4. Roles of substance P and NK1R

Substance P and NK1R have been implicated in a wide variety of functions. In terms of their physiological response, substance P has been shown to act as a potent vasodilator (Klede et al., 2003), to stimulate cell growth (Reid et al., 1993), and to regulate glucose metabolism (Karagiannides et al., 2011). However, the most widely known role of substance P and NK1R is arguably in nociception (although, as discussed below, NK1R antagonists have proved ineffective as analgesics). NK1R antagonists are also already licensed for the treatment of emesis. The presence of substance P and NK1R in the limbic system and basal ganglia has prompted intensive research into their possible role in a wide variety of CNS disorders, such as anxiety, depression, substance dependence and schizophrenia. The following section summarises the main roles of substance P and NK1R known to date, finishing with a particular emphasis on psychiatric disorders.

1.1.4.1. Sensory system

As mentioned, one of the most well known roles of substance P is in sensory transmission, in particular nociception. Substance P is found in high concentrations in the dorsal horn of the spinal cord, where it is expressed by primary sensory afferent neurons (Hokfelt et al., 1975). Application of substance P to the dorsal horn specifically excites neurons that are activated by noxious stimuli (Henry, 1976). Furthermore, peripheral noxious stimulation (mechanical, thermal or chemical) increases the release of substance P within this spinal region (Duggan et al., 1988). A number of

studies have also demonstrated that intrathecal injection of either substance P or its related analogues increases sensitivity to noxious stimulation (Yasphal et al., 1982; Moochhala and Sawynok, 1984; Ma and Woolf, 1995). It is now widely accepted that substance P is involved in the process of "central sensitisation" (in response to noxious stimuli) and the resulting allodynia/hyperalgesia (e.g., Laird et al., 1993; Ma and Woolf, 1995; Neumann et al., 1996).

These observations led to the proposal that NK1R antagonists might be analgesic. In support of this, intravenous administration of an NK1R antagonist attenuates the nociceptive response (paw licking) to intraplantar formalin injection (Rupniak et al., 1996). Intracerebroventricular administration of an NK1R antagonist blocks the hypersensitivity to mechanical stimulation induced by nerve ligation (Cumberbatch et al., 1998). Electrophysiological studies have also shown that NK1R antagonists specifically prevent the slow, prolonged excitatory postsynaptic potential caused by noxious peripheral or prolonged electrical stimulation, but not the response to innocuous or single electrical stimulation (De Koninck and Henry, 1991). Furthermore, ablation of NK1R-expressing neurons in the lamina 1 region of the dorsal horn markedly attenuates the mechanical and thermal hyperalgesia caused by intraplantar capsaicin (Mantyh et al., 1997). Interestingly, mice lacking functional neurokinin 1 receptors (discussed below) do not exhibit any differences in pain sensitivity at baseline, but do show a loss of stress-induced analgesia (De Felipe et al., 1998).

Despite initial optimism from the above preclinical evidence, NK1R antagonists have proved disappointing in clinical trials (for review, see: Borsook et al., 2012). However, it has been proposed that NK1R antagonists could be used as adjuvants to existing analgesics, especially when other stress-related factors are involved (Hill, 2000).

1.1.4.2. Emesis

Within the brain stem, the nucleus tractus solitarius and the area postrema are two regions that are involved in the control of emesis. Both regions contain a high concentration of substance P (Armstrong et al., 1981), prompting interest in whether NK1R antagonists might be useful as antiemetics. Application of substance P into the area postrema induces retching in ferrets, whereas NK1R antagonists attenuate retching induced by a broad range of emetogens (Bountra et al., 1993; Tattersall et al., 1994; Gardner et al., 1995; see: Rupniak and Kramer, 1999). Nausea and vomiting are common side-effects in patients undergoing chemotherapy. In preclinical studies, pretreatment with an NK1R antagonist prevents emesis induced by the chemotherapy agent cisplatin (Rudd et al., 1996; Tattersall et al., 1996) and blocks conditioned taste aversion induced by apomorphine and amphetamine (McAllister and Pratt, 1998). Prompted by this evidence, a number of clinical trials have now been carried out which have demonstrated both the safety and efficacy of NK1R antagonists in the treatment of chemotherapy-induced nausea and vomiting (e.g., Abidi et al., 2012; for review, see: Munoz and Covenas, 2014). As a result, two NK1R antagonists (aprepitant and fosaprepitant) are currently licensed for the prevention of emesis in cancer patients.

1.1.4.3. Psychiatric disorders

1.1.4.3.1. Stress and anxiety

There have been many studies that have investigated the effects of central substance P administration on anxiety-related behaviours. For example, increases in anxiety-related behaviours have been reported following intracerebroventricular injection of substance P in both rats and mice (Teixeira et al., 2004; Duarte et al., 2004) and following local administration into the

periaqueductal grey (De Araujo et al., 1999, amygdala (Ebner et al., 2004) and the lateral septal nucleus (Gavioli et al., 1999) of rats. Conversely, systemic administration of the NK1R antagonist, GR-205171, has been found to have anxiolytic effects in the elevated plus maze and a contextual fear test in gerbils (Heldt et al., 2009). There is also evidence that the NK1R antagonist, L-703606, reduces aggressiveness and violence in rats (Halasz et al., 2008), whilst the NK1R antagonist, L-760735, attenuates fear conditioning in gerbils (Rupniak et al., 2003). However, other studies in both rats and mice have also found no effects of NK1R antagonism on anxiety (e.g., Rodgers et al., 2004; Malkesman et al., 2007).

There is also evidence for changes in substance P content in a number of discrete brain regions following a bout of stress. For example, an increase in substance P immunoreactivity in rats has been reported in the periaqueductal grey following extended periods of isolation, subcutaneous saline injection or following restraint stress (Rosen et al., 1992; Brodin et al., 1994) as well as in the dentate gyrus and medial septum following foot shock (Siegel et al., 1984). In the basolateral amygdala of the guinea pig, an increase in NK1R receptor internalization has also been reported following neonatal separation, indicating increased release of endogenous substance P (Kramer et al., 1998). Furthermore, NK1R antagonism in rats attenuates the increase in Fos expression in response to stress exposure in brain areas such as the locus coeruleus, periaqueductal gray, and prefrontal cortex (Hahn and Bannon, 1999; Baulmann et al., 2000).

However, despite considerable preclinical evidence that substance P and NK1R are involved in the physiological response to stress and anxiety, a number of studies have found that treatment with an NK1R antagonist is ineffective at alleviating symptoms of anxiety in humans, including a large clinical trial of the NK1R antagonist LY686017 (Tauscher et al., 2010; Mathew et al., 2011; Michelson et al., 2013).

1.1.4.3.2. Depression

In a broad range of preclinical tests, the effects of NK1R antagonists resemble those of established antidepressants. In the chronic mild stress model in rats, treatment with an NK1R antagonist resulted in reduced anhedonia, an effect that was comparable to that of imipramine but with a faster onset of action (Papp et al., 2000). Treatment with an NK1R antagonist also decreases the duration of immobility in both rats and mice in the forced swim test (Zocchi et al., 2003; Dableh et al., 2005) and in gerbils in the tail suspension test (Varty et al., 2003). In guinea pig and mouse pups, NK1R antagonists have also been shown to reduce vocalisation caused by maternal separation with similar efficacy as the selective serotonin reuptake inhibitor (SSRI), fluoxetine (Molewijk et al., 1996; Rupniak et al., 2000).

In general, evidence suggests that a deficit in functional NK1R augments serotonergic transmission (see: Stanford, 2014). For example, serotonergic efflux in the frontal cortex of mice is increased by administration of an NK1R antagonist (Guiard et al., 2005). Furthermore, both long- and short-term treatment with an NK1R antagonist in mice causes an increase in the spontaneous firing activity of serotonergic neurons in the dorsal raphé nucleus (Santarelli et al., 2001). This was also associated with selective desensitisation of 5-HT_{1A} autoreceptors, resembling chronic antidepressant treatment (Santarelli et al., 2001).

The influence of NK1R on noradrenergic and dopaminergic transmission is less clear. In the frontal cortex of rats, administration of an NK1R antagonist has been found to increase both noradrenergic (Millan et al., 2001) and dopaminergic (Lejeune et al., 2002) transmission. However, another study investigating the same brain region in mice found no effect of NK1R antagonism on either of these neurotransmitters (Zocchi et al., 2003). Other studies in rats and gerbils have found that NK1R antagonism attenuates the noradrenergic and dopaminergic response to stress (Hutson

et al., 2004; Renoldi and Invernizzi, 2006). One possibility is that NK1R antagonism increases neurotransmission during low states of arousal but decreases transmission during high states of arousal (see: Stanford, 2014). Whatever the precise role, it is clear from this evidence that substance P and NK1R regulate monoaminergic transmission in brain regions implicated in depression.

In two separate reports, an increase in substance P concentration has been identified in the cerebrospinal fluid of patients with major depressive disorder (Rimon et al., 1984; Geracioti et al., 2006). A post-mortem study also found a reduction in NK1R binding in the prefrontal cortex of depressed patients (Stockmeler et al., 2002). In an early clinical study, patients with moderate to severe depression were treated with the NK1R antagonist aprepitant, which produced antidepressant effects similar to paroxetine, but with reduced side effects (Kramer et al., 1998). This was later replicated with a second NK1R antagonist, L-759274 (Kramer et al., 2004). However, despite initial optimism that NK1R antagonists might provide a novel therapeutic target for depression, in a subsequent clinical trial that compared the effects of aprepitant to paroxetine, aprepitant failed to show any effects in patients with major depressive disorder (Keller et al., 2006). Nonetheless, it is obvious that substance P and NK1R interact with the function of several neurotransmitters that are targeted by established antidepressants, and so research into their possible role in mood disorders is ongoing.

1.1.4.3.3. Schizophrenia

Established antipsychotics such as amisulpride or aripiprazole are thought to act through the mesolimbic dopamine system. In rat and mouse brain, substance P is found in high concentration in nerve terminals that form synaptic contact with dopaminergic neurons of the ventral tegmental

area (VTA) (Brownstein et al., 1976; Kanazawa and Jessel, 1976; Tamiya et al., 1990). There is evidence from studies using rats that substance P stimulates dopamine release from mesolimbic and mesocortical projection neurons: local administration of substance P into the VTA increases the concentration of dopamine and its metabolites in both the nucleus accumbens and prefrontal cortex (Deutch et al., 1985; Cador et al., 1989; Barnes et al., 1990c). By contrast, systemic administration of an NK1R antagonist decreases the spontaneous firing activity of dopaminergic neurons in rat ventral tegmental area (Minabe et al., 1996). Furthermore, treatment of rats with antipsychotic drugs, such as chlorpromazine, haloperidol, clozapine or sulpride, modifies the expression of PPT mRNA in several regions that are rich in dopaminergic innervation, such as the dorsal striatum and nucleus accumbens (Shibata et al., 1990).

However, there are no apparent differences in the concentration of substance P in the cerebrospinal fluid of patients suffering with schizophrenia compared with medical controls (patients with no family history of psychiatric illness, but who required a neurological examination for other reasons) (Miller et al., 1996). Furthermore, an exploratory, four-week placebo- and haloperidol-controlled clinical trial of inpatients with schizophrenia also found that the NK1R antagonist aprepitant lacks efficacy in schizophrenic patients (Rupniak and Kramer, 1999).

1.1.4.3.4. Drug addiction and reward

Finally, there is considerable interest in the role of substance P and NK1R in reward and addiction. This is supported by their presence in, and modulation of, brain regions associated with reward, such as the mesolimbic pathway (see above). A number of studies have found that NK1R activation has a positive reinforcing effect on behaviour. For example, studies in rats found that injection of substance P into the amygdala, globus pallidus and the nucleus basalis magnocellularis, produced

a positive reinforcing effect in a conditioned place preference paradigm, an effect which is blocked by pretreatment with an NK1R antagonist (Kertes et al., 2009, 2010; Hasenohrl et al., 1998). Injection of substance P into the ventromedial caudate putamen of rats has also shown positive reinforcing effects in a self-administration paradigm (Krappman et al., 1994). Finally, local infusion of the substance P analogue DiMe-C7 into the ventral tegmental area induces the reinstatement of cocaine-seeking behaviour in rats following a period extinction (Placenza et al., 2004).

Conversely, several studies have shown that disruption to NK1R function can lead to abnormalities in the reinforcing effects of drugs known to be rewarding. For example, co-administration of an NK1R antagonist abolishes conditioned place preference in mice treated with either morphine or cocaine (Jasmin et al., 2006). Bilateral ablation of NK1R-expressing neurons in the amygdala reduces conditioned place preference for morphine in mice (Gadd et al., 2003), whereas treatment with an NK1R antagonist in mice attenuates the rewarding effects of morphine in an intracranial self-stimulation protocol (Robinson et al., 2012). Intracerebroventricular injection of NK1R antagonists also reduced certain naloxone-induced withdrawal symptoms in rats (Maldonado et al., 1993). Treatment with an NK1R antagonist also reduces voluntary alcohol intake in mice (Thorsell et al., 2010), as well as suppresses the increase in alcohol self-administration observed in rats following stress (Schank et al., 2011).

The above findings led to a randomised controlled study, which found that the NK1R antagonist LY686017 suppressed cravings and improved feelings of wellbeing in recently detoxified alcoholic patients (George et al., 2008). Furthermore, fMRI revealed that NK1R antagonism seemed to benefit the brain's response to affective (negative and positive) stimuli (George et al., 2008). NK1R antagonism has been proposed as a novel treatment for both alcohol and opiate dependence (George et al., 2008; Walsh et al., 2013).

1.1.5. Neurokinin-1 receptor knockout ('NK1R-/-') mice

A common and useful way of investigating the role of a protein is to create a genetically modified 'knockout' mouse strain in which the gene encoding for that protein has been inactivated. In order to investigate the role of NK1R, de Felipe et al. (1998) created a mouse strain with functional disruption to the neurokinin-1 receptor gene ('NK1R-/- mice'). These mice were derived using homologous recombination on a 129/Sv x C57BL/6 genetic background. These were subsequently crossed, once, with an outbred MF1 strain to dilute the 129/Sv component, as 129/Sv mice demonstrate deficits in cognitive tests such as the Morris water maze (Wolfer et al., 1997). Whilst both genotypes derive from the same genetic background, NK1R-/- mice and their wildtype counterparts (NK1R+/+) have been maintained as separate, inbred homozygous lines. This breeding approach has the ethical benefit of reducing over-breeding, but there is the risk that genetic drift, or differences in their early life environment (e.g., the quality of maternal care and/or interaction with littermates), is influencing their behavioural phenotype. This forms the basis of some of the work presented in this thesis, and is discussed in more detail in subsequent chapters.

Since their creation, NK1R-/- mice have been studied extensively in relation to a wide variety of CNS disorders, such as pain (de Felipe et al., 1998), addiction (Murtra et al., 2000; Ripley et al., 2002; George et al., 2008), and depression (Rupniak et al., 2000, 2001). However, more recently, a series of interesting findings has led to the proposal that NK1R-/- mice offer a novel murine model of Attention Deficit Hyperactivity Disorder (ADHD) (for review, see: Yan et al., 2009). The following section provides a summary of the previous research carried out on NK1R-/- mice that led to this proposal.

1.1.5.1 Early behavioural testing in NK1R-/- mice – a model of 'antidepression'

In 2000, Rupniak and colleagues demonstrated that stress-induced vocalisation, caused by maternal separation, was markedly reduced in NK1R-/- mice compared with their wildtype counterparts, mimicking the effects of several established anxiolytic and antidepressant drugs (Rupniak et al., 2000). Similarly, it was shown that NK1R-/- mice display increased struggling in the tail suspension test, reduced immobility in the forced swim test, and increased attack latency in the resident intruder test (Rupniak et al., 2001). Again, this resembled the effects of antidepressant drugs. These results were in line with evidence (outlined above) that NK1R antagonists might be antidepressant (Kramer et al., 1998). Based on this evidence, it was suggested that NK1R-/- mice might provide a model of 'antidepression', and this prompted a series of studies investigating the monoaminergic function of these animals.

1.1.5.2. Serotonergic function in NK1R-/- mice

Early reports found that the firing-rate of serotonergic neurons in the dorsal raphé nucleus was increased by more than 2-fold in NK1R-/- mice compared with their wildtype counterparts (Santarelli et al., 2001). Furthermore, the potency of the $5HT_{1A}$ receptor agonist ipsapirone in inhibiting firing of serotonergic firing in the dorsal raphé nucleus was reduced approximately 10-fold in NK1R-/- mice (Froger et al., 2001). Cortical serotonergic efflux following treatment with the SSRI paroxetine was also 4- to 6-fold higher in freely moving NK1R-/- mice compared with wildtypes, whilst radioligand binding of the $5HT_{1A}$ receptor found that receptor density was significantly reduced in the dorsal raphé nucleus of NK1R-/- mice, as was the concentration of $5HT_{1A}$ mRNA in the anterior raphé area (Froger et al., 2001). It was concluded that NK1R

dysfunction is associated with selective downregulation/desensitisation of $5HT_{1A}$ autoreceptors, resembling the effects of chronic antidepressant treatment.

Following this, Guiard et al. (2007) investigated the effects of intraraphé injection of substance P on serotonin efflux in the frontal cortex, ventral hippocampus and dorsal raphé nucleus (Guiard et al., 2007). Substance P dose-dependently decreased cortical serotonin efflux, an effect that was blocked by NK1R antagonism and completely absent in NK1R-/- mice. This finding suggests that activation of NK1R normally blunts serotonergic efflux, and is line with the above evidence that NK1R antagonism increases serotonergic transmission. In the same study, genetic and pharmacological inactivation of $5HT_{1A}$ autoreceptors, through the use of 5HT1A-/- mice and $5HT_{1A}$ antagonism, blocked the response to substance P, indicating that substance P limits cortical serotonin efflux through (indirect) activation of $5HT_{1A}$ autoreceptors.

1.1.5.3. Noradrenergic function in NK1R-/- mice

At the same time, two studies were carried out that investigated noradrenergic function in anaesthetised NK1R-/- mice (see: Herpfer et al., 2005; Fisher et al., 2007). Initially, *in vivo* microdialysis of anaesthetised animals revealed that, in the prefrontal cortex, noradrenaline was increased 2- to 4-fold at baseline. However, systemic administration of the noradrenaline reuptake inhibitor, desipramine, increased noradrenaline efflux in this brain region to a similar extent in both NK1R-/- and wildtype mice. Furthermore, western blot analysis confirmed that, in both the locus coeruleus and frontal cortex, the amount of noradrenaline reuptake transporter protein is similar in both genotypes. These results suggest that the increased noradrenaline in the prefrontal cortex of NK1R-/- mice is caused by increased transmitter release, rather than reduced clearance.

Infusion of the α_2-adrenoceptor antagonist, RX 821002, into the prefrontal cortex using retrodialysis increased noradrenaline to a similar extent in both NK1R-/- and wildtype mice, suggesting that α_2-adrenoceptors on nerve terminals in the prefrontal cortex of NK1R-/- mice are not functionally impaired. Western blot analysis and radioligand binding further revealed that the density of α_{2A}-adrenoceptors is not reduced in either the frontal cortex or locus coeruleus of NK1R-/- mice. Finally, binding of [^{35}S]GTPgammaS to activated α_{2A}-adrenoceptors did not differ in the frontal cortex, but was reduced by 70% in the locus coeruleus. Combined, these findings suggest that somatodendritic α_{2A}-adrenoceptors in the locus coeruleus are present, but functionally desensitised, in anaesthetised NK1R-/- mice (Herfper et al., 2005; Fisher et al., 2007).

1.1.5.4. Dopaminergic function in NK1R-/- mice, the response to *d*-amphetamine and further behavioural abnormalities

In the prefrontal cortex, basal dopaminergic efflux is reduced by more than 50% in NK1R-/- mice compared with wildtypes (Yan et al., 2010). Moreover, this study found that treatment with an NK1R antagonist gradually reduces the efflux of dopamine in wildtype mice to that seen in NK1R-/- mice. Whilst basal dopamine efflux in the dorsal striatum was similar in both genotypes, an increase in striatal dopamine in response to amphetamine was absent in NK1R-/- mice. Furthermore, pretreatment with an NK1R antagonist prevented the increase in striatal dopamine efflux in response to amphetamine in wildtype mice.

In the same study, the effects of the psychostimulants, *d*-amphetamine and methylphenidate, on the behaviour of NK1R-/- mice were investigated (Yan et al., 2010). Previous reports had shown that the locomotor activity of NK1R-/- mice was greater than that of their wildtype counterparts (Herpfer et al., 2005; Fisher et al., 2007), so two possible outcomes were

predicted: (a) the locomotor activity of NK1R-/- mice would be at a ceiling, and so the psychostimulants would increase the locomotor activity of wildtype mice only; or (b) the psychostimulants would increase the locomotor activity of both genotypes (see: Yan et al., 2009). In fact, the hyperactivity of NK1R-/- mice was blunted by both *d*-amphetamine and methylphenidate (Yan et al., 2010). This, combined with the fact that NK1R-/- mice were hyperactive, provided the first evidence that NK1R-/- mice display behavioural abnormalities that echo those in patients with ADHD.

This prompted a case-control genetic study of 450 ADHD patients and 600 healthy controls investigating the *TACR1* gene (the human equivalent of the mouse *Nk1r* gene), which found that four polymorphisms of this gene were strongly associated with the disorder (Yan et al., 2010). Following this, NK1R-/- mice were tested using the 5-Choice Serial Reaction-Time Task (5-CSRTT), a commonly used test in rodents that measures both attentional performance and impulse control (Robbins, 2002). It was found that NK1R-/- mice displayed both greater impulsivity and greater inattentiveness than their wildtype counterparts (Yan et al., 2011). Consequently, NK1R-/- mice displayed all three core behavioural features of ADHD: hyperactivity, inattentiveness, and impulsivity. As a result of these studies, it has been proposed that polymorphism of the *TACR1* gene could contribute to the development of ADHD symptoms (Yan et al., 2010; Sharp et al., 2014). Further investigation of the NK1R-/- mouse model of ADHD forms the basis of the work presented in this thesis, and the following section provides an overview of the disorder.

1.2. Attention Deficit Hyperactivity Disorder

1.2.1. Background

Attention Deficit Hyperactivity Disorder (ADHD) is a highly debilitating neurobehavioural disorder with an estimated worldwide prevalence of 8 – 12% (Faraone et al., 2003). Internationally, there are two classification systems used to diagnose ADHD: the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases (ICD). In both, diagnosis is based around three core behavioural symptoms: hyperactivity, impulsivity and inattentiveness. Epidemiological studies have demonstrated that male sex, lower socioeconomic status, and young age are all associated with a greater risk of suffering from ADHD (Scahill and Schwab-Stone, 2000; Doyle, 2004). However, ADHD is prevalent in both males and females, and is increasingly recognised as a disorder that persists into adulthood (Nutt et al., 2007).

Despite being one of the most widely studied psychiatric disorders in children, the cause(s) of ADHD remain unknown. There are four drugs currently licensed for the treatment of ADHD (discussed below). However, approximately 10 – 30 % of ADHD patients either do not respond to these or they do not tolerate them well (Banaschewski et al., 2004). There are also occasionally marked side-effects (such as insomnia, loss of appetite, and motor tics), and the long-term consequences of repeated psychostimulant administration on brain development remains a major concern (see: Fone and Nutt, 2005). Consequently, there is a clear and urgent unmet need for a better understanding of the underlying pathophysiology of ADHD and the development of therapeutic alternatives.

1.2.2. Current treatments

In 2008, the National Institute of Clinical Excellence (NICE) published guidelines for the diagnosis and treatment of ADHD across the lifespan (NICE, 2008). These guidelines detail how the recommended course of treatment depends on factors such as patient age and symptom severity. For very young patients or those with only moderate ADHD symptoms, first-line treatment includes non-pharmacological approaches such as cognitive and behavioural therapy or parent/education intervention. However, for adult patients or those with more severe ADHD symptoms, the first-line treatment is always pharmacological medication. In turn, the choice of drug depends on factors such as side effects, coexisting conditions and abuse liability. The guidelines also outline the importance of the transition from childhood/adolescence services to adult services, and the ongoing monitoring of patient response and any possible side-effects. The NICE guidelines are discussed in detail as part of the evidence-based guidelines for the treatment of ADHD provided by the British Association for Psychopharmacology (Bolea-Alamanac et al., 2014).

All currently licenced drugs used in the treatment of ADHD target monoaminergic transmission. These include: the psychostimulants, *d*-amphetamine (e.g., Adderall) and methylphenidate (e.g., Ritalin); the α_2-adrenoceptor agonist, guanfacine (Tenex); and the selective noradrenaline reuptake inhibitor, atomoxetine (Strattera) (Fone and Nutt, 2005; Nutt et al., 2007). Tricyclic antidepressants are also sometimes used as second-choice drugs, but are less effective and can potentially cause cardiotoxicity (Kutcher et al., 2004; Elia et al., 1999; Wilens et al., 2002). Clonidine is an α_2-adrenoceptor agonist that is sometimes used, but is associated with more marked side-effects, such as hypotension and sedation. Finally, the antidepressant buproprion is

also sometimes prescribed, but mainly for those suffering with comorbid depression (Wilens et al., 2002).

Psychostimulants remain the first drug of choice and the most commonly prescribed treatment. However, the mechanism of action of *d*-amphetamine and methylphenidate are quite different (Fone and Nutt, 2005; see: Stanford, 2014). Both bind preferentially to the noradrenergic (NAT) and dopaminergic (DAT) transporters to prevent reuptake into the nerve terminal. *D*-amphetamine interacts with the substrate site of the transporter and competes with the monoamines for reuptake into the nerve terminal. Methylphenidate binds to a distinct, possibly overlapping, site and is not transported across the terminal membrane. Once within the nerve terminal, *d*-amphetamine also interacts with the vesicular monoamine transporter-2 (VMAT-2), which is responsible for sequestering neurotransmitters present within the cytoplasm of the nerve terminal. At higher doses (not used clinically) amphetamine inactivates VMAT2, which leads to leakage of transmitter into the neuronal cytosol from where it is extruded from the neurones by reverse transport. This process occurs independently of neuronal firing, bypassing normal auto-regulatory mechanisms and causing a more rapid increase in extracellular monoamine following treatment with *d*-amphetamine compared with methylphenidate. This is thought to explain the increased liability for abuse with *d*-amphetamine over methylphenidate.

1.2.3. Aetiology of ADHD

1.2.3.1. Aetiology of ADHD – neuronal circuits

It is widely believed that ADHD involves abnormalities in cortico-limbic-striatal circuitry (for reviews, see: Winstanley et al., 2006; Dalley et al., 2008; Sharma and Couture, 2014). This proposal is supported by a number of neuroimaging studies of patients with ADHD, which have found abnormalities in the volume, activation, and asymmetry of several areas across this network (Casey et al., 1997; Vaidya et al., 1998; Rubia et al., 1999; Hesslinger et al., 2002; Uhlikova et al., 2007; Dang et al., 2014). For instance, studies using functional MRI have demonstrated that, under baseline (resting) conditions, patients with ADHD show reduced connectivity between cortical regions such as the anterior and posterior cingulate cortex, as well as across fronto-temporal, fronto-striato-thalamic and sensorimotor networks (Cao et al., 2009; Wang et al., 2009; Fair et al., 2010; Sun et al., 2012; Sato et al., 2013). Furthermore, these abnormalities are subtype-specific, since patients of the Predominantly Inattentive Subtype show different abnormalities to those of the Combined Subtype (Fair et al., 2013).

Abnormalities in the activation and connectivity of various brain regions within this network have also been demonstrated during the performance of various cognitive tasks. For instance, patients with ADHD show: reduced functional connectivity between regions such as the right inferior frontal cortex, parietal lobe, cerebellum and basal ganglia during the performance of a continuous performance task (Rubia et al., 2009); reduced activation of regions such as the basal ganglia, anterior cingulate gyrus and ventral prefrontal cortex during performance of a Go/No-go task (Durston et al., 2003); and reduced activation of regions such as the left inferior occipital lobe and cerebellum during performance of a verbal working memory task (Valera et al., 2005).

Functional neuroimaging studies have also demonstrated how these abnormalities can be corrected by treatment with ADHD medication. For instance, acute treatment with methylphenidate has been shown to normalize functional abnormalities in regions such as inferior frontal cortex, parietal lobe, cerebellum, and striatum during the performance of tasks involving sustained attention and motor inhibition, such as a stop signal task or time discrimination task (Rubia et al., 2009, 2011a,b, 2014). The most consistent of these findings is an increase in the activation of the right inferior frontal cortex, normalizing the dysfunction normally seen in this brain region in ADHD patients (Rubia et al., 2014). Chronic treatment with methylphenidate in patients with ADHD has also been shown to increase the activation of regions such as the inferior frontal cortex, anterior cingulate cortex, medial prefrontal cortex, cerebellum and striatum during a Go/No-go task (Vaidya et al., 1998; Epstein et al., 2007).

Further support of the proposal that abnormalities in cortico-limbic-striatal circuitry contributes to the symptoms of ADHD is provided by evidence that patients with damage to these brain regions commonly show behavioural and cognitive deficits that resemble those seen in ADHD, such as problems with attentional performance and impulse control (Wilkins et al., 1987; Rolls et al., 1994; Chao and Knight, 1995). Furthermore, there have been a number of preclinical studies that have highlighted the potential importance of these brains regions in the aetiology of ADHD: lesions to areas such as the medial prefrontal cortex, anterior cingulate cortex, subthalamic nucleus and striatum all induce behavioural deficits similar to those seen in ADHD patients (Muir et al., 1996; Baunez and Robbins, 1997; Rogers et al., 2001; Muir et al., 1996).

1.2.3.2. Aetiology of ADHD – neurotransmitters

1.2.3.2.1. The dopamine system

The theory that ADHD involves abnormalities in the dopaminergic system was first proposed by Levy (1991), who suggested that the symptoms of ADHD are the result of deficits in dopamine function in specific regions of the brain, such as the cortex and striatum. Since then, dopamine is arguably the neurotransmitter that has received most interest with regards to ADHD's underlying pathophysiology. Brain imaging studies have highlighted abnormalities in dopaminergic function in patients with ADHD, such as reduced neuronal uptake of dopamine in the prefrontal cortex (Ernst et al., 1998), increased expression of the dopamine transporter in the basal ganglia and striatum (Dougherty et al., 1999; Krause et al., 2000; Spencer et al., 2007), and abnormalities in the activity of DOPA decarboxylase in the prefrontal cortex (Ernst et al., 1998). It is also striking that many of the animal models of ADHD, which show behavioural and cognitive abnormalities that resemble those seen in ADHD patients, also demonstrate abnormalities in dopaminergic function (Van der Kooij and Glennon, 2007). For example, two commonly used animal models of ADHD are rats with neonatal lesions using 6-hydroxydopamine and mice with genetic disruption to the dopamine transporter, both of which demonstrate hyperactivity that is prevented by treatment with a psychostimulant (Sontag et al., 2010; see: Section 1.2.7).

There has been debate over whether ADHD is associated with a hypo-dopaminergic or hyper-dopaminergic state. Early suggestions that dopaminergic function is reduced in ADHD came from the observation that *d*-amphetamine and methylphenidate are effective at alleviating ADHD symptoms (see: Sagvolden et al., 2005). Preclinical studies have also demonstrated that lesions to the dopaminergic system induce behavioural deficits such as hyperactivity and impulsivity (Luthman et al., 1989; Cardinal et al., 2001). However, these behavioural abnormalities have also

been linked with elevated dopaminergic function, in particular in mice with reduced expression of the dopamine transporter (e.g., Zhuang et al., 2001; discussed below). Increased basal dopamine release is also evident in the prefrontal cortex and nucleus accumbens of the Spontaneously Hypertensive Rat, currently the most extensively studied model of ADHD (Carboni et al., 2003, 2004; discussed below). The precise role of dopamine in ADHD remains unclear, and the same data have been interpreted in terms of both hypodopaminergic and hyperdopaminergic hypotheses (Ohno, 2003). It is possible that the influence of dopamine differs for each behavioural aspect of the disorder. It has also been proposed that dopamine may be 'hypo' with respect to serotonin, but 'hyper' with respect to noradrenaline (Oades, 2002).

1.2.3.2.2. The serotonin system

Interest in the role of serotonin in the aetiology of ADHD largely stems from the strong evidence that serotonin plays a key role in impulse control (see: Winstanley et al., 2006; Oades, 2002). However, reports concerning abnormalities in serotonergic function in ADHD patients have been inconsistent. For example, in one study a correlation was found between lower levels of plasma 5HT and symptom severity (Spivak et al., 1999). By contrast, in another study, a higher concentration than normal of 5HIAA in the CSF of patients with ADHD was reported (Oades, 2002). Furthermore, whereas some studies have shown tryptophan depletion to be beneficial in treating ADHD patients (Zepf et al., 2008), others have found that tryptophan depletion increases impulsivity in tests of response inhibition (Quintin et al., 2001; Walderhaug et al., 2002; Crean et al., 2002). It is also striking that SSRIs, whilst being effective in the treatment of other impulse control disorders (Hollander and Rosen, 2000), are either ineffective or exacerbate symptoms in ADHD patients (Popper, 1997).

One possible explanation for the above inconsistencies is that the effect of serotonin on impulsivity depends on the receptor subtype on which it is acting. For example, bilateral infusion of a 5-HT2A antagonist into the nucleus accumbens decreases impulsive behaviour, whereas bilateral infusion of a 5HT2C antagonist increases impulsive behaviour (Robinson et al., 2008a). This suggests that activation of these receptors increases and decreases impulsivity, respectively. It has also been proposed that the effects of serotonin on behaviours relevant to ADHD are dependent on its activity relevant to other neurotransmitters, such as dopamine and noradrenaline (see: Oades, 2002). It is also possible that the effects of serotonin on impulsive behaviour follow a bell-shaped curve.

1.2.3.2.3. The noradrenaline system

There is considerable evidence that inattentiveness is associated with abnormalities in noradrenergic transmission. In 2005, Aston-Jones and Cohen proposed the 'adaptive gain theory', which describes how deficits in selective attention could be explained by both excessive tonic activity and deficient phasic activity of noradrenergic neurons that project from the locus coeruleus to the prefrontal cortex (Aston-Jones and Cohen, 2005). In other words, the optimal noradrenergic phasic response is dependent on its background tonic activity, and abnormalities in either can affect attentional performance.

As with dopamine, support for a role of noradrenaline in the aetiology of ADHD is provided by the observation that many drugs that target the noradrenergic system are effective at alleviating ADHD symptoms, including *d*-amphetamine, methylphenidate, guanfacine and atomoxetine (Fone and Nutt, 2005). There is also evidence for dysfunctional noradrenergic regulation in the prefrontal cortex of the Spontaneously Hypertensive Rat (Russell et al., 2000).

1.2.3.3. Aetiology of ADHD – genetics

Many studies have shown that ADHD is highly heritable. First-degree relatives of patients with ADHD are two- to eight-times more likely to suffer from ADHD than relatives of unaffected individuals (Faraone et al., 2005). Data from a number of twin studies estimate heritability of ADHD to be in the range of 71-90%, suggesting that genetics plays an important role in the development of the disorder (Thapar et al., 1999; Biederman and Faraone, 2005; Nikolas and Burt, 2010). This has received further support from several published adoption studies, which further demonstrate a strong inherited contribution to the disorder (Cantwell, 1975; Morrison and Stewart, 1973; Alberts-Corush et al., 1986; Sprich et al., 2000).

In line with the above evidence, there have been a number of candidate gene association studies carried out which have implicated the monoamines as central to the aetiology of ADHD (see: Faraone et al., 2005). Based on meta-analytic evidence, the most consistent findings include genes for the dopamine D4 receptor (*DRD4*), dopamine D5 receptor (*DRD5*), dopamine transporter (*DAT1*), serotonin transporter (*5HTT*), serotonin 1B receptor (*HTR1B*), and synaptosomal-associated protein 25 (*SNAP-25*) (Li et al., 2006; Yang et al., 2007; Gizer et al., 2009; Smith, 2010; Thapar et al., 2013). However, in general, the genetic risks for ADHD are of small effect size, and also often increase the risk for a number of other psychopathologies, not simply ADHD. Consequently, as with other complex, heterogeneous psychiatric disorders, it is increasingly believed that ADHD is caused by complex interactions between genes and the environment.

1.2.3.4. Aetiology of ADHD – environment

Complications during pregnancy and childbirth have been associated with ADHD by a number of studies (Mick et al., 2002; Hack et al., 2004). It is thought that the resulting hypoxia, particularly to the basal ganglia, causes enduring effects on dopaminergic function (Boksa and El-Khodar, 2003) and that this causes the abnormalities observed in patients with ADHD. Maternal alcohol and nicotine intake during pregnancy have also been extensively studied and are thought to contribute to the risk of developing ADHD (Boyd et al., 1991; Thapar et al., 2003). In preclinical studies, chronic exposure to nicotine has been shown to correlate with hyperactivity in offspring (van de Kamp and Collins, 1994). As nicotinic receptors modulate dopaminergic activity (for review, see: Faure et al., 2014), it is theoretically possible that exposure to nicotinic during pregnancy could result in abnormalities to dopaminergic function later in life.

Psychosocial adversity has also been studied in relation to risk of developing ADHD. Low maternal education, lower socioeconomic status, and single parenthood have all been implicated as potentially important factors. Furthermore, children are more likely to suffer from ADHD if their parents suffer from some form of psychopathology, whilst chronic conflict and reduced cohesion in the family have also been implicated (see: Elia et al., 2014). The influence of early life environment on the behavioural abnormalities of NK1R-/- mice is explored as part of this thesis.

1.2.4. Sex differences in ADHD

For many years, research into ADHD has focussed almost exclusively on males, with little effort being made to determine any potential sex differences in the disorder (see: Staller and Faraone, 2006). This is most likely because the prevalence of ADHD was often thought to be much higher in males, with a ratio as high as 9:1 in some estimates (Anderson et al., 1987; Bird et al., 1988).

However, it is increasingly recognised that ADHD is a disorder that is evident in both sexes, and a number of studies have suggested that females with ADHD are currently underdiagnosed (e.g., Robison et al., 2002; see: Rucklidge, 2010).

The typical symptom profile also differs in male and female ADHD patients. Females are more likely to present with the Predominantly Inattentive Subtype, whereas males are more likely to present with the Predominantly Hyperactive/Impulsive or Combined Subtype (see: Staller and Faraone, 2006). Nonetheless, females are capable of expressing all three behavioural symptoms of the disorder, as well as all three clinical subtypes (Gaub and Carlson, 1997; Hudziak et al., 1998; Biederman et al., 1999; Graetz et al., 2005). Interestingly, evidence suggests that symptoms are typically less severe in females than they are in males (Gaub and Carlson, 1997; Gershon, 2002). This could explain the underdiagnosis of females with ADHD, since inclusion/exclusion criteria used in diagnosis are currently based on research that has been largely carried out on male subjects. There is also some evidence that males and females respond differently to psychostimulant medication, with a higher dose of methylphenidate required to produce a comparable effect in females (Markowitz et al., 2003). However, reports on sex-specific effects of ADHD medication are limited and somewhat inconsistent (Pelham et al., 1989; Sharp et al., 1999).

As with male patients, females with ADHD are also more likely to experience impaired IQ, disruption to academic performance and overall lower levels of achievement (Gershon, 2002; Biederman et al., 1999; Graetz et al., 2005). In addition, female patients are more likely to suffer from a number of other comorbidities compared with healthy controls, such as depression, anxiety or conduct disorder (Biederman et al., 1999). Therefore, the current under-representation of females in ADHD research is a serious issue, and this is a topic that will be addressed as part of this thesis.

1.2.5. Continuous Performance Tests: the assessment of ADHD symptoms

A number of different methods are used in the assessment of ADHD (see: Riccio and Reynolds, 2002). Among these is the Continuous Performance Test (CPT), which is possibly the most popular clinic-based tool used to measure sustained attention and "vigilance" (DuPaul et al., 1992). A number of different types of CPT exist, but all are based around the presentation of two different types of stimuli: one that requires the subject to respond (target/Go trials); and one that requires the subject to withhold from responding (non-target/No-go trials). Patients with ADHD perform worse than healthy controls in terms of both attentional performance and impulse control (Losier et al., 1996). The performance of ADHD patients is also improved in this test by treatment with ADHD medication (Losier et al., 1996; Riccio et al., 2001).

In preclinical research, one of the most commonly used tools for assessing sustained attention and response control is the 5-CSRTT (see: Robbins, 2002). This test has been used extensively to investigate a number of different CNS disorders, including ADHD (e.g., Patel et al., 2006; Loos et al., 2010; Yan et al., 2011). More recently, an adapted version of the 5-CSRTT, The 5-Choice Continuous Performance Test (5-CCPT), has also been developed (Young et al., 2009). In contrast to the 5-CSRTT, this test contains both target and non-target trials, and so is argued to be more analogous to human CPTs used in the assessment of ADHD symptoms. Furthermore, this also allows the measurement of multiple aspects of impulsivity simultaneously. This is important, because it is now widely accepted that impulsivity is not a unitary construct, but that various types of impulsivity exist with different underlying neurobiological mechanisms (Evenden, 1999; Moeller et al., 2001; see: Winstanley et al., 2006). This topic is discussed further in subsequent chapters, and the 5-CSRTT and 5-CCPT are both used as part of experiments reported in this thesis.

1.2.6. Memory deficits in ADHD

The role of memory deficits in ADHD has received considerable interest in recent years. There have been many reports of patients demonstrating impaired performance in tests of both working and long-term memory (e.g., Westerberg et al., 2004; Rhodes et al., 2012; Hammerness et al., 2014). In fact, it has been proposed that general problems with executive functioning, including memory impairments, are a core mechanism of the disorder, and contribute to the development of the three core behavioural symptoms (Wetsby and Watson, 2004). Memory deficits have also received interest as a potential endophenotype, which could offer a useful objective measure used in diagnostic procedures (Kasper et al., 2012).

In preclinical research, a common approach for assessing memory is through the use of "novel object" paradigms. These tests have been used in relation to a number of different CNS disorders associated with memory impairments (e.g., Sood et al., 2011; Li et al., 2013a; Balducci et al., 2014), including ADHD (Heyser et al., 2004; Langen and Dost 2011). Given that memory deficits are increasingly thought to play a central role in the mechanisms underlying ADHD, the performance of NK1R-/- mice in these behavioural paradigms will also form part of the research presented in this thesis.

1.2.7. Animal models of ADHD

The following summaries include all animal models of ADHD that demonstrate at least two or more aspects of Face, Construct or Predictive validity. A more extensive summary of animal models of ADHD is provided in Table 1.3 (for review, see: Sontag et al., 2010).

1.2.7.1. Spontaneously Hypertensive Rat

The most widely studied rodent model of ADHD is the Spontaneously Hypertensive Rat (SHR). The SHR was originally developed by Okamoto and Aoki (1963) by inbreeding Wistar-Kyoto (WKY) rats that exhibited high systolic blood pressure and high reactivity to stress. The SHR was subsequently found to demonstrate hyperactivity in a number of behavioural paradigms, as well as motor and cognitive impulsivity, and deficits in sustained attention (see: Sagvolden, 2000). The SHR therefore displays all three core behavioural deficits of ADHD and can be argued to display good face validity. There is also evidence for disruption to both dopaminergic and noradrenergic frontostriatal circuitry in the SHR (Russell et al., 1995, 2000), indicating construct validity, although reports on whether transmission is increased or decreased are inconsistent (see: Heal et al., 2008).

Whilst some studies have found that the hyperactivity of the SHR is attenuated by treatment with *d*-amphetamine (Myers et al., 1982), others have found that *d*-amphetamine actually increases activity of these animals (McCarty et al., 1980; Hynes et al., 1985; Tsai and Lin, 1988). Furthermore, methylphenidate has also been found to increase, rather than decrease, the activity of the SHR (Wultz et al., 1990; Amini et al., 2004; Yang et al., 2006). Limited predictive validity is therefore an issue with this model. In addition, there is criticism of the use of WKY rats as control animals, as WKY rats have themselves been shown to display abnormalities in both neurochemistry and behaviour (Pare, 1989; McCarty and Kirby, 1982; Diana, 2002; Drolet et al., 2002). Finally, a potentially confounding factor in the SHR is their hypertension, as it is possible that the behavioural deficits of these animals are related to brain dysfunction or even damage caused by their high blood pressure. It is not clear, for example, whether the beneficial effects of antihypertensive drugs in this model, such as clonidine and guanfacine, are due to their direct central actions or their effects on reducing blood pressure.

1.2.7.2. TR-β1 Transgenic Mouse

The Thyroid Hormone Receptor-beta 1 (TR-ß1) Transgenic Mouse carries a mutant gene originally derived from a patient diagnosed with Resistance to Thyroid Hormone (RTH) syndrome. Patients with RTH syndrome suffer from elevated levels of thyroid hormones, normal or elevated levels of thyroid stimulating hormone, tachycardia, hearing loss and short stature (Weiss and Refetoff, 2000). In addition, approximately 70% of children with RTH also suffer from ADHD (Burd et al., 2003), implicating the thyroid system in the disorder. Whilst thyroid abnormalities are rare in patients with ADHD (Weiss et al., 1993), abnormalities in the levels of thyroid hormone are known to severely disrupt neurodevelopment and cognition (Thompson and Potter, 2000). Moreover, one study has also suggested that subclinical maternal thyroid abnormalities may contribute to the development of ADHD (Haddow et al., 1999).

Early reports found that the TR-ß1 mouse is hyperactive, but not impulsive or inattentive (McDonald et al., 1998). However, Siesser et al. (2006) have since been able to induce all three behavioural deficits of ADHD through the use of another promoter for the TR-ß1 gene. This evidence provides good face validity and some construct and predictive validity for this model.

1.2.7.3. DAT-/- Mouse

As discussed, there is a lot of evidence that disruption to dopaminergic transmission underlies, at least in part, the pathophysiology of ADHD. The dopamine transporter (DAT) is responsible for the clearance of dopamine from the extracellular space and is targeted by a number of drugs used to treat ADHD, including *d*-amphetamine and methylphenidate, and abnormalities in the DAT gene have been implicated in the aetiology of ADHD (e.g., Sharp et al., 2009).

The Dopamine Transporter Knockout (DAT-/-) Mouse lacks the gene for this transporter. DAT-/- mice are hyperactive, and this hyperactivity is reduced by treatment with *d*-amphetamine and methylphenidate, as well as cocaine (see: Sontag et al., 2010). These mice also demonstrate poor behavioural inhibition in the eight-arm radial maze test, a common test of spatial memory in rodents (Gainetdinov and Caron, 2001). The efficacy of psychostimulants in reducing the activity of these mice suggests their efficacy in patients with ADHD is not due to action on the DAT. Furthermore, given that the majority of dopamine in the PFC is cleared by the noradrenaline transporter (NAT) rather than the DAT (Moron et al., 2002), and that reuptake by the NAT is also inhibited by psychostimulants, it seems likely that the reduction in activity in DAT-/- mice caused by these agents is due to action on the noradrenergic system.

Unsurprisingly, DAT-/- mice have been shown to express reduced dopamine clearance (Jones et al., 1998). There are several compensatory mechanisms that also occur as a result of a lack of DAT function, including a decrease in dopamine release from dopaminergic nerve terminals (Gainetdinov et al., 1998; Jones et al., 1998). It is thought that this is why there is only a 5-fold increase in spontaneous dopamine efflux in the striatum in these animals (Giros et al., 1996). In the basal ganglia, there are also ~50% postsynaptic decreases in D_1 and D_2 receptor protein and mRNA (Gainetdinov et al., 1998; Jaber et al., 1996, 1999).

Although there is evidence for an association between polymorphisms of the *DAT1* gene and ADHD (Brookes et al., 2006), there is no indication that DAT is reduced in patients with ADHD. In fact, a number of studies have found increased DAT density in the striatum of adults and children with ADHD (Dougherty et al., 1999; Krause et al., 2000; Cheon et al., 2003). Even if DAT function is abnormal in patients with ADHD, the transporter protein will still be present, unlike in DAT-/- mice. This model will also not be able to help with any drug development that involves

drugs targeting the DAT. Further issues with this model are that DAT-/- mice suffer from growth retardation (Bosse et al., 1997) and a higher incidence of premature death, with as few as only 68% surviving after 10 weeks old.

1.2.7.4. Coloboma Mouse

The coloboma mouse was developed by Searle and colleagues (1966) through the use of neutron irradiation. The model has a mutation in the gene for SNAP-25, a protein involved in the fusion of neurotransmitter vesicles to the presynaptic membrane during transmitter release. Any behavioural abnormalities are therefore likely related to SNAP-25 dysfunction and the consequential abnormalities is neurotransmitter function (Hess et al., 1992, 1996; Steffensen et al., 1996).

This mouse suffers from delayed neurodevelopment, and is hyperactive and impulsive, with impaired performance in a delayed reinforcement task (Hess et al., 1994, 1996; Heyser et al., 1995; Wilson, 2000; Bruno et al., 2007). The hyperactivity of these mice is reduced by *d*-amphetamine but not methylphenidate (Hess et al., 1996; Wilson, 2000). Striatal dopaminergic release in these mice is almost completely absent (Raber et al., 1997). Also, D_2 receptor expression is elevated in the ventral tegmental area and substantia nigra (Jones et al., 2001). Neurotoxin (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride, 'DSP-4')-induced depletion of noradrenaline reduces the hyperactivity, but not impulsivity, of these mice (Bruno et al., 2007; Jones and Hess, 2003), whereas adrenergic receptor antagonists have also been found to reduce their hyperactivity (Bruno and Hess, 2006). These latter results suggest that the hyperactivity of this model is due to hyperactivity of the noradrenergic system.

1.2.7.5. Other models

There are several other proposed animal models of ADHD. These models all demonstrate hyperactivity but not impulsivity or inattentiveness, although all show some form of construct and/or prediction validity through alterations in monoaminergic or other brain systems and a reduction in hyperactivity in response to drugs that target these systems. These models are summarised in Table 1.3 (for review, see: Sontag et al., 2010).

Species:	Type:	Name:	Face Validity:			Predictive Validity:	Construct Validity:
			Hyp	Imp	Inat		
Rat	Genetic	Spontaneously Hypertensive Rat				AMP: inconsistent; MPH: increases activity	Abnormalities in the fronto-striatal system
		Naples High-Excitability Rat		n/d	n/d	n/d	Abnormalities in the DA system
	Pharmaco-logical	6-OHDA Lesioned Rat		n/d	n/d	Hyperactivity reduced by methylphenidate	Abnormalities in the DA system
		Neonatal Hypoxia		n/d	n/d	All symptoms reduced by amphetamine	Abnormalities in NA, 5HT and DA systems
		Developmental Cerebellar Stunting		n/d	n/d	n/d	Reduced cerebellar volume in ADHD patients
Mouse	Genetic	TR-β1 Transgenic Mouse				All behaviours reduced by methylphenidate	Abnormalities in the DA system
		Coloboma Mouse			n/d	n/d	Abnormalities in DA and NA systems
		DAT Knockout Mouse			n/d	Hyperactivity prevented by psychostimulants	Abnormalities in DA system
		Acallosal Mouse		n/d	n/d	n/d	Reduced callosal regions in ADHD patients
		Alpha-synuclein lacking mouse		n/d	n/d	n/d	Increased release of DA
	Other	Maternally Stressed Mice		n/d	n/d	Hyperactivity prevented by DA antagonists	n/d

Table 1.3. Rodent models of Attention Deficit Hyperactivity Disorder

Hyp, hyperactivity; *Imp*, impulsivity; *Inat*, inattentiveness; *n/d*, no data; DA, dopamine; NA, noradrenaline, 5-HT, 5-hydroxytryptamine.

Adapted from Sontag et al., 2010.

1.2.8. NK1R-/- mouse model of ADHD

As discussed above, a number of previous studies have identified behavioural, cognitive and neurochemical abnormalities of NK1R-/- mice that resemble those seen in ADHD. These include hyperactivity, impulsivity and inattentiveness (Yan et al., 2010; Yan et al., 2011; Dudley et al., 2013), as well as abnormalities in dopaminergic, serotonergic and noradrenergic transmission (Froger et al., 2001; Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010). These are summarised in Table 1.4 in terms of the model's face, construct, and predictive validity.

	ADHD Patients	***NK1R-/- mice***	***Technique***
Face Validity	Hyperactivity	Hyperactivity	Activity chamber; LDEB
	Impulsivity	Impulsivity	5-CSRTT
	Inattentiveness	Inattentiveness	5-CSRTT
Construct Validity	Abnormalities in noradrenergic transmission	Increased cortical NA efflux in PFC (anaesthetised subjects)	*In vivo* microdialysis
		Desensitised α_2-adrenoceptors	Autoradiography; Radioligand binding
	Abnormalities in dopaminergic transmission	Reduced dopamine efflux in the frontal cortex	*In vivo* microdialysis
		No effect of amphetamine on striatal dopamine	*In vivo* microdialysis
	Abnormalities in serotonergic transmission	Increased serotonergic release in prefrontal cortex	*In vivo* microdialysis
		Desensitised $5HT_{1A}$ receptors	Autoradiography
	Polymorphisms at the TACR1 gene in patients with ADHD	Lack of functional NK1R	
Predictive Validity	Psychostimulants used as first-line treatments	Hyperactivity prevented by *d*-AMP and MPH	LDEB

Table 1.4. Validation of the NK1R-/- mouse model of ADHD
LDEB, light/dark exploration box; 5-CSRTT, 5-Choice Serial Reaction-Time Task
(Froger et al., 2001; Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010; Dudley et al., 2013; Sharp et al., 2014)

1.3. The Brain Renin Angiotensin System

1.3.1. Background

The discovery of a renin-angiotensin system (RAS) began more than a century ago with Tigerstedt and Bergman's (1898) demonstration that injection of renal extracts produced profound effects on blood pressure. The active substrate of this extract, named 'renin', was subsequently determined to be a protease enzyme responsible for a key step in the biosynthetic pathway that ultimately leads to the production of the physiologically active peptide angiotensin II (AngII). AngII was first discovered more than 70 years ago by two groups independently: the first group isolated AngII from the ischemic kidneys of Goldblatt hypertensive dogs (Braun-Menendez et al., 1940); the second isolated AngII after injecting cats intravenously with renin (Page and Helmer, 1940). Early research into the effects of AngII found that intracerebroventricular injection induced a centrally-mediated pressor response (Bickerton and Buckley, 1961) and drinking response (Epstein et al., 1970). Renin and AngII were later isolated in dog brain (Ganten et al., 1971; Fisher-Ferraro et al., 1971), whilst angiotensin (AT) receptors were identified and localized in rat brain (Sirett et al., 1977). This led to the hypothesis that a central RAS may exist in the CNS, separate to that in the periphery. Today, it is well established that the CNS contains an intrinsic brain RAS (BRAS) with all the necessary components to carry out a variety of physiological functions.

1.3.2. Biosynthetic pathway of angiotensin II and its peptide fragments.

Classically, the main effector peptide of the BRAS is AngII, which is derived from the precursor protein angiotensinogen (Fig. 1.3). Angiotensinogen is cleaved by renin to produce the decapeptide angiotensin I (AngI). AngI is thought to be inactive, but is a substrate for angiotensin-converting enzyme (ACE), a zinc metalloprotease glycoprotein that hydrolyzes AngI to produce the octapeptide, AngII (Johnston, 1990). AngII is subsequently broken down by the action

of glutamyl aminopeptidase A (Am-A), which cleaves AngII to produce the heptapeptide angiotensin III (AngIII) (Rich et al., 1984; Ramirez et al., 1990; Wilk and Healy, 1993; Chauvel et al., 1994). AngIII is cleaved by membrane alanyl aminopeptidase N (Am-N) to form angiotensin IV (AngIV), which can then be further converted by carboxypeptidase P (Carb-P) and propyl oligopeptidase (PO) to Ang(3-7). Further cleavage of AngIV and Ang(3-7), by endopeptidases such as chymotrypsin and dipeptidyl carboxypeptidase, produces inactive peptide fragments and amino acids (Unger et al., 1988; Saavedra, 1992; Speth et al., 2003; Reudelhuber, 2005; Banegas et al., 2006). AngII can also be cleaved by Carb-P or monopeptidase ACE_2 to produce Ang(1-7) (Wright and Harding, 1997; Ferrario and Chappell, 2004). Ang(1-7) can also be produced from Ang(1-9) by the action of ACE (Vauquelin et al., 2002). Finally, Ang(1-7) can be cleaved further by Am-A to produce Ang(2-7) (Mentlein and Roos, 1996).

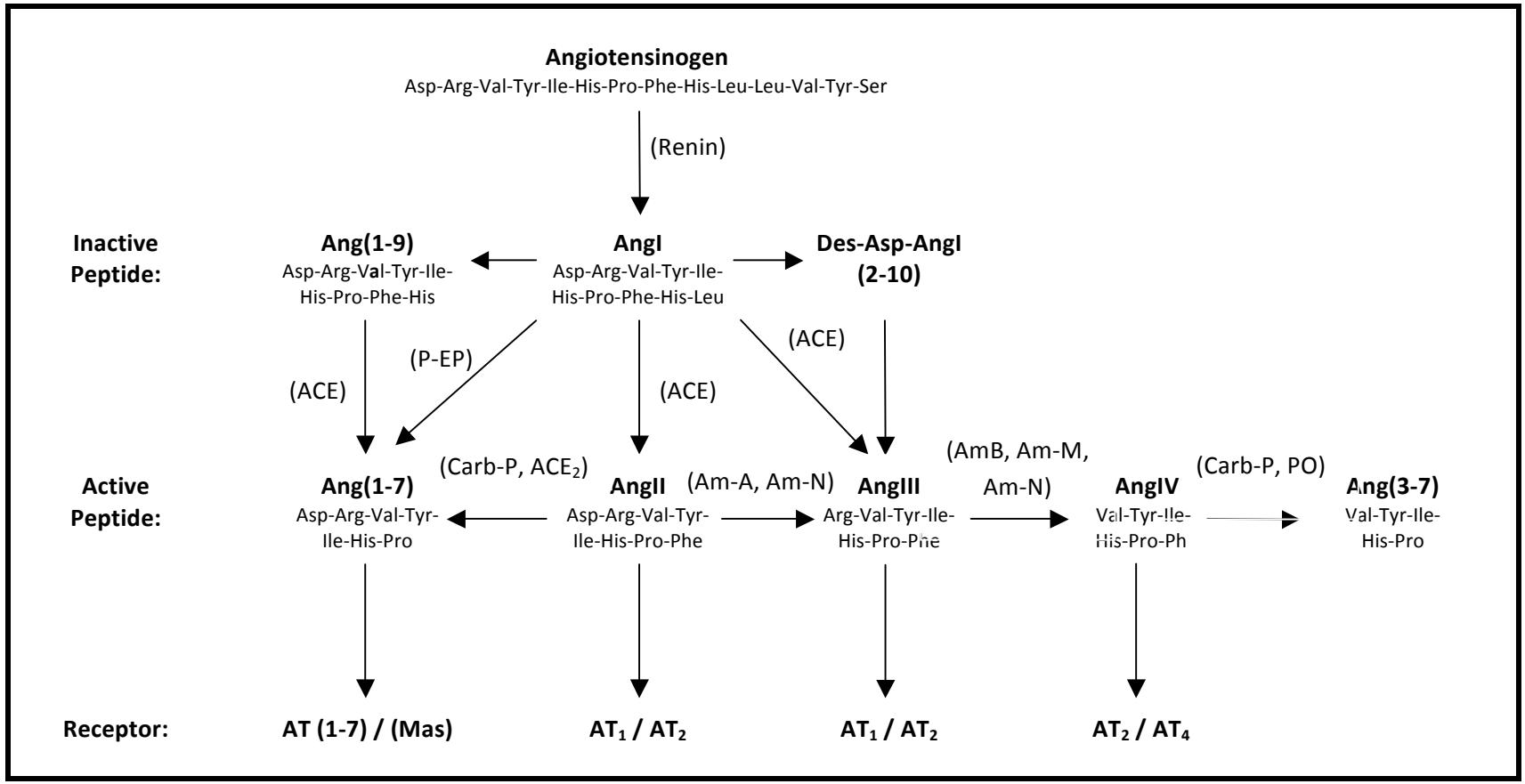

Figure 1.3. Simplified flow diagram of the biosynthesis of the angiotensin peptides.
ACE, angiotensin-converting enzyme; Am, aminopeptidase; PE-P, prolylendopeptidase; Carb-P, carboxypeptidase P; PO, propyl oligopeptidase. Adapted from: Ciobica et al. 2009.

1.3.3. Components of the BRAS

As mentioned, AngII was traditionally considered the end-product of these biosynthetic pathways and the only active ligand. However, there is now considerable evidence that many of the shorter peptide fragments of AngII, outlined above, are also biologically active. Furthermore, the actions of these peptides are sometimes functionally antagonist to those of AngII. This is explained, at least partly, by differences in their affinity for the several different types of angiotensin receptor that exist. In the following section, the relationship between each of these angiotensin peptides and their receptor subtypes is outlined.

1.3.3.1. AngII and AT_1 / AT_2 receptors

AngII binds to two main subtypes of angiotensin receptor: the angiotensin type 1 (AT_1) receptor and the angiotensin type 2 (AT_2) receptor. In humans, the AT_1 receptor is a 359-amino acid GPCR that is encoded by a gene located on chromosome 3q (Murphy et al., 1991; Sasaki et al., 1991). Upon activation, AT_1 receptors undergo a process of desensitization and internalization similar to that seen with many GPCRs (Hunyady et al., 1994; de Gasparo et al., 2000). AT_1 receptors are predominantly coupled to $G_{q/11}$, and their activation results in several different intracellular processes, such as the activation of phospholipase C, MAP kinase, or transcription pathways (Richards et al., 1999; Guo et al., 2001; Sumners et al., 2002). As a consequence, AngII is capable of producing signals that range from seconds to minutes, and even hours, in length, and the activation of a single receptor subtype can result in a number of different functional outcomes.

The AT_2 receptor is encoded by a gene located on the X chromosome, and shares 30% of its amino acid sequence with the AT_1 receptor (Kambayashi et al., 1993; Nakajima et al., 1993; Gard, 2002). However, unlike AT_1 receptors, activation of AT_2 receptors does not result in receptor internalization (Wright and Harding, 2004). Furthermore, whilst most AT_2 receptors are also GPCRs,

which signal via G_i alpha subunits (Kang et al., 1995; Gelband et al., 1998), there is also evidence that AT_2 receptors can signal via a G-protein independent mechanism (Kang et al., 1993; Buisson et al., 1995; Gallinat et al., 2000).

Within the peripheral nervous system, receptors for AngII are found in both the sympathetic and parasympathetic ganglia, where they are thought to potentiate sympathetic transmission and inhibit acetylcholine release, respectively (Lewis and Reit, 1965; Potter, 1982; Castren et al., 1987; Saito et al., 1987; Allen et al., 1990). AT_1 and AT_2 receptors are also found within the adrenal medulla, where they potentiate the release of adrenaline and noradrenaline (Marley et al., 1989; Zhuo et al., 1996). In terms of primary sensory afferent neurons, AT_1 receptors are found over the cell bodies of the dorsal root ganglia and in the terminal regions of these neurons in the dorsal laminae of the spinal cord (Oldfield et al., 1994**;** Allen et al., 1998). They are also found over the cell bodies of the nodose ganglion and in the terminal regions of the nucleus solitary tract, where they are thought to play a pivotal role in the baroreceptor reflex (Allen et al., 1998). However, not all primary sensory afferent neurons express AT_1 receptors, with no receptors expressed in the facial, cochlear, vestibular or visual sensory afferent neurons (for review, see: Allen et al., 1998).

Within the central nervous system, AT_1 receptors predominate in regions implicated in the regulation of blood pressure, body fluid homeostasis, and neuroendocrine function (Millan et al., 1991; Song et al., 1991; Tsutsumi and Saavedra, 1991). For example, they are found in regions such as the circumventricular organs, median preoptic nucleus, and paraventricular nucleus, where they regulate the cardiovascular system. They are also found in regions such as the lamina terminalis, subfornical organ and preoptic region, where they regulate the dipsogenic effects of AngII. In addition, they are also found in regions such as the supraoptic nucleus, where they

regulate pituitary hormone release, and in the spinal nucleus of the trigeminal nerve, where they regulate sensory information (for reviews, see: Shanmugam and Sandberg, 1996; Dinh et al., 2001).

By contrast, AT_2 receptors are predominantly found distributed within the limbic system and in regions involved in sensory information and motor control (Millan et al., 1991; Song et al., 1991; Tsutsumi and Saavedra, 1991). For example, they are found within the medial geniculate body, which relays auditory information, and in the ventrolateral and dorsolateral geniculate nuclei, which process visual information from the retina. They are also found in regions such as the superior colliculus and the accessory and inferior olivary nuclei, which regulate motor control. In addition, they are also found in limbic regions such the lateral septal nucleus and medial amygdaloid nucleus (for reviews, see: Shanmugam and Sandberg, 1996; Dinh et al., 2001)

Of particular interest in this thesis is the presence of these receptors within the basal ganglia (Allen et al., 1991, 1992). As discussed in Section 1.1 and 1.2, the basal ganglia have been heavily implicated in the aetiology of ADHD, and are thought to play a central role in the control of movement. Radioligand binding studies in humans, using the (non-specific) AT receptor ligand ^{125}I-[Sar^{1},Ile^{8}]AngII, have shown that regions such as nigrostriatal nerve terminals, the globus pallidus and the substantia nigra pars compacta, all express AngII receptor binding sites (Allen et al., 1991, 1992). This strongly suggests that AngII and the BRAS regulate the function of this network, and this topic is discussed further in Chapter 4. Other regions of interest in this thesis that also express AT_1 and AT_2 receptors include the locus coeruleus, which is thought to be important in attentional performance, the hippocampus, which is thought to play a central role in memory, and cortical regions, such as the entorhinal cortex and piriform cortex (Reagan et al., 1994; von Bohlen und Halbach and Albrecht, 1998a, 2006). The presence of these receptors in these brain regions and its potential implications for experiments reported in this thesis are discussed further in the relevant subsequent chapters.

An important consideration regarding the distribution of AT_1 and AT_2 receptors is that a number of studies have demonstrated that the expression of these receptors changes during the course of development (Millan et al., 1991; Tsutsumi and Saavedra, 1991). For instance, many areas of the fetal brain that express AT_2 receptors show a marked decline, or complete loss, in adulthood, such as the cingulate cortex, the cerebellum, geniculate bodies, thalamic nuclei, and the nuclei of the third and twelfth cranial nerves. Other areas that show low levels of AT_1 receptor expression in the fetal brain show higher levels in adulthood, such as the dentate gyrus, nucleus of the solitary tract, choroid plexus and the suprachiasmatic nucleus. However, not all brain areas undergo developmental changes and, in general, AT_1 receptor expression is thought to be more stable than that of AT_2 receptors (see: Shanmugam and Sandberg, 1996; Dinh et al., 2001).

Another important consideration is that there are marked species differences in the distribution of these receptors, in particular for the AT_2 receptor. For example, in humans AT_2 receptors are only found on the molecular layer of the cerebellum (Barnes et al., 1993; MacGregor et al., 1995), whereas in rats these receptors are found in a number of brain regions, such as the thalamic nuclei, the inferior colliculus and medial geniculate nucleus (Song et al., 1992). As discussed, a human study using the non-selective AT receptor agonist [^{125}I][Sar^1,Ile^8]Angiotensin II found that receptor expression was high within the nigrostriatal pathway (Allen et al., 1991). By contrast, studies in rat brain have found that receptor expression of both AT_1 and AT_2 receptors in this brain region was below the level of detection (Gehlert et al., 1991; Tsutsumi and Saavedra, 1991).

There are also reports of species differences in the distribution of the various receptor subtypes. For example, in the locus coeruleus receptor expression in humans is exclusively of the AT_1 receptor subtype, whereas in the rat this region shows high levels of the AT_2 receptor subtype (MacGregor et al., 1995; Song et al., 1992). These species differences could have important

implications for the translational value of any research investigating the behavioural effects of drugs that target this system (for review, see: Dinh et al., 2001).

Through activation of AT_1 and AT_2 receptors, there is evidence that AngII influences the function of a number of other neurotransmitter systems. These include noradrenergic (e.g., Gelband et al., 1998), serotonergic (e.g., Nahmod et al., 1978) and dopaminergic (e.g., Tchekalarova and Georgiev, 1998) transmission. There is also evidence that AngII influences the function glutamate (Barnes et al., 2003), GABA (Li et al., 2003), adrenocorticotropic hormone (Ferguson et al., 1999) and corticotrophin-releasing hormone (Sumitomo et al., 1991). In light of this evidence, it is unsurprising that AngII and its related peptides are increasingly implicated in a wide variety of psychiatric disorders (discussed below).

1.3.3.2 AngIV and the AT_4 receptor

As described above, AngIV is a biologically active peptide fragment of AngII produced by the sequential action of several aminopeptidase enzymes. However, unlike AngII, AngIV binds with only low affinity to AT_1 receptors and AT_2 receptors, and instead binds preferentially to a separate receptor termed the angiotensin type 4 (AT_4) receptor (Swanson et al., 1992; de Gasparo et al., 2000). Neither AngII nor specific AT_1 or AT_2 receptor antagonists are capable of binding to this receptor with high affinity (Harding et al., 1992; Swanson et al., 1992).

Unlike AT_1 and AT_2 receptors, the AT_4 receptor is not a GPCR (Hall et al., 1993; Miller-Wing et al., 1993), but instead is identical to the enzyme insulin-regulated aminopeptidase ('IRAP'; Albiston et al., 2001). Activation of AT_4 receptors results in a number of different intracellular pathways, such as tyrosine phosphorylation (Chen et al., 2001), an increase in mitogen activated protein (MAP) kinase phosphorylation and the activation of p38 kinase (Handa, 2001), an increase

in intracellular Ca^{2+} concentration (Dulin et al., 1995), the activation of immediate early genes (Roberts et al., 1995), and an increase in nitric oxide synthase and cGMP activity (Patel et al., 1998).

1.3.3.3 Ang(1-7) and the Mas receptor

Finally, another prominent biologically active angiotensin peptide in the CNS is Ang(1-7). This peptide is thought to act via a GPCR, Mas, encoded for by the *Mas* protooncogene (Young et al., 1986; Brown, 1989; Santos et al., 2003). The Mas receptor is located in several different tissues, including the brain where it is found in moderate or high densities in regions such as the hippocampus, amygdala and cortical regions such as the piriform cortex and frontal cortex (Bunnemann et al. 1990; Metzger et al. 1995). Little is known about the intracellular signalling of Mas.

In many cases, the effects of Ang(1-7) are opposite to those of AngII. For example, AngII is a well known vasoconstrictor, whilst Ang(1-7) produces a vasodilator effect (Ueda et al., 2000). Furthermore, AngII inhibits the sensitivity of the baroreceptor reflex, whereas Ang(1-7) facilitates it (Campagnole-Santos et al., 1992). AngII is also known to facilitate cell growth, and yet Ang(1-7) has an antiproliferative effect (Freeman et al., 1996). These functionally antagonist effects of different angiotensin peptides are an important consideration when interpreting the behavioural effects of drugs that target the BRAS.

1.3.3.4. Angiotensin converting enzyme

As described above, the classical biosynthetic pathway of the BRAS involves the conversion of the decapeptide AngI to the octapeptide AngII by the action of angiotensin converting enzyme (ACE). One of the most widely studied mammalian peptidases, ACE is found in both membrane-bound form, facing extracellularly, and in a soluble form within the extracellular space itself (Corvol et al.,

1995). ACE is capable not only of converting AngI to AngII, but also converts Ang(1-7) to Ang(1-5) and des-Asp-AngI to AngIII. Furthermore, ACE inactivates the vasodilator peptide, bradykinin, and is involved in the metabolism of a number of other peptides, including neurotensin, dynorphin, enkephalin and, as mentioned above, substance P. However, one peptide that ACE is not capable of hydrolyzing is AngII itself. This is thought to be due to its inability to cleave the His^6-Pro^7 bond (Rice et al., 2004).

In 2000, a second form of ACE was discovered termed 'ACE_2', a carboxypeptidase responsible for the conversion of AngI to the inactive Ang(1-9) (Donoghue et al., 2000; Tipnis et al., 2000), which is subsequently cleaved by other peptidase enzymes to produce the biologically active Ang(1-7). ACE_2 is also capable of producing Ang(1-7) by the direct metabolism of AngII. This ability of a separate and distinct enzyme to produce Ang(1-7) rather than AngII is important, due to the functionally antagonist effects of these two peptides, described above. However, as ACE inhibitors are well-established antihypertensives, it seems unlikely that ACE inhibitors such as captopril produce a discernible effect on ACE_2 activity.

ACE has a wide distribution in the CNS. It is found in high densities in the circumventricular organs (Saavedra and Chevillard, 1982), where it is thought to act on peripherally circulating AngI by converting it to AngII, which then acts on receptors located within these regions (Thunhorst et al., 1989; McKinley et al., 1997). ACE has also been identified in a number of other brain regions, such as the caudate putamen, substantia nigra, nucleus of the tractus solitarius, dorsal motor nucleus and the median preoptic nucleus (Saavedra and Chevillard, 1982; Chai et al., 1987a,b, 1990, 1991; Rogerson et al., 1995).

1.3.4. Roles of the BRAS in behaviour and cognition

Traditionally, the RAS has been associated with the regulation of the cardiovascular and renal systems, regulating blood pressure and the maintenance of water and electrolyte homeostasis. There is also evidence for a role in the regulation of reproductive and pituitary gland hormones. However, there is now extensive evidence that the BRAS acts as a crucial mediator in a number of other 'novel' functions, including mood, stress and learning and memory (for review, see: Wright et al., 2008). The following section provides an outline of the findings that have been made concerning the role of the BRAS in behaviour and cognitive performance.

1.3.4.1. Depression

Preclinical studies have revealed that treatment with the ACE inhibitor, captopril, reduces immobility in the forced swim test to the same extent as the antidepressants imipramine and mianserin (Giardina and Ebert, 1989). Furthermore, treatment with captopril prevents learned helplessness induced by foot-shock to the same extent as imipramine (Martin et al., 1990). Interestingly, in both these studies, the protective effects of captopril were reversed by treatment with naloxone, suggesting that the antidepressant effects of captopril are exerted, at least in part, via the opioid system. More recently, genetically modified mice lacking angiotensinogen have also been shown to exhibit reduced immobility in the forced swim test (Okuyama et al., 1999), whereas AT receptor antagonism has a positive effect in both the forced swim and tail suspension tests (Gard et al., 1999; Gard, 2002; Vijayapandi and Nagappa, 2005; Nayak and Patil, 2008).

In humans, there are reports that treatment with an ACE inhibitor produces an antidepressant effect in hypertensive patients with comorbid depression (Zubenko and Nixon, 1984; Croog et al., 1986; Deicken, 1986; Germain and Chouinard, 1988, 1989). There is also

evidence that polymorphisms of genes for angiotensinogen, AT receptors or ACE may contribute to the risk of developing mood disorders such as bipolar affective disorder or major depressive disorder (Arinami et al., 1996; Meira-Lima et al., 2000; Hishimoto et al., 2006; Saab et al., 2007).

Despite the above evidence, the precise role of the BRAS in mood and affective disorders remains poorly understood. This topic will not be the focus of the present thesis, but is reviewed in detail by several authors elsewhere (e.g., Gard, 2002; von Bohlen und Halbach and Albrecht, 2006; Wright et al., 2008).

1.3.4.2. Stress and anxiety

There is considerable evidence that the BRAS plays an important role in the physiological response to stress. Exposure to stress increases the concentration of circulating AngII, as well as tissue concentration of AngII in key brain regions involved in the stress response, such as the hypothalamus and medulla oblongata (Yang et al., 1996). In turn, AngII facilitates the release of other important transmitters that are involved in the response to stress, such as noradrenaline (Chevillard et al., 1979; Garcia-Sevila et al., 1979; Meldrum et al., 1984; Schacht, 1984) and adrenocorticotropin hormone (Ganong and Murakami, 1987). High densities of AT receptors are found in the adrenal medulla, anterior pituitary gland, median eminence, paraventricular nucleus, and zona glomerulosa (Tsutsumi and Saavedra, 1991; see: Wright and Harding, 1992). Furthermore, stress exposure increases the expression of AT receptors in several of these brain regions (Castren and Saavedra, 1988; Jezova et al., 1998; Leong et al., 2002).

Treatment with an ACE inhibitor reduces anxiety-like behaviours in a number of different paradigms, such as the elevated plus-maze and social interaction test (Costall et al., 1990). Similar

results follow treatment with the AT_1 receptor antagonist, losartan (Barnes et al., 1990a; Kaiser et al., 1992). By contrast, intracerebroventricular administration of AngII increases anxiety in the elevated plus-maze, an effect that is abolished by pretreatment with an AT_1 receptor or AT_2 receptor antagonist (Braszko et al., 2003). In line with this, the transgenic rat TGR (mREN2)27, which has an elevate concentration of angiotensin peptides in the brain, demonstrates an increased anxiogenic profile in another test of anxiety, the elevated X maze (Senanayake et al., 1994; Wilson et al., 1996). Combined, these results suggest that AngII increases anxiety-related behaviours by a mechanism that involves AT_1 and/or AT_2 receptor activation.

However, not all findings have supported this proposal. In one study, intracerebroventricular of AngII produced an anxiolytic effect in a defensive burying paradigm in rats (Tsuda et al., 1992), whilst in another study, treatment with an AT receptor antagonist failed to produce an effect on anxiety at all (Shepherd et al., 1996). Consequently, as with depression, the precise role of the BRAS in anxiety remains unclear. It has been proposed that this role could involve an interaction with either dopaminergic or GABAergic transmission (Georgiev et al., 1987; 1995). Again, this will not be a major focus of the present thesis but is reviewed in detail elsewhere (see: Gard, 2002).

1.3.4.3. Learning and memory

There is a considerable body of literature that suggests the BRAS plays a role in learning and memory. In preclinical research, the effects of AngII administration, ACE inhibitors and AT receptor antagonists have all been investigated using a number of behavioural paradigms. For example, intracerebroventricular administration of AngII facilitates learning and memory retention in a conditioned avoidance test (Georgiev and Yonkov, 1985). This effect was prevented by treatment

with the non-selective AT receptor antagonist, saralasin, indicating that AngII facilitates associative memory in a mechanism that directly involves AT receptor activation. Administration of AngII also improves memory consolidation in a spatial discrimination T-maze task (Braszko et al., 1987, 1988) and enhances memory recall in an object recognition task (Kulakowska et al., 1996). Similarly, this effect was prevented by treatment with the AT_1 receptor antagonist, losartan, indicating that AngII facilitates memory via a mechanism that directly involves AT_1 receptor activation.

However, perhaps paradoxically, there have also been several studies in which AngII administration has been found to disrupt, rather than facilitate, learning and memory processes. For example, administration of AngII into the striatum disrupts memory retention in a passive avoidance task (Morgan and Routtenberg, 1977), whereas administration of AngII into the hippocampus disrupts memory formation and retrieval in step-down inhibitory avoidance task (Kerr et al., 2005; Bonini et al., 2006). Furthermore, most preclinical investigations into the effects of ACE inhibitors have generally found that these drugs improve performance in tests of cognitive function: treatment with an ACE inhibitor prevents cognitive deficits induced by scopolamine in both a T-maze task and water maze task (Costall et al., 1989; Barnes et al., 1992), as well as improves performance in tests of active and passive avoidance (DeNoble et al., 1991; Nikolova et al., 2000).

In humans, early indications that the BRAS might be involved in learning and memory came from evidence that the ACE inhibitor, captopril, improves cognitive performance in patients with mild to moderate hypertension (Croog et al., 1986), as well as in healthy volunteers (Currie et al., 1990). The AT_1 receptor antagonist, losartan, has also been shown to improve cognitive performance in hypertensive patients (Tedesco et al., 1999). More recently, the BRAS has been implicated in Alzheimer's disease, with an increase in ACE activity and abnormalities in

components of the BRAS observed in patients with the disorder (Ohrui et al., 2004) and polymorphisms of the ACE gene being associated with increased susceptibility (Alvarez et al. 1999; Amouyel et al. 2000; Lehmann et al. 2005). Both ACE inhibition and AT receptor antagonism might also improve memory function in the elderly (Fogari et al., 2003).

A longstanding hypothesis regarding the cellular correlates of learning and memory is that of long-term potentiation (LTP). Injection of AngII above the CA1 region of the hippocampus prevents the induction of LTP in perforant path-stimulated neurons of the dentate gyrus (Denny et al., 1991). This effect is entirely blocked by administration of an AT_1 receptor antagonist, indicating this process involves activation of AT_1 receptors (Wayner et al., 1993a,b). Activation of AT_1 receptor also suppresses LTP in the lateral nucleus of the amygdala, in a process that is thought to involve an inhibitory effect on glutamatergic N-methyl-D-aspartate (NMDA) receptors (von Bohlen und Halbach and Albrecht, 1998b; Albrecht et al., 2003). There is also evidence that inhibition of LTP by ethanol involves AT_1 receptor activation, possibly via pathways that project from the hypothalamus to the hippocampus (Wayner et al., 1997).

In contrast to AngII, administration of AngIV to rat hippocampal slices facilitates, rather than disrupts, LTP in the CA1 region (Kramer et al., 2001). Moreover, administration of an AT_4 receptor antagonist interferes with the stabilization of LTP in this brain region (Davis et al., 2006). Similar findings have also been observed *in vivo* in the dentate gyrus (Wayner et al., 2001). Similarly, Ang(1-7) facilitates LTP in the CA1 region of the hippocampus (Hellner et al., 2005), an effect that is dependent on activation of the Mas receptor, as genetic deletion of this receptor abolishes this effect. These studies provide another example of how the actions of smaller peptide fragments may exert the opposite effect of AngII itself, and, as mentioned, is an important consideration when interpreting the behavioural effects of drugs that target the BRAS.

1.4. Aims of thesis

The first major theme of this thesis is to further phenotype the NK1R-/- mouse model of ADHD. Initially, the behaviour of NK1R-/- mice bred from two different breeding strategies is compared, to determine whether the behavioural abnormalities of NK1R-/- mice are a direct result of a lack of functional NK1R or whether other contributory factor(s), such as early life environment, are influencing their behaviour (Chapter 1). Following this, the locomotor activity of male and female animals is compared, to determine whether or not the hyperactivity of NK1R-/- mice is sex-specific (Chapter 2). Subsequently, the performance of NK1R-/- mice in the 5-CCPT is investigated, to determine whether NK1R-/- mice display an inattentive or impulsive phenotype in this test (Chapter 6). Finally, NK1R-/- mice are tested in novel object recognition / novel object location paradigms, to explore whether or not NK1R-/- mice display any memory deficits (Chapter 6).

The second major theme of this thesis is to test the behavioural effects of drugs that target the BRAS and investigate the possibility that this system interacts with NK1R in the regulation of behaviours associated with ADHD. This was prompted by preliminary evidence that the BRAS and NK1R may interact in the regulation of locomotor activity (discussed in Chapter 4). Initially, the effects of both ACE inhibition and AT receptor antagonism on locomotor activity are investigated (Chapter 4). Following this, the effects of ACE inhibition on the performance of NK1R-/- mice in the 5-CSRTT are explored, to determine whether the BRAS influences the other behavioural abnormalities of these mice (Chapter 5). Finally, the effects of AT_1 receptor antagonism on performance in the 5-CCPT, as well as two test of memory, are investigated (Chapter 6).

Chapter 2

Methods and materials

2.1. Ethics statement

All experiments were licensed under the Animals (Scientific Procedures) Act, 1986 (UK) (2010/63/EU) and received local ethical approval at University College London, UK.

2.2. Animals

2.2.1. Neurokinin-1 receptor gene knockout mice

Neurokinin-1 receptor gene knockout ('NK1R-/-') mice were originally developed by de Felipe et al. (1998). Homologous recombination was used to incorporate a gene cassette with targeted disruption of the *Nk1r* gene into the DNA of embryonic stem cells of mice with a 129/Sv background strain. Cells containing the gene cassette were then injected into blastocysts and implanted into female mice with a C57BL/6 background strain. Male chimeric offspring were mated with C57BL/6 female mice to produce mixed litters containing wildtype (NK1R+/+) and heterozygous (NK1+/-) offspring. The heterozygous offspring were subsequently mated to produce mixed litters of wildtype, heterozygous and NK1R-/- mice of a 129/Sv X C57BL/6 background strain. Finally, these mice were subsequently crossed, once, with mice from an outbred MF1 background strain, with the aim of diluting the influence of the 129/Sv background. Consequently, mice used in this thesis are derived from a 129/Sv X C57BL/6 background strain crossed with an outbred MF1 strain.

2.2.2. Housing conditions

Mice were bred and maintained in the Biological Services facility at University College London (UK) and housed in groups of 2 – 5 mice per cage, with environmental enrichment supplied routinely. Bedding was supplied by 3Rs Bedding Pty, Ltd. Unless stated otherwise, both food (Harlan Tekland

TRM Rat / Mouse Diet, Harlan, Bicester, UK) and water were available *ad libitum*. The colony room was set on a 12:12 h light/dark cycle (lights turned on in steps between 07.00 – 08.00 h and turned off in steps between 19.00 – 20.00 h), with temperature and humidity maintained at 20 ± 2°C and 45 ± 5%, respectively.

2.2.3. Genotype verification

2.2.3.1. Tissue digestion

Ear samples were taken from animals using a 2 mm ear punch and placed in an eppendorf tube over ice. For tissue digestion, 75 µL alkaline lysis reagent was added (25 mM NaOH, 0.2 mM disodium EDTA, dissolved in water) and samples were heated at 95 °C for 30 min. Following this, samples were allowed to cool to 4 °C, and 75 µL of neutralising reagent (40 mM Tris-HCl, dissolved in ultrapure (18MΩ) water) added. Samples were then stored at -20°C until required.

2.2.3.2. Polymerase chain reaction – DNA amplification

Following digestion, the polymerase chain reaction (PCR) was used to confirm the genotype of each animal. Once tissue samples were defrosted, a PCR 'master mix' was added to each sample. This master mix contained a buffer, a dNTP mix, three PCR primers (NeoF, NK1-F, NK1-R), magnesium chloride, and nuclease-free (ultrapure) water. The master mix was also added to three control tubes (1 x wildtype mouse, 1 x NK1R-/- mouse, 1 x nuclease-free water). All tubes were placed in a thermocycler and run through a pre-programmed cycle of temperatures for DNA amplification.

2.2.3.3. Electrophoresis

Amplified samples were loaded into wells of a 2% agarose gel and run for approximately 60 min at 115 V, after which the gel was visualised using an ultraviolet transilluminator plate. For wildtype mice, a single 350-base band is seen; for NK1R-/- mice, a single 260-base band is seen; for heterozygous mice (NK1R+/-), both a 350-base band and 260-base band are seen.

Full details of the genotyping protocol are provided in Appendix I.

2.3. 5-Choice Serial Reaction-Time Task

2.3.1. Background

The 5-Choice Serial Reaction-Time Task (5-CSRTT) is a widely used preclinical test of sustained visual attention and response control. It was initially developed by Carli et al. (1983) and is based on the analogous human task of sustained attention originally devised by Leonard (1959). Since then, it has been used extensively in both rats and mice to study a wide range of neurological disorders (see: Robbins, 2002; Bari et al., 2008). The following protocol is based on that described by Yan et al. (2011), with some refinements.

2.3.2. Apparatus

The apparatus (Med Associates, St. Albans, VT, USA) comprised four mouse operant chambers (21.6 x 17.8 x 12.7 cm), each housed within a ventilated sound-attenuated box (55.9 x 38.1 x 35.6 cm) (Figure 2.1). One of the walls of each chamber is curved and contains five equally-spaced 'nose-poke' apertures (1 cm diameter). Inside each of these holes is a light, to illuminate the hole,

and an infrared detector, which monitors 'nose-pokes' by the mouse. On the opposite wall, a hole (2.2 cm diameter) provides access to a magazine that delivers a liquid milk reward (0.01 mL of 30% condensed milk solution), which is signalled by illumination of the hole. Head entries into this hole to collect the milk reward from the magazine are similarly monitored by interception of an infrared detector by 'nose-pokes' by the mouse. The chamber can be illuminated by a house-light mounted above the magazine hole. The presentation of the light stimuli and monitoring of the animals' responses are controlled and recorded using a Smart Ctrl Package 8IN/16OUT with an additional interface by MED-PC for Windows (Med Associates, St. Albans, VT, USA).

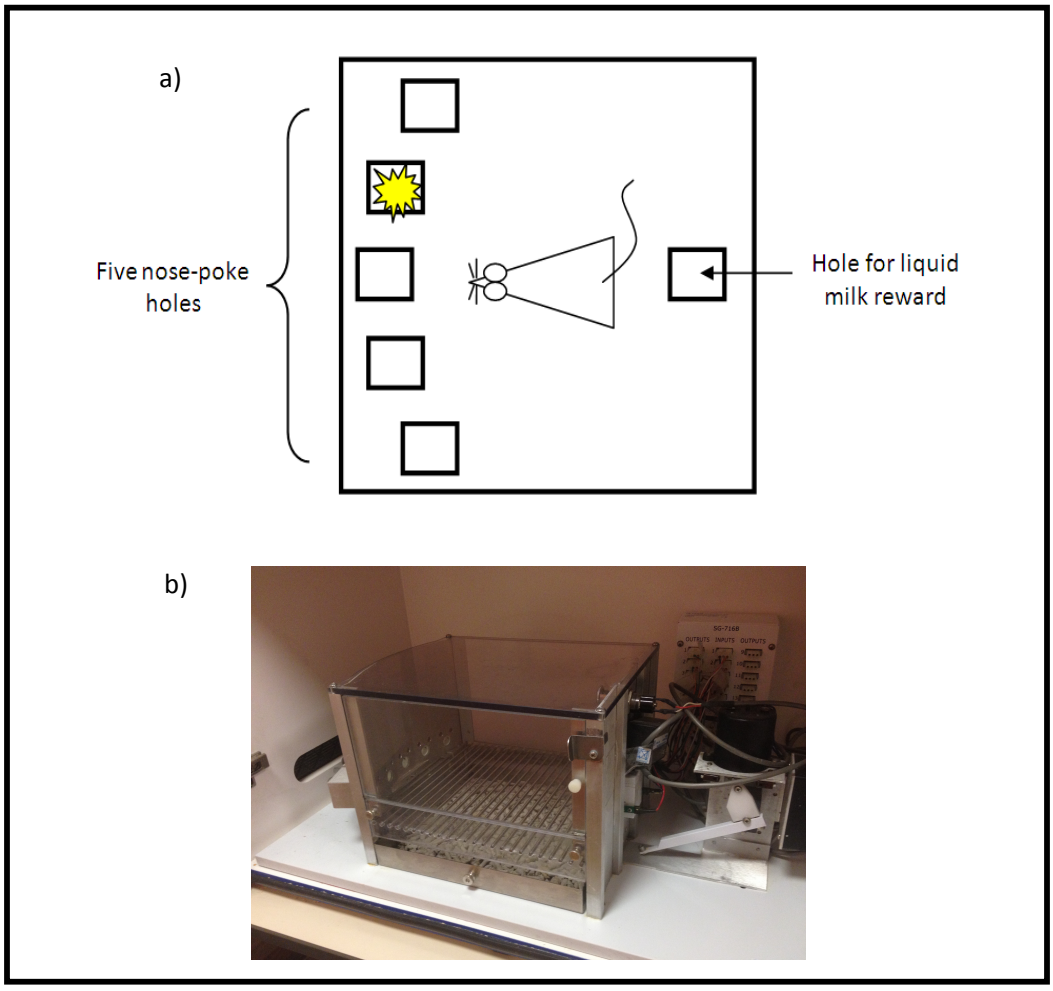

Figure 2.1. (a) Illustration and (b) photograph of the 5-Choice Serial Reaction-Time Task apparatus.
(illustration not to scale)

2.3.3. Protocol

Daily protocol: For the duration of the 5-CSRTT, animals are brought to the laboratory and weighed between 09.00 and 09.30 h (Monday to Friday), and trained/tested in either a morning (10.00 – 12.00 h) or afternoon (13.00 – 15.00 h) session. Half of all animals from each experimental group are assigned to the morning session and half are assigned to the afternoon session, and are placed in the operant chamber at the same time every day for the duration of the experiment.

Food deprivation: Each experiment starts when the animals are between 6 – 8 weeks old. The weight of each animal is recorded whilst food is still available *ad libitum*, referred to as their 'free-feeding body weight'. From this point, access to food is then restricted and each cage is fed once a day at 16.30 h (Harlan Tekland TRM Rat / Mouse Diet, Harlan, Bicester, UK). The first week of an experiment involves gradually reducing the amount of food given each day until animals reach 90% of their free-feeding body weight. This remains their target weight and is fixed for the duration of the experiment by adjusting the amount of food given each day. Mice are not placed in the operant chamber during this week, but instead remain in their home-cages. Following this initial week of food deprivation, mice proceed to the habituation phase of the 5-CSRTT. Water is available *ad libitum* for the duration of the experiment.

Habituation: Animals are placed in the operant chambers for 30 min, once-daily, for three consecutive days. During this habituation, all lights in the chamber (house light, the five nose-poke apertures and food magazine hole) remain switched on, whilst the liquid milk reward is given on a continuous reinforcement schedule and remains available for 10 s after each nose poke, after which the dipper is retracted and refilled. During the first two of these days, condensed milk is also placed in each of the five apertures to encourage the animal to nose-poke into these holes. The

number of head entries into the magazine and number of reinforcers earned is recorded by the MED-PC software.

After three days, animals are then habituated on a non-spatial reinforcement schedule, during which the liquid reinforcer is only given when animals nose-poke into one of the five apertures. All lights in the five nose-poke holes remain switched for the duration, with a nose-poke into any of these holes resulting in a reward. Animals are required to earn more than 50 reinforcers for two consecutive days (maximum of 10 days) before proceeding onto the training phase of the 5-CSRTT.

Training (Stages 1 to 6): Training in the 5-CSRTT involves a series of six stages, which the animals must pass before being tested. A single day's training session involves a series of 'trials' (Figure 2.2). At the start of each trial, the animal must wait a certain length of time ('intertrial interval', ITI) for a light stimulus to appear in one of the five nose-poke holes. This light stimulus remains on for a length of time known as the 'stimulus duration', and the animal must respond by nose-poking into the hole within a specific time-frame, referred to as the 'limited hold'. If the animal correctly responds to the light stimulus by nose-poking into the same hole within the limited hold ('correct response'), a liquid milk reinforcer is provided by a liquid dipper in the magazine hole in the wall opposite. Collection of the liquid milk reinforcer starts the next trial. If the animal responds before the onset of the light stimulus ('premature response'), or responds in the incorrect nose-poke hole ('incorrect response'), or fails to respond at all ('omission'), a 'time out' occurs, in which the house light is turned off for 5 s and no reward is available (Table 2.1a). Each daily training session lasts 100 trials or 30 min, whichever occurs first.

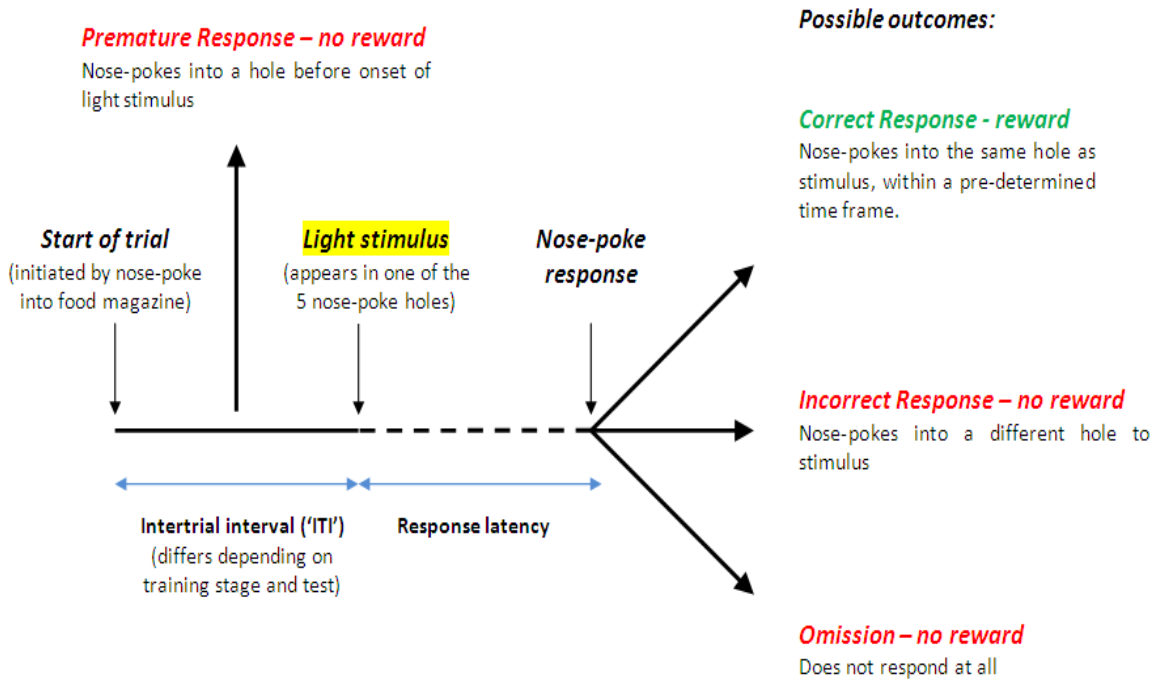

Figure 2.2. Basic design of a single trial in the 5-Choice Serial Reaction-Time Task
(adapted from: Robbins, 2002)

At the end of each session a set of performance variables are calculated (Table 2.1b), including: *premature responses/100 trials* (an index of motor impulsivity), *%omissions* (an index of inattentiveness), *perseveration score* (repetitive responses into a nose-poke hole), *%accuracy* (to measure an animal's understanding of the task), *total number of trials completed* (to measure motivation), and their mean *latencies to respond* and *collect the reward* (to measure motivation). An animal must achieve a set of performance criteria based on these variables in order to progress from one stage of training to the next, with each stage becoming progressively more difficult by decreasing the stimulus duration and limited hold, and increasing the intertrial interval (Table 2.2a). Once animals pass Stage 6, this becomes their 'baseline performance' during the testing phase on days when they are not being tested (Monday to Thursday). On these days,

animals must continue to achieve stable baseline performance criteria in order to be tested, once-weekly, on Fridays.

Exclusion criteria: Animals that failed to achieve baseline performance criteria for three consecutive days before testing (i.e., Tuesday – Thursday) were not tested and were kept on training until baseline performance criteria were achieved. Animals that failed to achieve stable baseline performance criteria at Stage 6 for the duration of the experiment were not included in any statistical analysis, including training.

(a) Response – recorded per trial	
Correct (reward)	animal nose-pokes into the same hole as the light stimulus within the required time-frame
Incorrect (time out)	animal nose-pokes into a different hole to the light stimulus within the required time-frame
Premature (time out)	animal nose-pokes into one of the five holes before the onset of the light stimulus
Omission (time out)	animal fails to nose-poke into any hole within the required time-frame
(b) Performance Variables – calculated per session	
Premature Responses / 100 trials	[premature responses / (correct + incorrect responses + omissions)] x 100
%Omissions	[omissions / (correct + incorrect responses + omissions)] x 100
Perseveration Score	total number of nose-pokes into the same hole following a correct response
%Accuracy	[correct responses / (correct + incorrect responses)] x 100
Total number of trials completed	correct responses + incorrect responses + omissions
Latency to Correct Response	mean latency to nose-poke into the correct hole after the onset of the light stimulus
Latency to Collect Reward	mean latency to collect the milk reward following a correct response

Table 2.1. (a) Possible responses by an animal on a single trial, and (b) how these are used to work out the performance variables at the end of each session.

Testing (VITI & LITI): There are two main tests in the 5-CSRTT: the variable intertrial interval ('VITI') and the long intertrial interval ('LITI') (Table 2.2b). In the VITI, the ITI is variable (2, 5, 10 or 15 s) and delivered on a randomised schedule. In the LITI, the ITI is longer compared with that experienced during training (e.g. 7 s) but remains constant. For both, the stimulus duration and limited hold remain fixed for all trials, and the session length is increased to 45 min. The test session lasts for 100 trials or 45 min, whichever occurs first. The two tests challenge cognitive performance in different ways. In particular, the VITI prevents subjects from using 'interval timing' to predict when the light stimulus will appear. Testing is carried out, once-weekly, on Fridays, provided animals have achieved stable baseline performance criteria during the week. All performance variables that were calculated for training are also calculated for each test session.

In this thesis, where the effects of a drug on task performance were investigated, the experiment was carried out in a randomised (pseudo-Latin-square) repeated-measures design. The number of testing sessions was limited to no more than 12 weeks. This was for several reasons: (i) this was the maximum number of test sessions, involving drug-treatment, permitted under the Home Office project licence; (ii) it prevented animals from being over-trained/over-tested; (iii) it prevented animals from being tested when they were too old; and (iv) it had the ethical benefit that animals were not injected too many times with drug/vehicle. As testing in the 5-CSRTT involves two different types of test (VITI and LITI), it was not possible to test the effects of more than one drug on a single cohort of mice.

(a) 5-CSRTT Training parameters (Stage 1-6)				
Stage	***SD (s):***	***LH (s):***	***ITI (s):***	***Progression Criteria:*** **(Stage 1 to 5: two consecutive days; Stage 6: three consecutive days)**
Stage 1	30	30	2	≥ 30 correct trials
Stage 2	20	20	2	≥ 30 correct trials
Stage 3	10	10	5	≥ 50 correct trials
Stage 4	5	5	5	≥ 50 correct trials; ≥ 75% accuracy; ≤ 25% omissions; total trials – premature = 100
Stage 5	2.5	5	5	≥ 50 correct trials; ≥ 75% accuracy; ≤ 25% omissions; total trials – premature = 100
Stage 6 ('baseline')	1.8	5	5	≥ 50 correct trials; ≥ 75% accuracy; ≤ 25% omissions; total trials – premature = 100 Stage 6 must be carried out for 7 days before testing can begin
(b) 5-CSRTT Test parameters (VITI & LITI)				
Test	***SD (s):***	***LH (s):***	***ITI (s):***	
VITI	1.8	5	2, 5, 10, 15	Baseline: Monday to Thursday Drug Testing (once weekly): Fridays
LITI	1	5	7 or 10	
SD, Stimulus Duration: length of time the light stimulus in one of the 5 nose-poke apertures is kept on LH, Limited Hold: length of time allowed for a nose-poke response after the onset of the light stimulus ITI, Intertrial Interval: length of time between the start of the trial and the onset of the light stimulus				

Table 2.2. (a) Training and (b) testing procedure in the 5-Choice Serial Reaction-Time Task

(adapted from: Yan et al., 2011)

2.4. 5-Choice Continuous Performance Test

2.4.1. Background

A continuous performance test (CPT) is an umbrella term used to describe a number of different tests used to measure attentional performance in humans (e.g., Rosvold et al., 1956; Conners, 1985; Servan-Schreiber et al., 1996). These tests involve a series of trials in which a subject must learn to respond to a 'Go' signal and withhold a response to a 'No-go' signal. Consequently, they are also useful measures of response inhibition, and are commonly used in the assessment of patients with ADHD (Huang-Pollock et al., 2012; Bart et al., 2014; Lopez-Vicente et al., 2014). The 5-Choice Continuous Performance Test (5-CCPT) is a preclinical equivalent of human CPTs and was originally developed by Young et al. (2009). It is similar to the 5-CSRTT, but involves both 'Go' trials (in which an animal must learn to respond when one of the five nose-poke holes is illuminated, as in the 5-CSRTT) and 'No-go' trials (in which an animal must learn to withhold a response when all five nose-poke holes are illuminated). This, it is argued, makes the 5-CCPT more analogous to human CPTs and therefore of more translational value to disorders such as ADHD. The following protocol is largely based on that described by Young et al. (2009).

2.4.2. Apparatus

The apparatus used is identical to that in the 5-CSRTT (see Section 2.3.2).

2.4.3. Protocol

The **daily protocol** and **food deprivation** are identical to those used in the 5-CSRTT (see Section 2.3.3.).

Habituation: Animals are placed in the operant chambers for 30 min, once-daily, for three consecutive days. During this habituation, the lights in the five nose-poke apertures and food magazine hole remain switched on, whereas the house light remains switched off. Liquid milk reward is given on a continuous reinforcement schedule and remains available for 10 s after each nose poke, after which the dipper is retracted and refilled. During the first two of these days, condensed milk is also placed in each of the five apertures to encourage the animals to nose-poke into these holes. The number of head entries into the magazine and number of reinforcers earned is recorded by the MED-PC software.

After three days, animals are then habituated on a non-spatial reinforcement schedule, during which the liquid reinforcer is only given when animals nose-poke into one of the five apertures. All lights in the five nose-poke holes remain switched for the duration, with a nose-poke into any of these holes resulting in a reward. Again, the house light remains switched off throughout. Animals are required to earn more than 70 reinforcers for two consecutive days (maximum of 10 days) before proceeding onto the training phase of the 5-CCPT.

Training (Stages 1 – 4): Training in the 5-CCPT involves a series of four stages, which the animals must pass before being tested (Table 2.3a). For all stages, each training session lasts 120 trials or 30 min, whichever occurs first. 'Go' trials and 'No-go' trials are introduced at different stages of training. Initially (Stage 1), animals are trained using 'Go' trials only and a fixed ITI (5 s), in which an animal is required to nose-poke in response to a light that appears in one of the five nose-poke holes. The animal's possible responses at this stage are identical those described in the 5-CSRTT ('correct response', 'incorrect response', 'premature response', and 'omission'; see: Section 2.3.3.). However, in the 5-CCPT a 'correct response' is also referred to as a 'Hit', whereas an 'incorrect response' and 'omission' are also both referred to as a 'Miss'. A Hit results in the

animal receiving the liquid milk reward, whereas a Miss results in a 'Time Out' punishment, during which the house light is turned on for 5 s and no milk reward is available. The stimulus duration starts at 20 s and the animal is required to have a mean correct response latency of less than half the stimulus duration (i.e., less than 10 s) for three consecutive days. The stimulus duration is then reduced to 10 s, and the animal is again required to have a mean correct response latency of less than half that of the stimulus duration (i.e., less than 5 s) for three consecutive days. This continues as the stimulus duration is further reduced to 8 s, 4 s and finally 2 s, where it remains for the remainder of training and throughout testing. Following this (Stage 2), the animal is switched to a variable ITI (ITI = 3, 4, 5, 6, and 7 s), which are delivered on a randomised schedule; again, only 'Go' trials are used at this stage. The animal is required to achieve greater than 80% accuracy and less than 40% omissions for three consecutive days in order to progress further.

Once the above performance criteria are achieved, 'No-go' trials are introduced (Stage 3; Fig. 2.3). Again, the animal is trained using a variable ITI (3, 4, 5, 6, and 7 s), but this time 80 out of the 120 trials in a session are 'Go' trials and 40 out of the 120 trials are 'No-go' trials (2:1 ratio). In 'No-go' trials, all five nose-pokes are illuminated and the animal is required to withhold a response in order to receive a reward. There are two possible responses: 'correct rejection', in which an animal correctly withholds from responding; and a 'false alarm', in which an animal responds by nose-poking into any of the five illuminated nose-poke holes. The animal is required to correctly withhold a response for more than 50% of the 'No-go' trials, for two consecutive days, in order to progress further. Once this is achieved, the number of 'Go' trials is increased to 100 out of 120 trials and the number of 'No-go' trials is reduced to 20 out of 120 trials (5:1 ratio). At this stage (Stage 4), the animal is required to achieve a positive 'sensitivity index'. This involves a calculation based on both the number of correct responses during 'Go' trials ('Hits') and the number of 'False Alarms' during 'No-go' trials (see Table 2.4). Once a positive sensitivity index is achieved for three

consecutive days, the animal is tested in the 5-CCPT. Stage 4 remains the animal's 'baseline performance' during the testing phase on days when they are not being tested (Monday to Thursday). On these days, animals must continue to achieve a positive sensitivity index in order to be tested, once-weekly, on Fridays.

Exclusion criteria: Animals that failed to achieve baseline performance criteria for three consecutive days before testing (i.e., Tuesday – Thursday) were not tested and were kept on training until baseline performance criteria were achieved. Animals that failed to achieve stable baseline performance criteria at Stage 4 for the duration of the experiment were not included in any statistical analysis, including training.

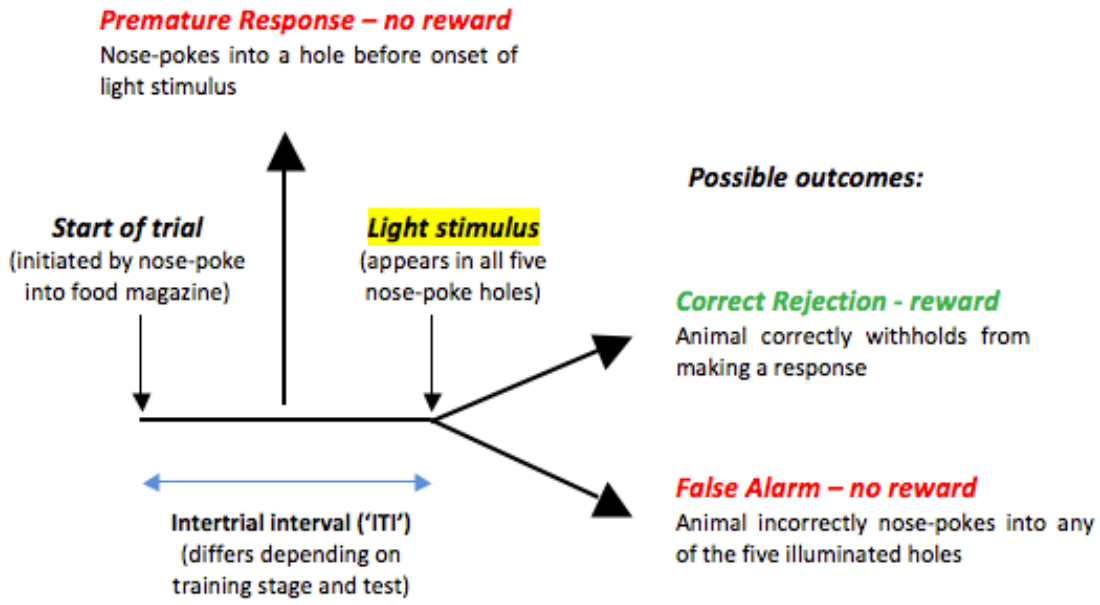

Figure 2.3. Basic design of a single 'No-Go' trial in the 5-Choice Continuous Performance Test.

Testing: Animals are tested, once-weekly, using a 5:1 extended VITI test session. In this test session, the ITIs are variable and extended (7, 8, 9, 10, and 11 s), and are delivered on a randomised schedule. The test session lasts for 250 trials or 60 min, whichever occurs first. Animals are tested on Fridays, provided animals achieve stable baseline performance (positive

sensitivity index) during the week. All performance variables that were calculated for both 'Go' and 'No-go' trials during training are also calculated for each test session.

Where the effects of a drug were investigated, the experiment was carried out in a randomised (pseudo-Latin square) repeated-measures design. As in the 5-CSRTT, the number of testing sessions was limited to no more than 12 weeks (see: Section 2.3.3). However, as there is only one type of test in the 5-CCPT (5:1 extended test session), a maximum of two different drugs can be investigated on a single cohort of mice. In the experiment reported in Chapter 6, the effects of both methylphenidate (reported elsewhere) and losartan are investigated.

(a) 5C-CPT Training parameters		
Stage	***Parameters***	***Progression Criteria***
Stage 1: **<u>Fixed ITI</u>** **(all 'Go' trials)**	ITI: fixed at 5 s SD: fixed at 20, 10, 8, 4 and finally 2 s LH: 2 s greater than SD (min. 5 s) 120 trials	In order to progress from one SD to the next: Mean correct latency (MCL) < 0.5 x SD (three consecutive days) At SD of 2 s, animals must achieve: at least 10 correct responses for 3 consecutive days
Stage 2: **<u>VITI</u>** **(all 'Go' trials)**	ITI: variable (3,4,5,6,7 s) SD: fixed at 2 s LH: fixed at 5 s 120 trials	%Accuracy > 80% %Omissions < 40%
Stage 3: **<u>2to1</u>** **(80 'Go' trials /** **40 'No-go' trials)**	ITI: variable (3,4,5,6,7 s) SD: fixed at 2 s LH: fixed at 5 s 120 trials	Probability of False Alarm (pFA) < 0.5
Stage 4 (baseline): **<u>5to1</u>** **(100 'Go' trials /** **20 'No-go' trials)**	ITI: variable (3,4,5,6,7 s) SD: fixed at 2 s LH: fixed at 5 s 120 trials	Sensitivity Index (SI) > 0
(b) 5C-CPT Test parameters		
Drug Testing	***Parameters***	
<u>Variable ITI</u> **(5:1 extended test session)**	ITI: variable (7,8,9,10,11 s) SD: fixed at 2 s LH: fixed at 5 s 250 trials	Baseline: Monday to Thursday Drug Testing (once weekly): Fridays
SD, Stimulus Duration: length of time the light stimulus is kept on LH, Limited Hold: length of time allowed for a nose-poke response after the onset of the (Go) light stimulus ITI, Intertrial Interval: length of time between the start of the trial and the onset of the light stimulus		

Table 2.3. (a) Training and (b) testing procedure in the 5-Choice Continuous Performance Test

2.4.4. Additional performance variables in the 5-CCPT

In addition to the behaviours also measured in the 5-CSRTT (Table 2.1), several additional performance variables are also calculated in the 5-CCPT based on performance during the 'No-go' trials (Table 2.4). These include: *probability of false alarm* (an index of behavioural disinhibition); *sensitivity index* (an index of an animal's sensitivity to the two (Go & No-Go) trials); and *response bias* (an index of an animal's 'tendency to respond').

Performance variable	Calculation	Range of outcomes
Probability of false alarm (pFA)	FA / [FA + CR]	0 (all No-go trials are correct rejections) 1 (all No-go trials are false alarms)
Sensitivity Index (SI)	$[pHR - pFA] / [2(pHR + pFA) - (pHR + pFA)^2]$	+1 (all Go trials are Hits / all No-go trials are correct rejection) 0 (chance) -1 (all Go trials are Misses / all No-Go trials are false alarms)
Response bias (RI)	$[pHR + pFA - 1] / [1 - (pFA - pHR)^2]$	0 ('liberal' responder) -1 ('conservative' responder)

Table 2.4. Additional performance variables in the 5-Choice Continuous Performance Test. FA, false alarm; CR, correct rejection; pFA, probability of false alarm; pHR, probability of hit rate (see: Young et al., 2009)

2.5. Light/Dark Exploration Box

2.5.1. Background

The light/dark exploration box (LDEB) consists of a large light compartment (two thirds) and a smaller dark compartment (one third), and was initially described by Crawley and Goodwin (1980). The original test was developed as a preclinical screen for sedative and anxiolytic drugs and is based on a rodent's natural aversion to brightly lit, unfamiliar environments. It involves measuring an animal's movement between a dark zone and a brightly lit 'aversive' zone, typically over a period of 3 min.

In the protocol described here, the conditions used are deliberately quite different. In particular, the light intensities are much lower than those often used in other studies using the LDEB. This is intended to make the light zone 'novel' rather than aversive. Furthermore, the animal is also allowed a period of 90 min habituation to the dark zone before being tested. Since the animal is allowed to habituate to the dark zone, it is possible to use the unfamiliar zone to measure active / passive avoidance (using latency to leave and return to the light zone, respectively). As a result, this protocol provides more information than other common tests of locomotor activity, such as the open field. The LDEB has been used extensively in this way to explore the behavioural phenotype of NK1R-/- mice (Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010). The following protocol is largely based on that described by Yan et al. (2010).

2.5.2. Apparatus

The LDEB apparatus comprises a 'dark zone' (length 15 cm, width 20 cm, height 25 cm and light intensity 4 lx) and a larger 'light zone' (length 30 cm, width 20 cm, height 25cm and light intensity 12 lx). The 'dark zone' consists of black walls and a black floor marked with a white grid of 5 x 5 cm squares. The 'light zone' consists of white walls and a white floor marked with a black grid of 5 x 5 cm squares. A removable partition door separates the two zones until the start of testing (Figure 2.4).

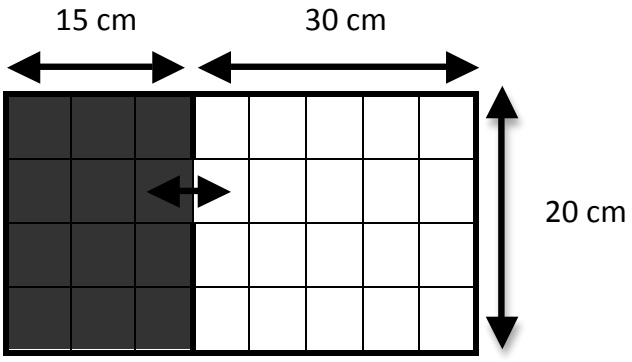

Figure 2.4. Illustration of the light/dark exploration box.
(not to scale)

2.5.3. Protocol

Animals are brought to the experimental room at 09.30 h on the day of testing and kept in home cages until the start of the experiment, with food and water available *ad libitum*. Two LDEBs are placed next to each other and a camera placed above them to record the behaviour of two mice (e.g., one of each genotype) simultaneously. At 13.00 h, mice are placed with minimal handling into the 'dark zone' of the LDEB for 90 min habituation. During this time, the partition door is kept closed and, if applicable, drug (or vehicle) is administered at the appropriate time. Following habituation, the camera is switched on and the mice are transferred with minimal handling to the

centre of the 'light zone', facing the wall opposite the partition door. The door is immediately raised, allowing free movement between zones, and the behaviour of the mice is recorded for 30 min. Additional experiments are started at 15.15 h, with subsequent times adjusted accordingly.

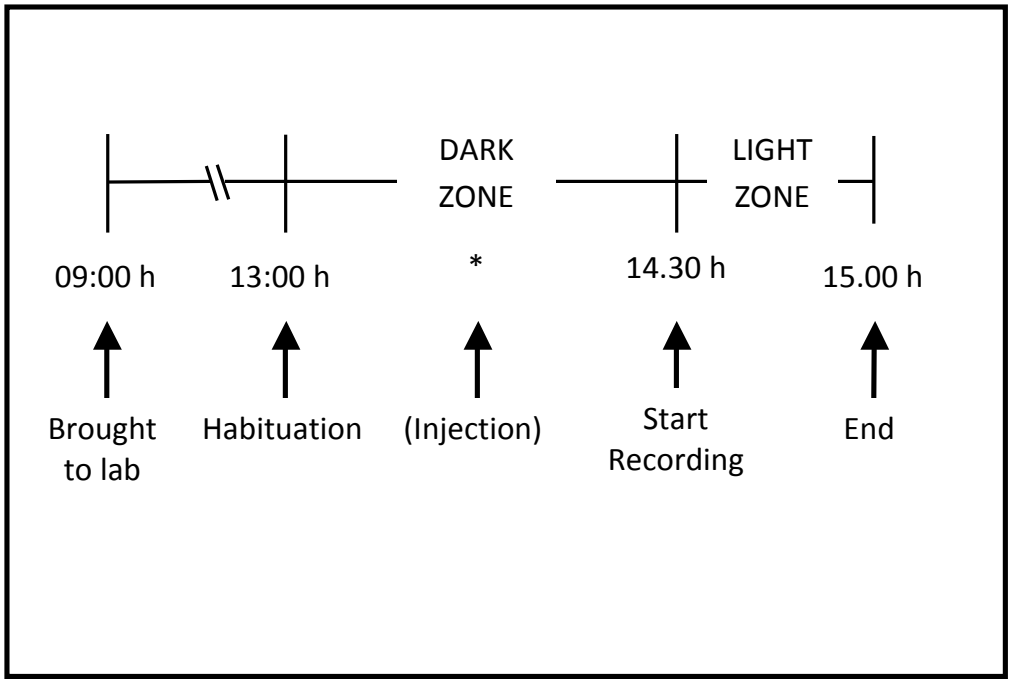

Figure 2.5. Experimental protocol used in the light/dark exploration box. Habituation was also started at 15.15 h, with subsequent times adjusted accordingly. * Injection time depends on drug treatment

2.5.4. Behavioural scoring

Following the experiment, several behaviours are scored 'blind' in 5 min time bins, described in Table 2.5. For all behaviours, the criterion for crossing a line or leaving a zone is the crossing of all four paws.

Behaviour	Measurement	Indication
Speed in Light Zone	Number of lines crossed divided by time spent in the light zone	Locomotor activity
Time in Light Zone	Total amount of time spent in the light zone in seconds	Inversely related to active avoidance
Latency to Leave the Light Zone	Time it takes to enter the dark zone after being placed in the light zone	Active avoidance
Latency to Return to the Light Zone	Time it takes to first return to the light zone after leaving for the first time	Passive avoidance
Number of Returns to the Light Zone	Number of times the animal enters the light zone	Exploratory activity / passive avoidance

Table 2.5. List of behaviours scored in the light/dark exploration box. For all behaviours, the criterion for crossing a line or leaving a zone is the crossing of all four paws.

2.6. Activity Sensors

2.6.1. Background

Part of the work presented in this thesis has involved the use of activity sensors that monitor the activity levels of animals within their home-cage environment across the entire 24 h cycle. This work was carried out in collaboration with Dr Stuart Peirson and Dr Laurence Brown (University of Oxford, UK).

2.6.2 Apparatus

The activity sensors work through the use passive infra-red technology (Panasonic AMN 32111, Premier Farnell UK Ltd). The sensors record the animals' movement every 100 ms, and these are sent as digital inputs to a microcontroller board (Ardunio Uno (Rev3). For each 60 s interval, percentage activity is calculated and, alongside a signal of the environmental light conditions measured using a light-dependent resistor, is sent as a serial message to a laptop. The activity data, light measurement, and time are saved to a tab-delimited text file.

To measure the activity of an animal in their home-cage environment, activity sensors are attached to the base of a standard home-cage that is positioned, as normal, in the colony rack of the animal holding room (Figure 2.6). In this position, the activity sensors are 20 cm above the floor of the cage below, and monitor the entire area of the floor that is accessible to the mice. The activity sensors are activated by gross ambulatory movement, as well as turns of the body and rearing, but not by finer movements, such as breathing.

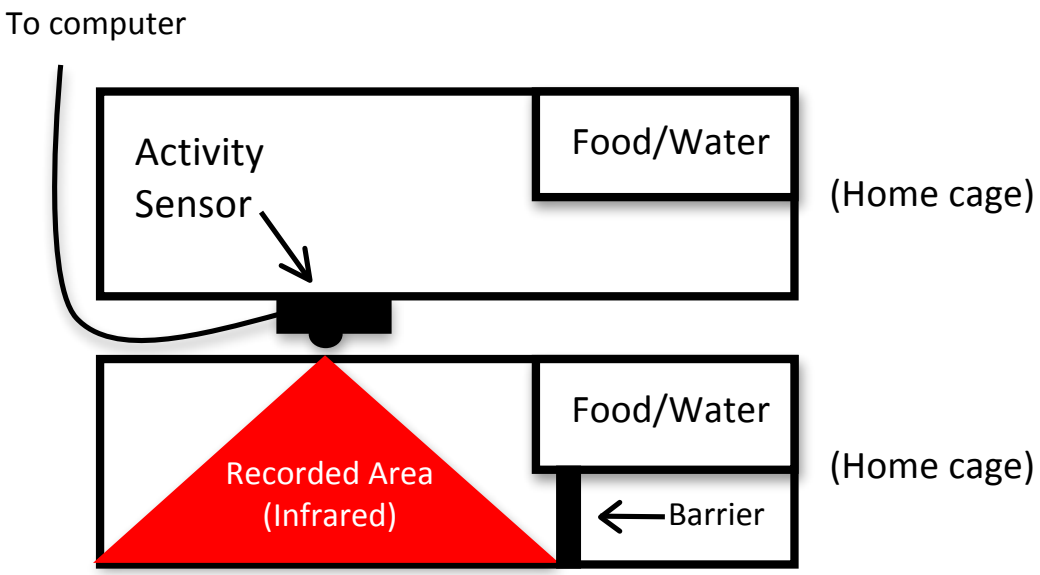

Figure 2.6. The position of the activity sensors in a rack of the animal holding room.
(illustration not to scale)

2.7. Novel Object Recognition / Location

2.7.1. Background

The final two behavioural paradigms that are used as part of this thesis are tests of novel object recognition and novel object location (NOR/NOL). These tests were first described by Ennaceur and Delacour (1988), and are now widely used measures of memory, attention and novelty seeking in rodents (e.g., Silvers et al., 2007; Goulart et al., 2010). They are useful because they require little training or habituation and can be designed to study short-term, intermediate, or long-term memory. Unlike other tests of memory, such as the Morris water maze, these procedures also inflict only mild stress that is unlikely to disrupt baseline cognition. The following protocol is based on one that has been devised in this laboratory based on published literature

(see: Cohen and Stackman, 2014), which has previously shown genotype differences in performance and/or drug treatment (unpublished observations).

2.7.2 Apparatus

The apparatus comprises a white plastic box (length 30 cm, width 20 cm, height 25cm, light intensity 12 lx) with a white floor marked with a black grid of 5 x 5 cm squares (Figure 2.7a). Three of the walls are also white, but a fourth wall is black, as a means of allowing the animals to orientate themselves in the box. Two objects were used in the object recognition task: (i) a black, plastic sharpener (length 3.5 cm, width 3.5 cm, height 5.5 cm); and (ii) a black, wooden penguin figure (length 2.5 cm, width 2.5 cm, height 9 cm) (Figure 2.7b). In the object location task, only the sharpener was used. These objects were chosen to be similar in height and width but differ in shape and texture.

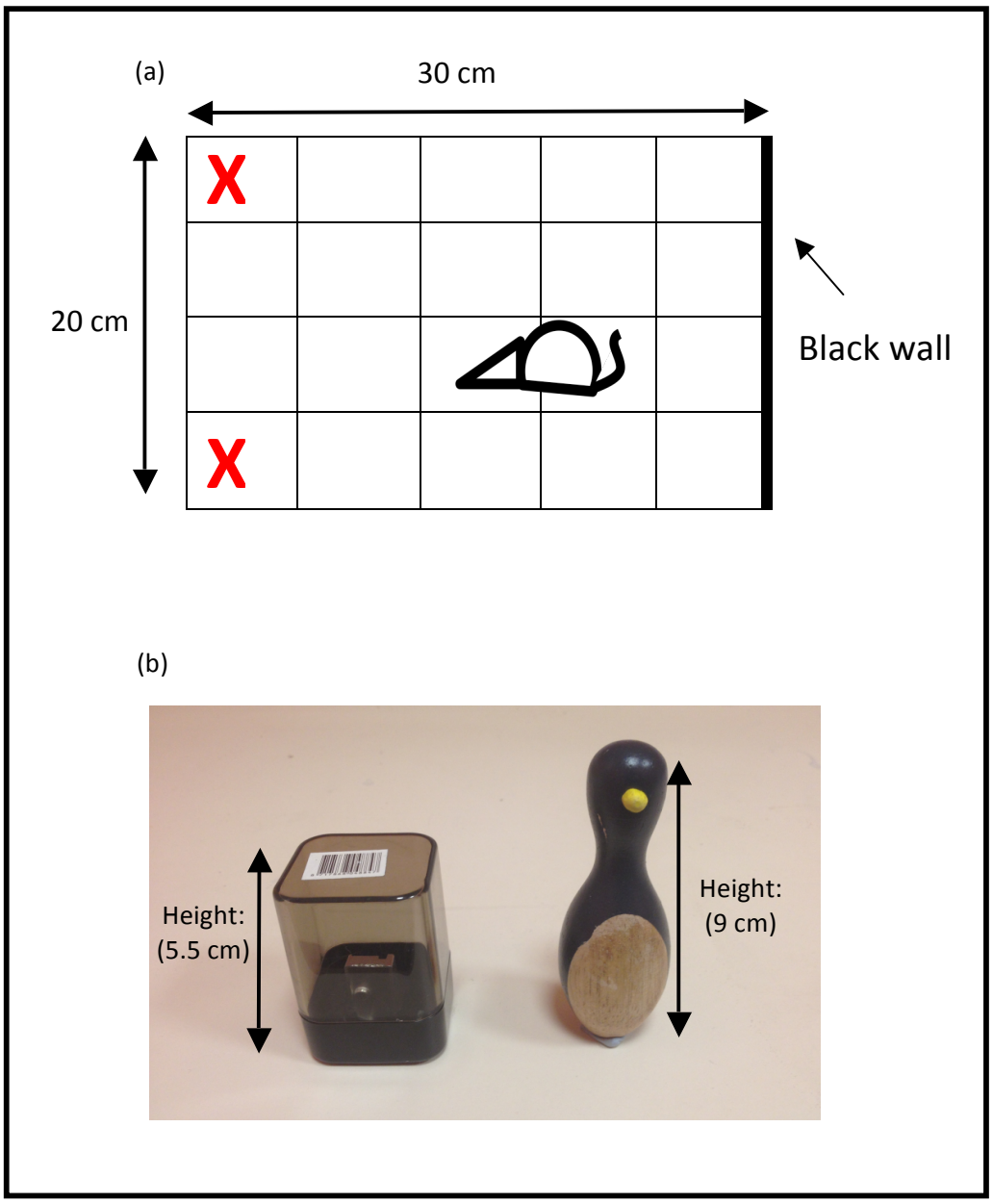

Figure 2.7. (a) Illustration of the box used in the novel object tests. (b) The two objects used in the novel object tests.
X denotes example of object positions (illustration not to scale)

2.7.3. Protocol

In both the NOR and NOL tasks, the experiment takes place over two days. On both days, animals are brought to the experimental room at 09.30 h and kept in their home cages until the start of the experiment, with food and water available *ad libitum*. Two white plastic boxes (described above) are placed next to each other and a camera placed above them to record the behaviour of two mice (e.g., one from each genotype) simultaneously. All experiments took place between 13.00 and 15.00 h, in order to match the timings used in the LDEB protocol (Section 2.5.3).

2.7.3.1. Novel Object Recognition test

On day one, animals are placed in the white box, which is empty, at 13.00 h for 30 min habituation. After this, they are placed back into their home-cage and returned to the animal holding room at 16.00 h. On day two, animals experience two trials. When applicable, animals receive drug treatment at the appropriate time prior to the first trial. In trial 1, animals are placed in the box at 13.00 h for 10 min. The box contains two identical objects, secured to the floor. The animal is then removed from the box and placed in its home-cage. In trial 2, animals are placed in the box at 14.00 h for 10 min. This time, one of the objects is replaced with a different object, but both remain in the same positions as the previous trial. The replaced object is referred to as 'novel', whereas the object that has not been replaced is referred to as 'familiar'.

The protocol for the novel objection recognition test is summarised in Table 2.6. and Figure 2.8.

Day	Time (h)	Action
One	13.00 (habituation)	Animals are habituated to the empty plastic boxes for 30 min.
Two	Drug Treatment*	If applicable, animals are injected with drug (i.p.) before being placed in the box for the first trial.
	13.00 (trial 1)	Animals are placed in the box for 10 min. Two of the same objects are placed at specific locations.
	14.00 (trial 2)	Animals are placed in the box for 10 min. One of the objects has been replaced by a different object

Table 2.6. Summary of the protocol used in the novel object recognition test.
Habituation was also started at 13.30 h and subsequent times adjusted accordingly.
* Injection time depends on drug treatment

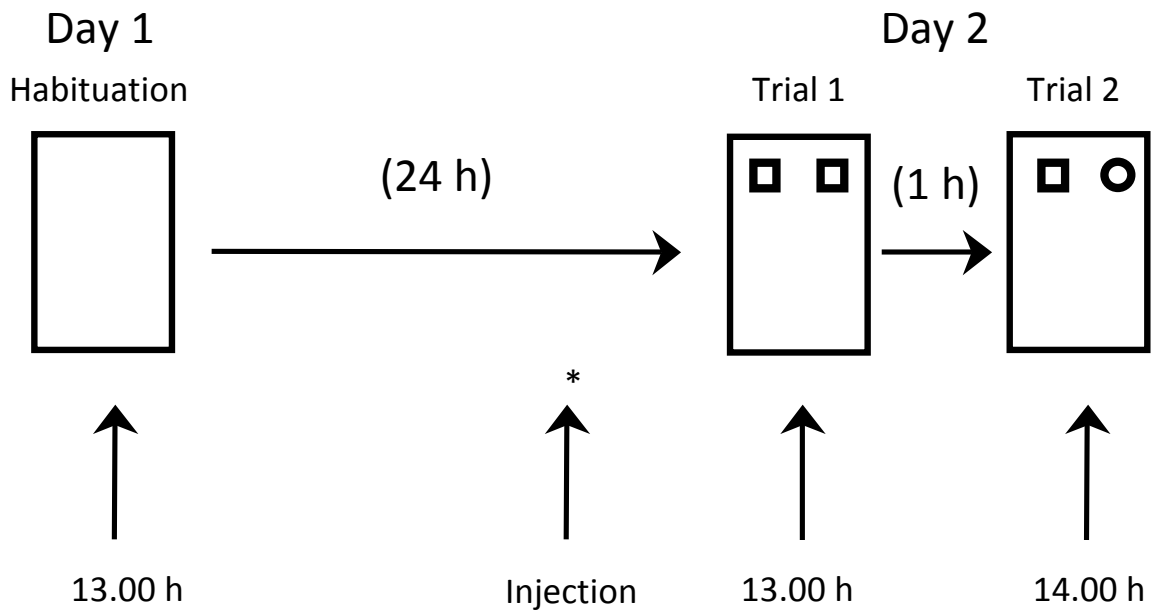

Figure 2.8. Schematic illustration of the protocol used in the novel object recognition test.
Habituation was also started at 13.30 h and subsequent times adjusted accordingly.
* Injection time depends on drug treatment

2.7.3.2. Novel Object Location test

On day one, animals are placed in the white box, which is empty, at 13.00 h for 30 min habituation. After this, they are placed back into their home-cage and returned to the animal holding room at 16.00 h. On day two, animals experience two trials. When applicable, animals receive drug treatment at the appropriate time prior to the first trial. In trial 1, animals are placed in the box at 13.00 h for 10 min. The box contains two identical objects, secured to the floor. The animal is then removed from the box and placed in its home-cage. In trial 2, animals are placed in the box at 14.00 h for 10 min. This time, one of the objects has been moved to a different position. The moved object is referred to as 'moved', whereas the object that has not been moved is referred to as 'stationary'.

The protocol for the novel objection location test is summarised in Table 2.7. and Figure 2.9.

Day	Time (h)	Action
One	13.00 (habituation)	Animals are habituated to the empty plastic boxes for 30 min.
Two	Drug Treatment*	If applicable, animals are injected with drug (i.p.) before being placed in the box for the first trial.
	13.00 (trial 1)	Animals are placed in the box for 10 min. Two of the same objects are placed at specific locations.
	14.00 (trial 2)	Animals are placed in the box for 10 min. One of the objects has been moved to a different location.

Table 2.7. Summary of the protocol used in the novel object location test.
Habituation was also started at 13.30 h and subsequent times adjusted accordingly.
* Injection time depends on drug treatment

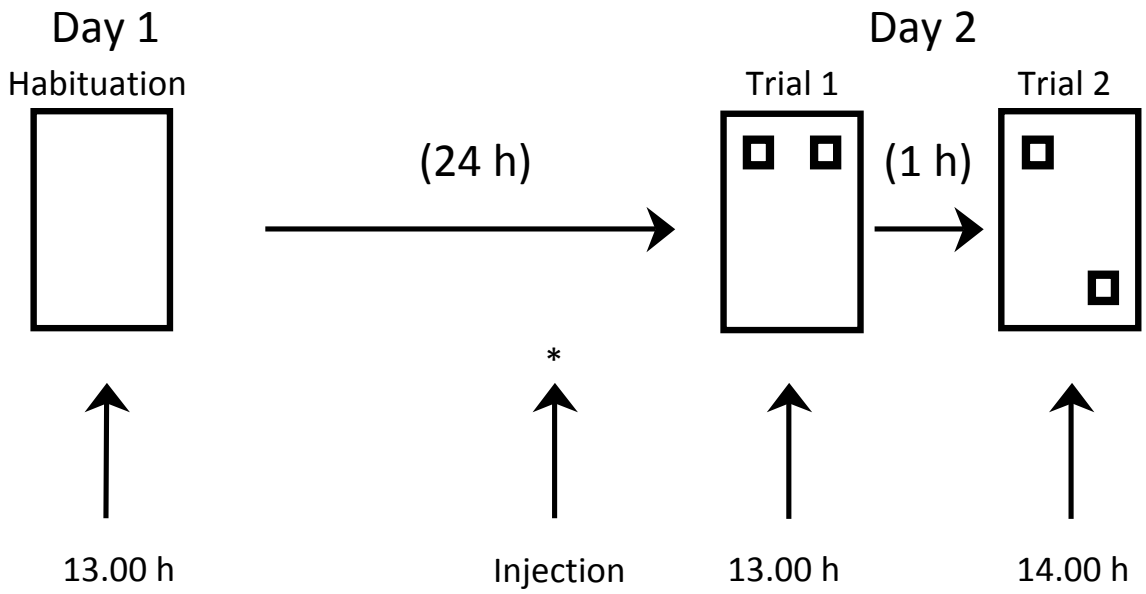

Figure 2.9. Schematic illustration of the protocol used in the novel object location test.
Habituation was also started at 13.30 h and subsequent times adjusted accordingly.
* Injection time depends on drug treatment

2.7.4. Behavioural scoring

Following the experiment, the amount of time spent exploring the novel/moved and familiar/stationary objects is scored, 'blind', in 5 min time bins. These are then used to work out a 'discrimination index':

NOR Discrimination Index =

[time spent at novel object – time spent at familiar object] / [time spent at novel object + time spent at familiar object]

NOL Discrimination Index =

[time spent at moved object – time spent at stationary object] / [time spent at moved object + time spent at stationary object]

The criterion for inclusion in scoring was to be facing the object within at least 1 cm distance. Time spent climbing on the object was not included in behavioural scoring.

2.8. Statistical analysis

For all experiments, statistical analyses were carried out using InVivoStat (Clark et al., 2012).

Data were analysed using Analysis of Variance (ANOVA), with *post-hoc* pairwise comparisons. Further details of the treatment factors and dependent variables used in each experiment are provided in each chapter. In all experiments, diagnostic plots for normality of the data-set and equality of the variance of the samples were checked and, when necessary, the data

were transformed (square-root(score) or Log_{10}(score+1)) to optimise the homogeneity of variance across the experimental groups before proceeding with subsequent analyses.

Due to the multifactorial/multivariate design of the experiments reported in this thesis, it was not possible to use power analysis to predict the number of animals needed or to determine whether sample sizes were adequate to detect statistical significance. This is because power analysis only considers a single treatment factor and a single variable (behaviour) at once. Consequently, multiple analyses would have to be performed that consider each treatment factor and each variable in turn, each with a different power. Also, in a multifactorial design all groups of data provide information about the population variance (making it a far more efficient design in terms of animal reduction), and so by comparing only two groups the use of power analysis would exclude a large amount of information. Finally, in many cases in this thesis this was the first time that the experiments were being performed, and so there was no information regarding effect size or sample variance. Because of these reasons, Mead's resource equation was instead used for all experiments to ensure that sample sizes were adequate; for all experiments, samples sizes were determined as adequate using this approach.

Data points more than 3 standard deviations from the mean were excluded from data analysis as outliers. Statistical significance was set at $P < 0.05$.

2.9. Statement of student supervision

As part of their laboratory projects, BSc or MSc students assisted in certain aspects of experiments that do not require a personal licence (e.g., in the 5-CSRTT: weighing the animals, setting up the computer, etc.). When students were involved in the running of an experiment, all work was carried out under my direct supervision.

Chapter 3

The different influences of genetic, environmental and/or epigenetic factors on the behavioural abnormalities of NK1R-/- mice

3.1. Introduction

As discussed in Chapter 1, the observation that NK1R-/- mice display a number of behavioural abnormalities that resemble symptoms of ADHD in humans (hyperactivity, impulsivity, and inattentiveness) has prompted the proposal that polymorphisms of the *TACR1* gene (the human equivalent of the mouse *Nk1r* gene) may be associated with increased vulnerability to ADHD (see: Yan et al., 2009; Yan et al., 2011). This led to a case-control human genetic study of the *TACR1* gene in 450 ADHD patients and 600 controls that identified four single-nucleotide polymorphisms which were strongly associated with the disorder (Yan et al., 2010). This association was subsequently confirmed with further human genetic studies on ADHD patients (Sharp et al., 2014). These findings suggest that disruption to NK1R function could be a contributory factor to the development of ADHD.

However, a potential caveat to this proposal is that the studies carried out on NK1R-/- mice and their wildtype counterparts were performed on mice bred from separate, homozygous breeding pairs. Although this approach has the ethical benefit of reducing over-breeding, there is the risk that genetic drift, whereby random genetic mutations and changes in allele frequency occur across generations, can change the genetic background of the two genotypes so that they differ in more ways than the single gene of interest (Bailey, 1982). Furthermore, this breeding method results in wildtype and NK1R-/- mice being raised separately, by dams of their respective genotypes, and housed with littermates of the same genotype only. Consequently, it is also possible that differences in early life environment, such as maternal care or interactions with littermates, could be influencing their behaviour as adults (Crews et al. 2004; Sasaki et al. 2014; Tarantino et al. 2011).

The aim of experiments reported in this chapter was to determine which of the behavioural abnormalities of NK1R-/- mice can be attributed directly to a lack of functional NK1R, and which involve an interaction between a lack of functional NK1R and other contributory (e.g., environmental) factor(s). To that end, the behaviour of wildtype and NK1R-/- mice bred from homozygous breeding pairs (the original '*Hom*' colony, described in Section 2.2) is compared with that of wildtype and NK1R-/- mice bred from heterozygous breeding pairs ('*Het*' colony, derived from the two genotypes from the *Hom* colony).

The first step was to establish whether the hyperactivity of NK1R-/- mice from the *Hom* colony is also evident in NK1R-/- mice from the *Het* colony. Previously, the locomotor activity of NK1R-/- mice has been measured using a light/dark exploration box (LDEB) (Fisher et al., 2007; Yan et al., 2010) or activity chamber (Herpfer et al., 2005), in which activity was measured, for 30 min, during the day (light phase of the 24 h cycle). Here, the locomotor activity of wildtype and NK1R-/- mice, from both the *Hom* and *Het* colonies, is measured for the first time in their home-cage environment using passive infra-red sensors. This method has the benefit of being able to measure the movement of these animals across the entire 24 h cycle. In light of evidence that the normal sleep / arousal pattern is disrupted in ADHD patients (e.g., Kooij and Bijlenga 2013; van Veen et al. 2010), it was also of interest to determine whether the diurnal rhythm of motor activity across the 24 h cycle is disrupted in NK1R-/- mice.

The second step was to establish whether the impulsivity and inattentiveness of NK1R-/- mice from *Hom* colony is also evident in NK1R-/- mice bred from the *Het* colony. In preclinical research, these cognitive measures are commonly assessed using the 5-Choice Serial Reaction-Time Task (5-CSRTT), which measures an animal's sustained visual attention and response control (Robbins, 2002; see: Section 2.3). Here, the performance of NK1R-/- and wildtype

mice from the *Hom* and *Het* colonies was compared, to determine whether the behavioural abnormalities of NK1R-/- mice reported previously in this test (Yan et al., 2011) can be attributed directly to a lack of functional NK1R or whether there are other contributory factor(s).

3.1.1. Objectives

1) Determine whether the hyperactivity of NK1R-/- mice from the *Hom* colony is also evident in NK1R-/- mice from the *Het* colony, and whether the 24 h cycle in motor activity is disrupted in these mice

2) Determine whether the impulsivity and inattentiveness of NK1R-/- mice from the *Hom* colony are also evident in NK1R-/- mice from the *Het* colony

The following experiments have been accepted for publication in *Genes, Brain, and Behavior* (Porter et al., 2015a).

3.2. Methods

3.2.1. Animals

Two colonies of mice were used in these experiments. In the first ('*Hom*') colony, wildtype (NK1R+/+; *'WT-Hom'*) and NK1R-/- ('*KO-Hom*') mice were derived from homozygous breeding pairs of the same genotype, as described in Section 2.2. The second (*'Het'*) colony was derived by cross-breeding wildtype and NK1R-/- mice from the *Hom* colony to produce heterozygous (NK1R+/-) offspring. These were then used as heterozygous breeding pairs to produce mixed litters containing wildtype (NK1R+/+; '*WT-Het*'), NK1R-/- ('*KO-Het*') and NK1R+/- mice. The genotype of these animals was confirmed using the PCR technique. The NK1R+/- mice were culled at weaning (age: 21 days).

In the *Hom* colony, *WT-Hom* and *KO-Hom* mice were housed separately in cages containing up to four littermates of the same genotype. In the *Het* colony, *WT-Het* and *KO-Het* mice were house together as mixed litters in cages containing up to four littermates, with at least one animal from each genotype. Other aspects of housing and husbandry were identical in the two colonies (see: Section 2.2.2).

3.2.2. Protocol: activity sensors

Further details of the activity sensors protocol are provided in Section 2.6.

Five male mice were used from each group (*WT-Hom*, *KO-Hom*, *WT-Het*, *KO-Het*; aged 8 - 14 weeks at the start of testing). Animals from the *Hom* colony were selected from two breeding pairs per genotype, and animals from the *Het* colony were selected from three breeding pairs. Animals were selected in order to age-match the four groups as closely as possible.

On the first day of the experiment, animals were removed from their home-cages between 09.00 - 10.00 h and placed in an individual home-cage positioned below an activity sensor attached to the cage above (see: Fig. 2.6). As discussed in Chapter 2, in this position the sensors monitored the entire area of the cage floor below, and recorded the animal's gross ambulatory movement, rears and turns of the body. They did not measure smaller movements, such as breathing. The activity of each animal was recorded, without interruption, for seven days. At the end of seven days, data produced by the activity sensors were transferred to a Microsoft Excel file for statistical analysis using InVivoStat (Clark *et al.* 2012).

3.2.3. Protocol: 5-CSRTT

Further details of the 5-CSRTT protocol are provided in Section 2.3.

Twelve male mice were used from each group (*WT-Hom*, *KO-Hom*, *WT-Het*, *KO-Het*; mean age: 6.5±0.1 weeks, 6.6±0.1 weeks, 7.9±0.2 weeks and 7.8±0.2 weeks, respectively; start weight: 34.55±0.71 g, 31.85±0.53 g, 38.40±1.00 g and 34.80±0.60 g, respectively). Animals from the *Hom* colony were selected from four breeding pairs per genotype, and animals from the *Het* colony were selected from six breeding pairs. Animals were selected in order to age-match the four groups as closely as possible (starting the experiment at 6 – 8 weeks old). As a result of the large number of animals needed for this study (four groups of 12 mice; 48 mice in total), the experiment was performance in two replicate steps, each involving the training and testing six animals from each group. As a precaution, animals from the *Het* colony used in the second half of the experiment were re-derived from the *Hom* colony, instead of using subsequent litters from the same (heterozygous) breeding pairs.

All animals were trained/tested in the 5-CSRTT as described fully in Section 2.3 (see also: Fig. 3.1). Half of all animals were assigned to the morning session (10.00 – 12.00 h) and half were assigned to the afternoon session (13.00 – 15.00 h) (balanced across the two colonies and two genotypes). Once stable baseline performance criteria had been achieved at Stage 6 of training, animals were tested using both the variable intertrial interval (VITI) and long intertrial interval (LITI) tests. In the VITI, the ITI was variable (2, 5, 10 and 15 s) and delivered on a random schedule; in the LITI, the ITI was fixed (7 s) and was longer than that experienced during training. In order to correct for any possible effect of previous test experience, the order of testing in these two tests was counterbalanced across all experimental factors (Colony, Genotype and Time-of-Day). Testing was carried out on Fridays, with stable baseline criteria required for at least three consecutive days prior to testing.

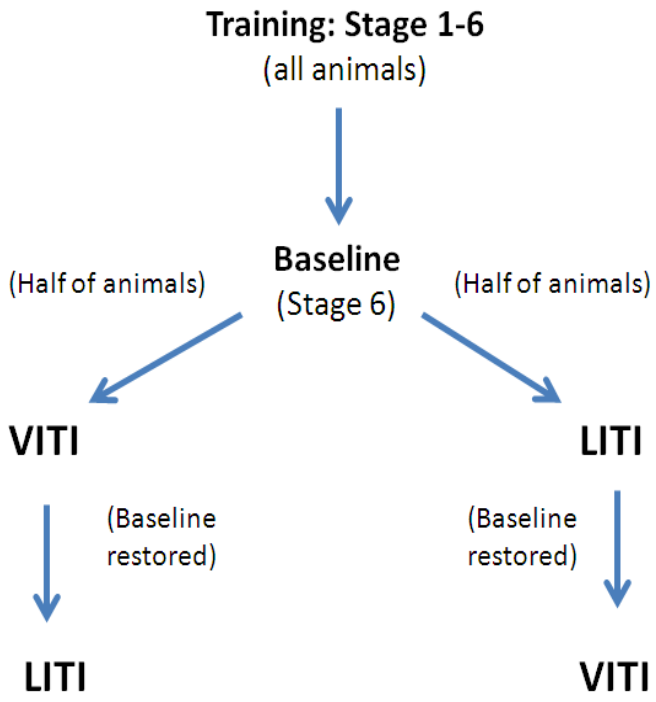

Figure 3.1. Schematic diagram of the 5-CSRTT protocol used in this study. The order of testing in the VITI and LITI was counterbalanced across all three experimental factors (Colony, Genotype and Time-of-Day).

3.2.4. Statistical analysis

Further details of statistical analysis are provided in Section 2.8.

Activity Sensors: For each animal, data captured by the activity sensors at 60 s intervals were pooled into 6 h time bins (07.00 – 12.59 h, 13.00 h – 18.59 h, 19.00 – 00.59 h, 01.00 – 06.59 h). These were chosen based on the lighting schedule in the holding room (lights turned on in steps from 07.00 – 08.00 h; lights turned off in steps from 19.00 – 20.00 h), giving activity levels during the 'early' and 'late' halves of the light and dark phases.

In order to allow for any possible effect of individual housing conditions on animals' behaviour, data collected across the first 24 h were excluded from analysis (only data collected across days 2 to 7 were used for statistical analysis). To determine whether activity changed across days 2 to 7, each 6 h time bin was initially analysed using mixed model ANOVA, treating 'Day' as the within-subjects factor and 'Colony' and 'Genotype' as between-subjects factors. For time bins where there was no interaction between Day and Colony and/or Genotype, the data were pooled across days 2 – 7 to produce a mean activity for that time bin (N = 1 for each animal). Mean activity was subsequently compared across time-bins using mixed model ANOVA, treating 'Time-Bin' as the within-subjects factor and 'Colony' and 'Genotype' as between-subjects factors. For time bins where there was an interaction between Day and Colony and/or Genotype, each day was analysed separately.

5-CSRTT: Data from the first day of each Stage of training were analysed using 4-way mixed model ANOVA, treating 'Colony', 'Genotype' and 'Time-of-Day' (morning or afternoon) as the between-subjects factors and 'Stage' as the within-subjects factor. Data from the VITI and LITI tests were first analysed using 3-way single measures ANOVA, treating 'Colony', 'Genotype' and 'Time-of-Day' as between-subjects factors. Any data points more than 3 standard deviations from

the mean were removed from analysis as outliers. This only occurred in the VITI test, with one mouse removed from analysis of *perseveration*, (1 x *WT-Hom*), *%accuracy* and *latency to correct response* (1 x *KO-Hom*), and *latency to collect reward* (1 x *KO-Hom*). Consequently, statistical analyses across training and testing in the 5-CSRTT were carried out on groups of N = 10 – 12.

In both experiments, a significant effect of one of the main factors, or an interaction between them, was the criterion for progressing on to *post hoc* 2-way or 1-way ANOVA, as appropriate, followed by pairwise group comparisons (LSD test). Four animals (1 x *WT-Hom*, 2 x *WT-Het*, and 1 x *KO-Het*) failed to reach the criteria necessary for testing in the VITI and LITI tests, and so were excluded from all statistical analysis, including training.

3.3. Results

3.3.1. Activity sensors: the hyperactivity of NK1R-/- mice is a direct consequence of dysfunctional NK1R

The activity of one mouse from each group (*WT-Hom*, *KO-Hom*, *WT-Het*, *KO-Het*) across days 1 - 7 is represented by double-plotted actograms in Figure 3.2.

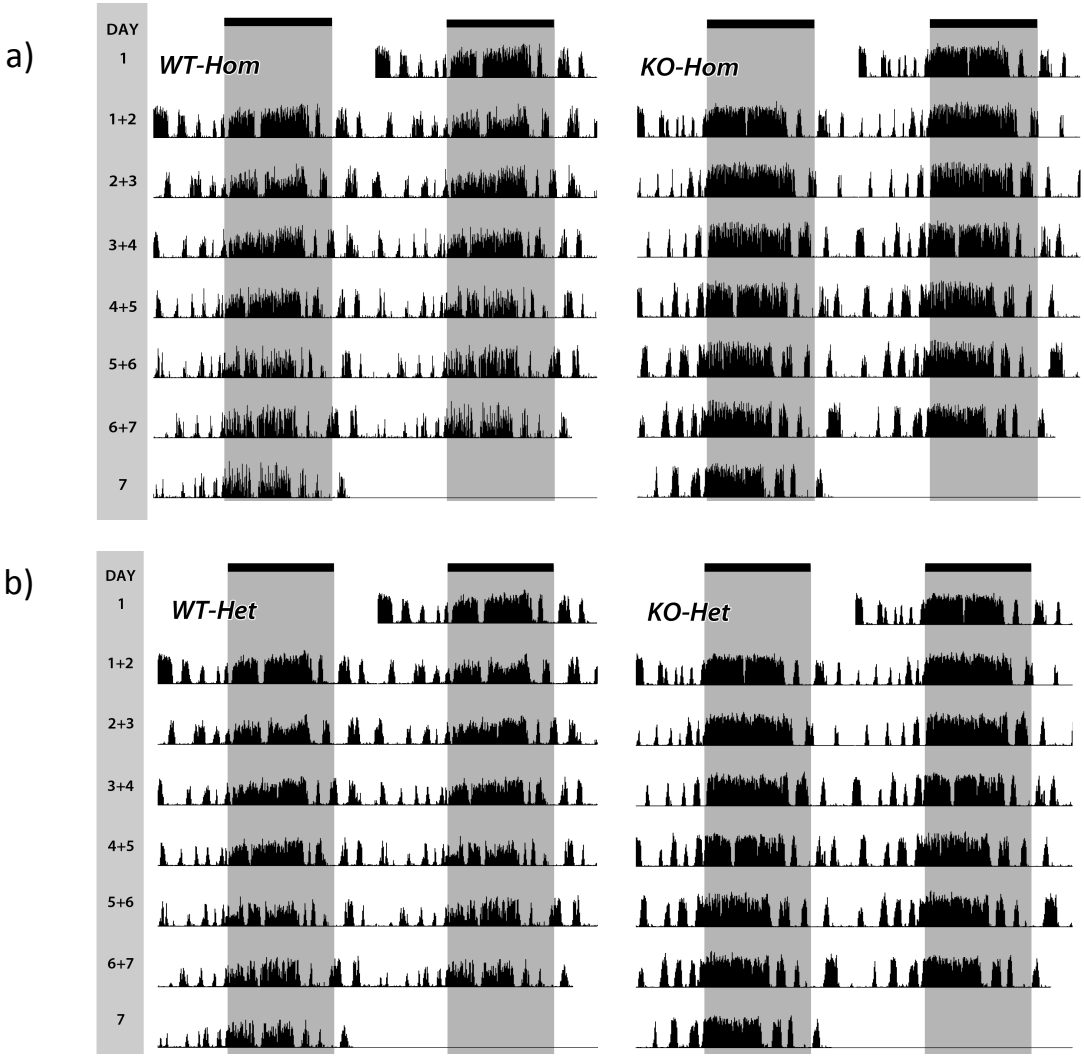

Figure 3.2. Double-plotted actograms representing activity across days 1 – 7 of (a) one *WT-Hom* and one *KO-Hom* mouse and (b) one *WT-Het* and one *KO-Het* mouse. Areas in white represent the light phase of the 24 h light : dark cycle (07.00 – 18.59 h), areas in grey represent the dark phase of the 24 h light : dark cycle (19.00 – 06.59 h). Lighting was increased or reduced in steps between 07.00 – 08.00 h and 19.00 – 20.00 h, respectively. Actograms provided by Dr Laurence Brown, Oxford University

Effect of 'Day' on activity levels: During the 'early' dark phase (19.00 – 00.59 h), activity did not change across days 2 to 7 ($F(5,80)=1.18$; $P = 0.326$) and so these data were pooled to produce a mean activity for that time bin. During both the 'late' dark phase (01.00 – 06.59 h) and 'early' light phase (07.00 – 12.59 h), activity did change across days 2 to 7 ($F(5,80)=10.96$, $P < 0.001$ and $F(5,80)=2.65$, $P =0.029$, respectively), but this did not interact with either Colony or Genotype, and so these data were again pooled to produce a mean activity for each time bin. By contrast, during the 'late' light phase (13.00 – 18.59 h), a change in activity across days 2 – 7 interacted with Colony ($F(5,80)=3.17$; $P=0.012$). Consequently, data across days 2 – 7 were kept separate for this time bin, and not included in the time bin comparisons.

Time bin comparisons (07.00 – 12.59 h, 19.00 – 00.59 h, 01.00 – 06.59 h): The activity levels of the two Colonies did not differ at any point during these time bins ($F(1,16) = 0.25$; $P = 0.626$; Fig. 3.3a,b). Differences in the two Genotypes depended on time bin ($F(2,32)=7.97$; $P = 0.002$). During both the 'early' light phase and 'early' dark phase, activity levels did not differ in wildtype and NK1R-/- mice, in either colony (LSD: *Hom*: $P=0.705$ and $P=0.951$, respectively; *Het*: $P=0.42$ and $P=0.958$, respectively; Fig. 3.4a,b). However, during the 'late' dark phase, activity levels were higher in NK1R-/- mice, from both colonies, compared with their wildtype counterparts (LSD: *Hom*: $P = 0.046$; *Het*: $P = 0.031$; Fig. 3.4a,b).

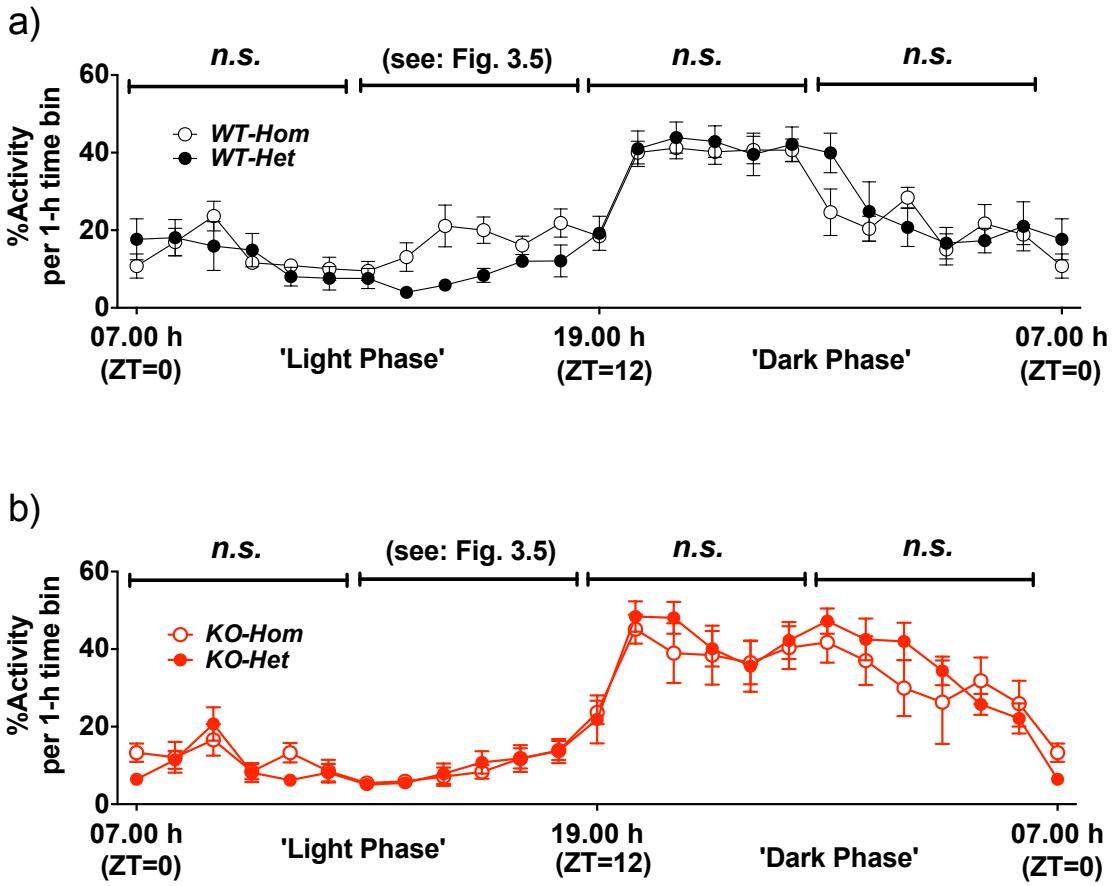

Figure 3.3. Locomotor activity across the 24 h cycle: colony comparisons.

24 h activity pattern of (a) *WT-Hom* and *WT-Het* mice and (b) *KO-Hom* and *KO-Het* mice. Circles show mean±s.e.m. for each hourly time-point. Lines above the graphs represent 6 h time bins used for statistical comparisons. N = 5 per group. *n.s.*, non-significant; ZT, zeitgeber time (light cue)

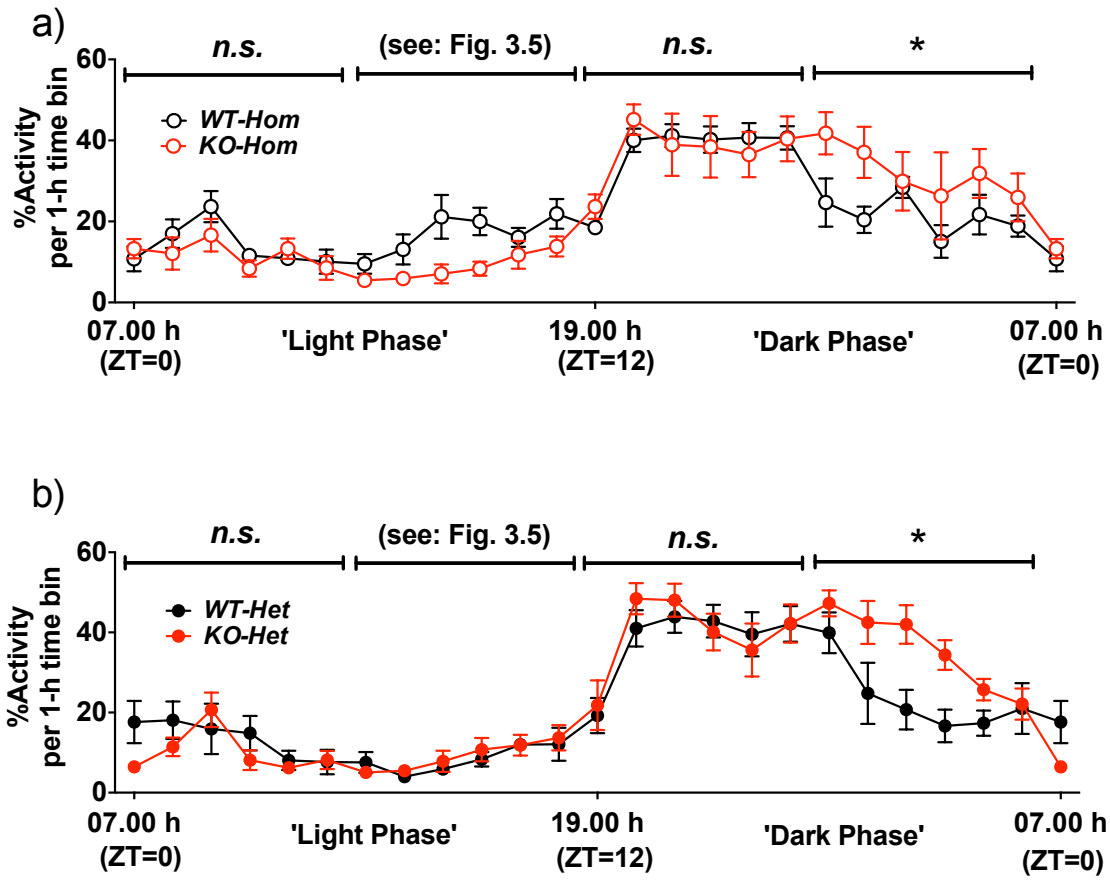

Figure 3.4. Locomotor activity across the 24 h cycle: genotype comparisons.

24 h activity pattern of (a) *WT-Hom* and *KO-Hom* mice and (b) *WT-Het* and *KO-Het* mice. Circles show mean±s.e.m. for each hourly time-point. Lines above the graphs represent 6 h time bins used for statistical comparisons. N = 5 per group. * $P<0.05$; *n.s.*, non-significant; ZT, zeitgeber time (light cue)

'Late' light phase (13.00 – 18.59 h): During this time bin, differences in activity levels in the two Colonies changed across days 2 to 7 ($F(5,80)=3.17$; $P = 0.012$; Fig. 3.5). Furthermore, differences in activity levels in the two Genotypes depended on Colony ($F(1,16)=6.26$; $P = 0.024$). Across days 2 to 7, activity levels of both *KO-Hom* mice and *WT-Het* mice decreased (LSD: $P<0.05$ for all), whilst activity levels of *KO-Het* mice increased (LSD: $P<0.05$ for all). During this time bin, the activity of *WT-Hom* mice was greater than all other groups of mice (LSD: $P < 0.05$ for all), but the other three groups did not differ from each other.

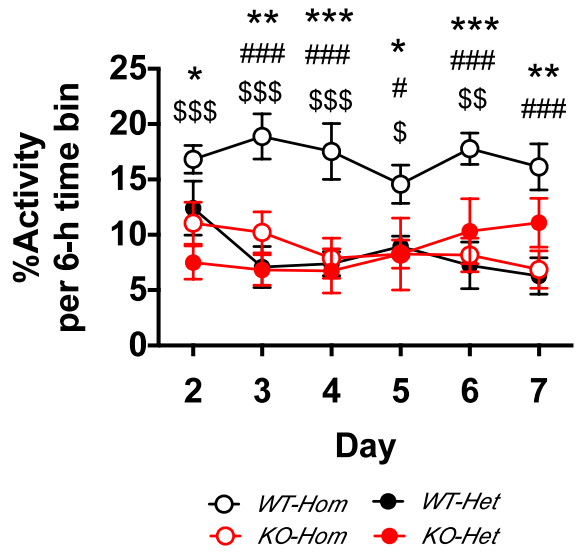

Figure 3.5. Locomotor activity during the 'late' light phase of the 24 h cycle across days 2 – 7. Circles show mean ± s.e.m. for this time-bin across days 2 – 7. N = 5 per group. * $P<0.05$, ** $P<0.01$, ***$P<0.05$ (*c.f.*, *WT-Hom* vs *KO-Hom*); # $P<0.05$, ## $P<0.01$, ### $P<0.001$ (*c.f.*, *WT-Hom* vs *WT-Het*); $ $P<0.05$, $$ $P<0.01$, $$$ $P<0.001$ (*c.f.*, *WT-Hom* vs *KO-Het*).

3.3.2. 5-CSRTT (training): the higher incidence of premature responses and perseveration seen in NK1R-/- mice are both a direct consequence of a lack of functional NK1R

Across stages 1 – 6, the *total number of sessions* required to pass training did not differ in the two Colonies ($F(1,36) = 0.09$, $P = 0.764$; Fig. 3.6). However, further analysis revealed that the effect of both Colony and Genotype on this behaviour depended on Stage ($F(5,180)=2.81$; $P=0.018$ and $F(5,180)=2.78$; $P=0.019$, respectively): at Stage 4, *WT-Hom* mice took longer than *KO-Hom* mice to pass (LSD: $P=0.037$); at Stage 5, *KO-Het* mice took longer than both *WT-Het* (LSD: $P=0.023$) and *KO-Hom* (LSD: $P=0.019$) mice to pass.

Throughout training, the two Colonies did not differ in *%omissions* ($F(1,36)=1.8$, $P=0.188$; Fig. 3.7a,b), *perseveration* ($F(1,36)=0.38$; $P = 0.543$; Fig. 3.7d,e) or *premature responses/100 trials* ($F(1,36)=0.01$; $P=0.943$; Fig. 3.7g-h). The two Colonies also did not differ in *%accuracy* or *latency to correct response*, but *latency to collect the reward* was slightly longer in mice from the *Hom* colony (c. 0.7 s) (see: Table 3.1).

Overall, the incidence of both *perseveration* ($F(1,36)=15.53$, $P<0.001$; Fig. 3.7f) and *premature responses/100 trials* ($F(1,36)=6.00$, $P=0.019$; Fig. 3.7i) was higher in NK1R-/- mice, from both colonies, across training. The incidence of *%omissions* did not differ in NK1R-/- mice and wildtypes, overall ($F(1,36) = 0.92$, $P = 0.343$), but the effect of Genotype on this behaviour depended on Stage ($F(5,180)=2.33$, $P=0.044$; Fig. 3.7c): At stage 1, this behaviour was higher in wildtypes, from both colonies, compared with NK1R-/- mice (LSD: $P = 0.014$); at later stages, this behaviour did not differ in the two genotypes. Finally, throughout training the *latency to correct response* was shorter in NK1R-/- mice, from both colonies, although this difference was small (c. 0.6 s; $F(1,36)=5.63$; $P=0.023$; for values, see: Table 3.1).

By Stage 6, there were no Colony or Genotype differences, in any behaviour.

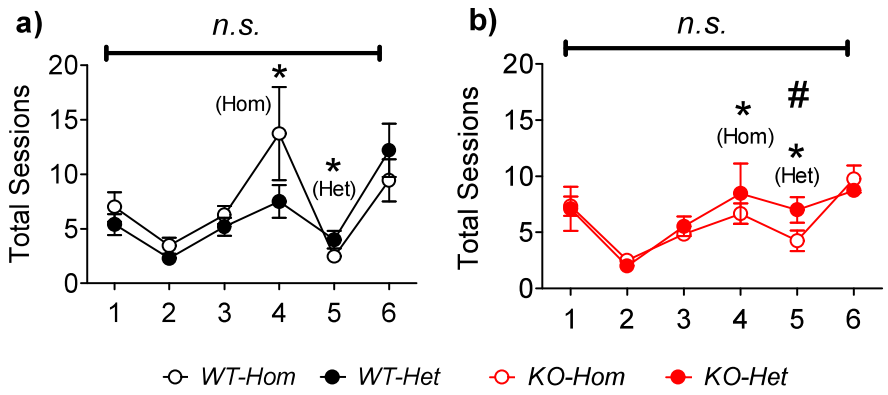

Figure 3.6. Total sessions required to pass each stage of training in the 5-CSRTT. Circles show mean ± s.e.m. Numbers underneath graphs indicate stage of training. Lines above the graph represent statistical comparison across all stages. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 (Genotype comparison). # *P*<0.05 (Colony comparison); *n.s.*, non-significant; N = 10 – 12 per group.

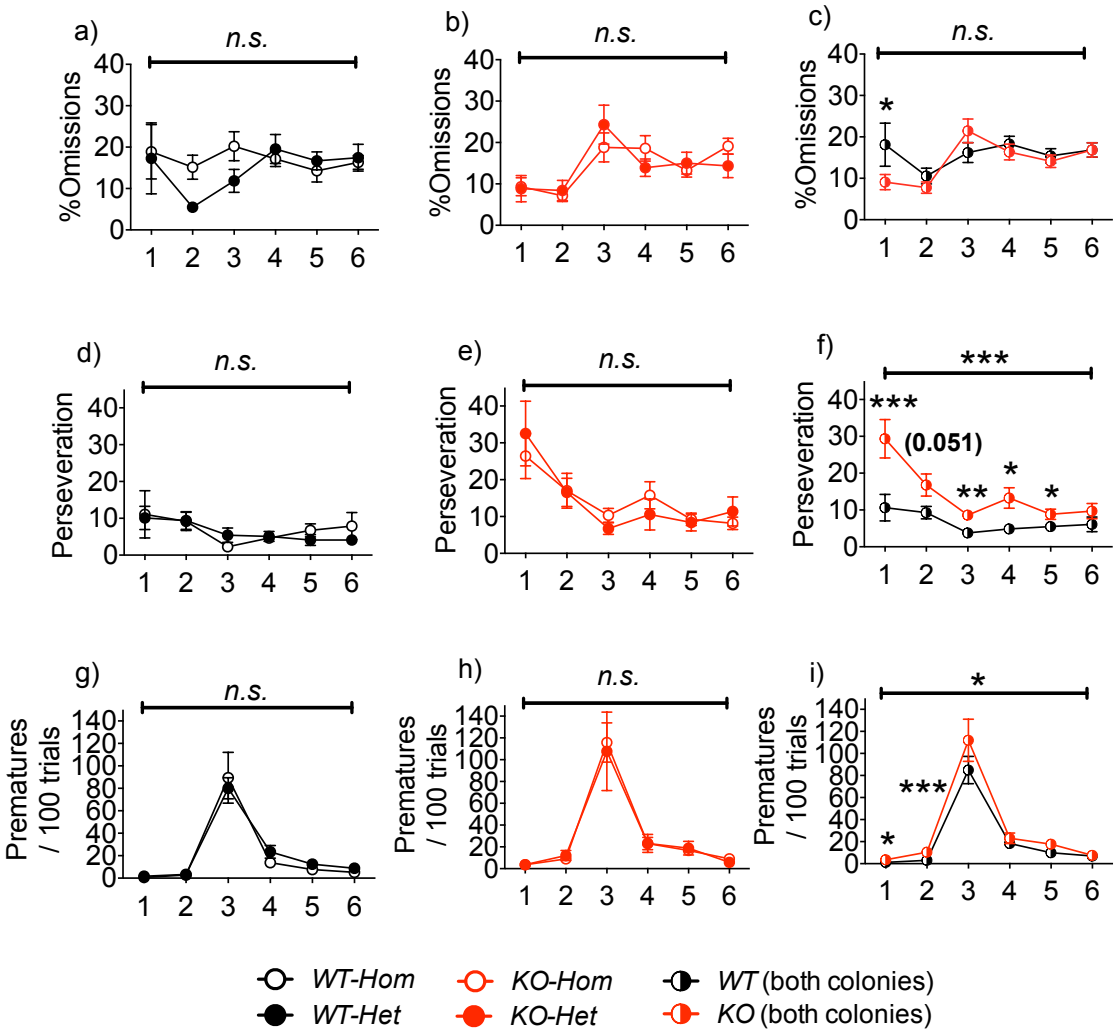

Figure 3.7. Training in the 5-Choice Serial Reaction-Time Task

Circles show mean ± s.e.m. Numbers underneath graphs indicate stage of training. Lines at top of graphs represent statistical comparisons across all stages of training. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 (Genotype comparison); *n.s.*, non-significant; N = 10 – 12 per group.

3.3.3. 5-CSRTT (VITI): the incidence of premature responses, but not omissions or perseveration, differs in NK1R-/- mice from the *Hom* and *Het* colonies in the VITI test

When tested in the VITI, the two Colonies did not differ in the incidence of either *%omissions* ($F(1,35)=0.31$, $P=0.579$; Fig. 3.8a) or *perseveration* ($F(1,34)=0.72$, $P=0.439$; Fig. 3.8b). As during training, the incidence of *perseveration* was higher in NK1R-/- mice, from both colonies ($F(1,34)=5.12$; $P=0.03$). Genotype differences in *%omissions* depended on Time-of-Day ($F(1,39)=8.28$, $P=0.006$). In the morning, *%omissions* did not differ in the two genotypes, but in the afternoon were higher in wildtypes (LSD: $P=0.001$). Moreover, *%omissions* were higher in NK1R-/- mice, from both colonies, when tested in the morning compared with the afternoon (LSD: $P=0.009$).

The effect of Genotype on *premature responses/100 trials* differed in the two Colonies ($F(1,39)=11.98$, $P = 0.001$; Fig. 3.8c). In the *Hom* colony, the incidence of *premature responses/100 trials* was higher in NK1R-/- mice compared with wildtype mice (LSD: $P = 0.006$). In the *Het* colony, there was no difference in the two genotypes, although an apparent reduction in NK1R-/- mice approached significance (LSD: $P = 0.054$).

The two Colonies also did not differ in *%accuracy*, *total number of trials completed, latency to correct response*, or *latency to collect reward* (Table 3.1). Moreover, none of these behaviours differed in wildtype and NK1R-/- mice. Across all groups, *%accuracy* was higher in animals tested in the afternoon compared with animals tested in the morning, although this difference was small (c. 3%; Table 3.1).

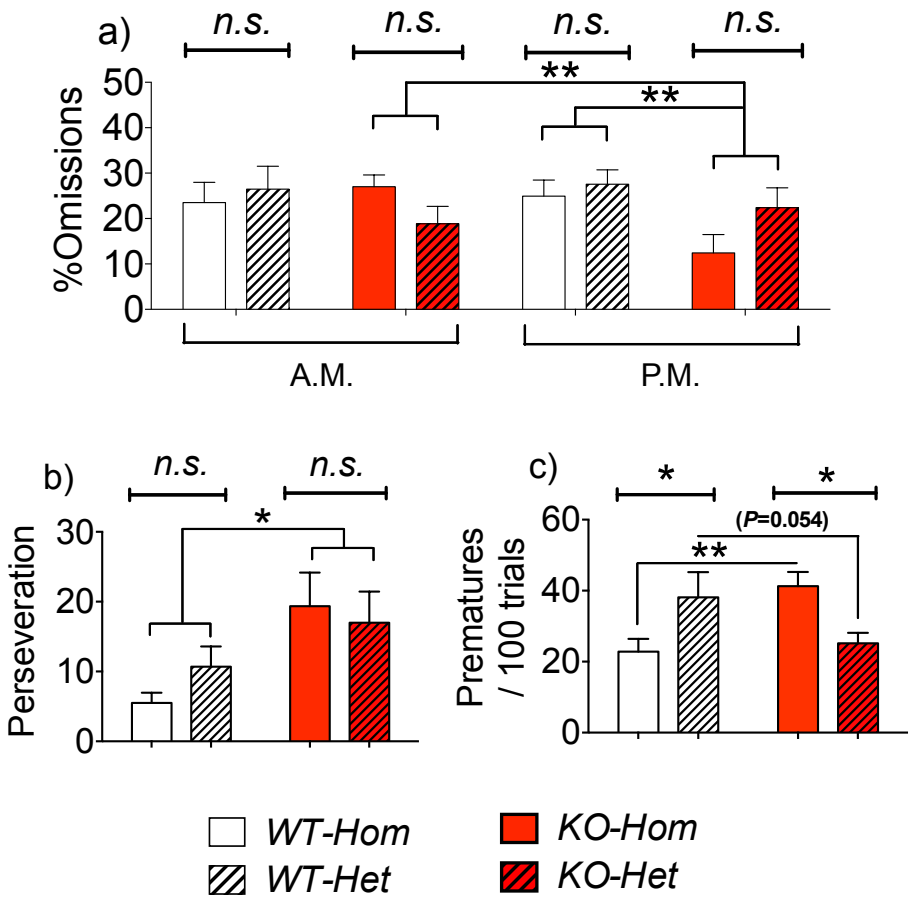

Figure 3.8. Variable Intertrial Interval (VITI) in the 5-Choice Serial Reaction-Time Task

Lines at the top of graphs indicate colony comparisons. Other lines indicate statistical comparisons between groups. Bars show mean ± s.e.m. * $P<0.05$, ** $P<0.01$; *n.s.*, non-significant. N = 10 – 12 per group.

3.3.4. 5-CSRTT (LITI): the performance of NK1R-/- mice from the two colonies does not differ in the LITI test

In the LITI, there were no differences in the two Colonies in *%omissions* (F(1,35)=0.01, *P*=0.903; Fig. 3.9a), *perseveration* (F(1,35)=0.01, *P*=0.91; Fig. 3.9b), or *premature responses/100 trials* (F(1,35)=0.33, *P*=0.567; Fig. 3.9c). Furthermore, in both colonies, neither *perseveration* (F(1,35)=0.33; *P*=0.567) nor *premature responses/100 trials* (F(1,35)=0.1; *P*=0.753) differed in the two Genotypes. However, as occurred in the VITI, the effect of Genotype on *%omissions* depended on Time-of-Day (F(1,39)=8.51; *P*=0.006). In the morning, *%omissions* were higher in NK1R-/- mice (LSD: *P*=0.035). In the afternoon, there was no genotype difference in *%omissions*, although a higher incidence of this behaviour in wildtype mice approached the criterion for significance (LSD: *P*=0.059). Furthermore, *%omissions* were higher in NK1R-/- mice tested in the morning compared with afternoon (LSD: *P*=0.003).

Finally, there were no Colony differences in *%accuracy*, *total number of trials completed*, *latency to correct response*, or *latency to collect reward* (Table 3.1.), and none of these behaviours differed in wildtype and NK1R-/- mice.

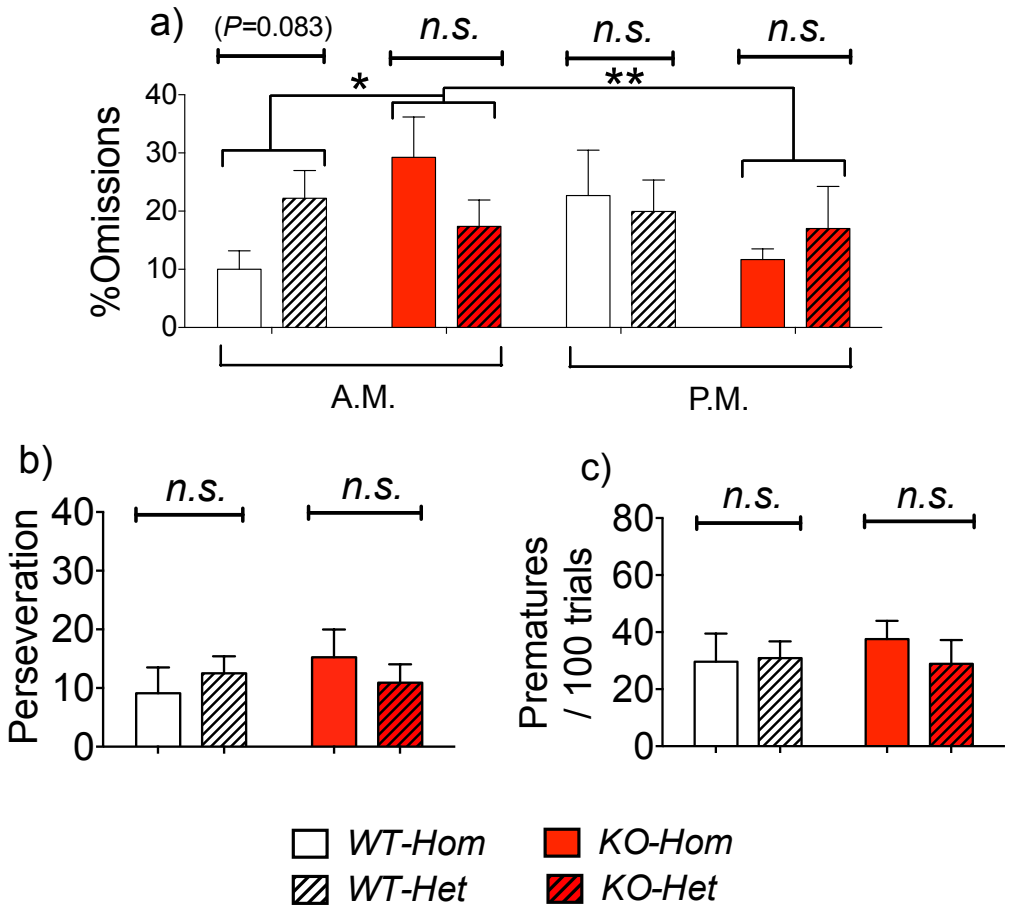

Figure 3.9. Long Intertrial Interval (LITI) in the 5-Choice Serial Reaction-Time Task. Lines at the top of graphs indicate colony comparisons. Other lines indicate statistical comparisons between groups. Bars show mean ± s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; *n.s.*, non-significant. N = 10 – 12 per group.

	(a) Training				(b) VITI				(c) LITI			
Behaviour	**Colony**	**Geno**	**Value ±s.e.m.**	**Statistic**	**Colony /ToD**	**Geno /ToD**	**Value ±s.e.m.**	**Statistic**	**Colony**	**Geno**	**Value ±s.e.m.**	**Statistic**
%Accuracy	*Hom*	*WT*	76.5±1.3	F(1,36)=0.06, *P*=0.815	*AM*		93.0±1.1	**F(1,34)=5.05, *P*=0.031**	*Hom*	*WT*	91.7±1.7	F(1,35)=0.19, *P* = 0.662
		KO	72.1±1.1							*KO*	90.2±2.0	
	Het	*WT*	74.3±2.2		*PM*		96.0±0.7		*Het*	*WT*	88.3±2.7	
		KO	74.9±1.5							*KO*	91.2±2.2	
Total Trials	*Hom*	*WT*	n/a	n/a	*Hom*	*WT*	99.2±0.8	F(1,35)=0.2, *P*=0.656	*Hom*	*WT*	97.6±2.4	F(1,35)=0.01, *P*=0.916
		KO	n/a			*KO*	98.6±0.7			*KO*	100.0±0	
	Het	*WT*	n/a		*Het*	*WT*	99.8±0.2		*Het*	*WT*	99.0±1.0	
		KO	n/a			*KO*	100.0±0			*KO*	95.8±3.1	
Latency to Correct Response (s)	*Hom*	*WT*	5.2±0.3	F(1,36)=2.2, *P*=0.147	*Hom*	*WT*	1.0±0.1	F(1,34)=0.63, *P*=0.434	*Hom*	*WT*	0.8±0.0	F(1,35)=1.69, *P*=0.203
		KO	4.6±0.2			*KO*	0.9±0.0			*KO*	0.7±0.0	
	Het	*WT*	5.0±0.2		*Het*	*WT*	1.0±0.0		*Het*	*WT*	0.8±0.1	
		KO	4.4±0.2			*KO*	1.0±0.0			*KO*	0.8±0.0	
Latency to Collect Reward (s)	*Hom*	*WT*	5.6±0.6	**F(1,36)=4.34, *P*=0.044**	*Hom*	*WT*	1.5±0.1	F(1,34)=3.94, *P*=0.055	*Hom*	*WT*	1.5±0.1	F(1,35)=1.19, *P*=0.283
		KO	4.5±0.2			*KO*	1.7±0.3			*KO*	1.4±0.1	
	Het	*WT*	4.2±0.4		*Het*	*WT*	1.3±0.1		*Het*	*WT*	1.4±0.1	
		KO	4.4±0.4			*KO*	1.3±0.1			*KO*	1.3±0.1	

Table 3.1. Summary of accuracy, total trials, latency to correct response and latency to collect reward during training and in the VITI and LITI tests of the 5-CSRTT. Values show mean±s.e.m. Training data show an average across the six stages of training. Statistic refers to Colony or Time of Day comparison, as appropriate. Geno, Genotype. ToD, Time of Day. n/a, not applicable

3.4. Discussion

The aim of the experiments reported in this chapter was to determine whether the behavioural abnormalities of NK1R-/- mice, reported previously (Yan et al., 2010; Dudley et al., 2013), are a direct result of a lack of functional NK1R or whether other contributory factors influence their behaviour. The results indicate that the *hyperactivity* and *perseveration* of NK1R-/- mice are both a direct consequence of dysfunctional NK1R. By contrast, the greater *impulsivity* of these mice appears to arise from an interaction between a lack of functional NK1R plus other (e.g., environmental) factor(s). Finally, the *inattentiveness* of NK1R-/- mice appears to be highly dependent on time-of-day.

3.4.1. The hyperactivity of NK1R-/- mice is a direct consequence of dysfunctional NK1R

The first finding from this study is that NK1R-/- mice, from both the *Hom* and *Het* colonies, displayed a higher level of activity than their respective wildtypes during the 'late' dark phase of the 24 h cycle. The fact that hyperactivity was evident in NK1R-/- mice from both colonies indicates that this behaviour is a direct consequence of dysfunctional NK1R.

It is not yet established how disruption to NK1R function results in locomotor hyperactivity. However, evidence suggests that motor activity is under the chronic inhibitory control of NK1R, as acute treatment with an NK1R antagonist induces hyperactivity in wildtypes (Yan et al., 2010). This also indicates that the hyperactivity of NK1R-/- mice is not due to any extraneous differences, such as developmental changes. It is highly likely that the hyperactivity of NK1R-/- mice involves abnormalities in corticostriatal circuitry and the basal ganglia. NK1R are densely expressed within the dorsal striatum, the main input nucleus of the basal ganglia, where they are primarily found on

cholinergic interneurons (Gerfen, 1991). These interneurons modulate the release of both acetylcholine and dopamine (Galarraga et al., 1999), and their functional integrity is necessary for the regulation of ongoing motor activity in response to salient environmental stimuli (e.g., Ding et al., 2010). A recent study from this laboratory found that the number of choline acetyltransferase (ChAT)-expressing neurons in the dorsal striatum is lower in NK1R-/- mice than wildtypes (Dudley, 2013). Therefore, one possible explanation for the hyperactivity of NK1R-/- mice is that striatal regulation of cholinergic and / or dopaminergic transmission is impaired in these mice. It would be interesting to carry out *in vivo* microdialysis studies of cholinergic efflux in the dorsal striatum of these mice. Another possibility is that the deficit in dopaminergic efflux in the PFC of NK1R-/- mice (Yan et al., 2010) results in disinhibition of glutamatergic afferents projecting from the PFC to the striatum (Le Moal and Simon, 1991; Ventura, et al., 2004).

Previous studies investigating the locomotor activity of NK1R-/- mice have been carried out over a 30 min period, during the light (inactive) phase, using a LDEB or activity chamber (Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010). In this study, the use of passive infrared sensors has, for the first time, allowed the measurement of locomotor activity across the entire 24 h cycle whilst animals are in their home-cage environment. It is interesting that the locomotor activity of NK1R-/- mice differs from wildtype mice at certain points of the 24 h cycle but not others. This could suggest that the diurnal regulation of motor activity is disrupted in these mice. Furthermore, it is striking that the hyperactivity of NK1R-/- mice is evident during the 'late' dark phase. There are clear equivalents to this in patients with ADHD, who commonly suffer from a delayed sleep onset/offset pattern ('delayed sleep phase syndrome') and are often typified as evening persons or 'night owls' (van Veen et al. 2010; Kooij and Bijlenga, 2013).

An animal's diurnal motor rhythm is regulated by a set of structures in the central nervous system that are influenced by environmental signals. The major synchroniser of an animal's diurnal rhythm is light (Cermakian and Sassone-Corsi, 2002). In mammals, light activates the retinohypothalamic tract (RHT), which projects to a region of the hypothalamus called the suprachiasmatic nucleus (SCN). This projection occurs both directly and via the thalamic intergeniculate leaflet (IGL; Morin et al., 2003). Substance P is found within both the core and/or in peripheral zones of the SCN (Hartwich et al. 1994; Piggins et al. 2001). Furthermore, both substance P and NK1R are expressed in the IGL (Morin et al. 1992; Piggins et al. 2001). In the hypothalamus, NK1R antagonism suppresses excitatory postsynaptic currents evoked by optic nerve stimulation of the SCN (Kim et al., 1999, 2001), indicating that substance P is an excitatory neurotransmitter in this brain region. There is also evidence that NK1R antagonism blocks the ability of light to induce phase advances in locomotor activity (Challet et al. 2001); interestingly, these phase advances occurred during the late subjective night, equivalent to the 'late' dark phase in which genotype differences in locomotor activity were found in this study.

Serotonergic receptors are also found in the SCN (Horikawa et al., 2000), and serotonergic transmission is also thought to regulate motor activity across the 24 h cycle (Jacobs and Azmitia, 1992; Edgar et al. 1997; Meyer-Bernstein et al. 1997). NK1R are expressed on some serotonergic neurones in the raphé nuclei (Lacoste et al. 2006), and so the abnormalities in serotonergic transmission in NK1R-/- mice (Froger et al. 2001) could also contribute to disruption of the 24 h cycle in motor activity.

The position of the activity sensors in this study was configured so as to measure the animals' gross ambulatory movement, as well as turns of the body and rearing. The sensors did not measure smaller movements, nor were they able to measure whether the animals were asleep

or awake. A logical next experiment would be to determine whether the normal sleep/wake cycle is disrupted in NK1R-/- mice, as in ADHD. This could be done, for example, by using a novel, non-invasive video-tracking technique used to measure sleep cycles in mice (Fisher et al., 2012). Finally, it would also be interesting to carry out pharmacological studies to test whether drugs that are used to treat ADHD reduce the hyperactivity of these mice during the 'late' dark phase of the 24 h cycle.

3.4.2. The impulsivity of NK1R-/- mice is influenced by both genetic and non-genetic factors

During training, a higher incidence of premature responses was evident in NK1R-/- mice, from both colonies, compared with wildtypes. This was particularly evident during the early stages of training. The fact that NK1R-/- mice from both colonies were more impulsive than wildtype mice during training suggests that, during early exposure to the task at least, a lack of functional NK1R alone causes impulsivity. However, this genotype difference dissipated at later stages of training, and there was no difference in impulsivity when animals were tested in the LITI. This suggests that a lack of functional NK1R does not prevent animals from ultimately matching the performance of wildtype mice.

When mice were tested in the VITI, a higher incidence of premature responses was only reinstated in NK1R-/- mice from the *Hom* colony. This indicates that a lack of functional NK1R also causes impulsivity when animals are tested in the VITI, but this influence rests on an interaction with other contributory factors. Consequently, it appears that both genetic and non-genetic factors contribute to the impulsivity of NK1R-/- mice in the 5-CSRTT, but in different ways at different stages of the training and testing procedure.

Impulsivity has long been associated with dysfunctional serotonergic transmission (e.g., Linnoila et al., 1983). Although the precise relationship remains unclear, manipulations of serotonergic transmission have consistently been shown to affect premature responding in the 5-CSRTT (e.g., Harrison et al., 1997; Dalley et al., 2002; Robinson et al., 2008a; Fletcher et al., 2013). NK1R are found within the raphé nuclei, located on both serotonergic neurons and on local glutamatergic neurons that regulate serotonergic function (Liu et al., 2002; Lacoste et al., 2006). There is extensive evidence that serotonin is regulated by NK1R, with all reports indicating that a deficit in functional NK1R augments serotonergic transmission (Guiard et al., 2005; Ebner et al., 2008; see: Stanford, 2014). In NK1R-/- mice (from the *Hom* colony), serotonergic efflux in the PFC is increased (Froger et al., 2001). It follows that one explanation for the greater impulsivity caused by a deficit in functional NK1R is due to disturbances in serotonergic function.

Dopamine has also been heavily implicated in impulsive behaviour (see: Oades, 2002; Winstanley et al., 2006). As with serotonin, manipulations of the dopamine system influence the incidence of premature responding in the 5-CSRTT (e.g., Bizarro and Stolerman, 2003; Bizarro et al., 2004; Loos et al., 2010; Fletcher et al., 2011; Lloyd et al., 2013). In NK1R-/- mice (from the *Hom* colony), dopamine efflux in the PFC is reduced by more than 50% (Yan et al., 2010), echoing the 'hypofrontality' seen in patients with ADHD (Zang et al., 2005). Therefore, it is also possible that abnormalities in dopaminergic transmission further contribute to the impulsivity of these animals.

However, the fact that in the VITI impulsivity was only evident in NK1R-/- mice from the *Hom* colony suggests that, at certain stages of the 5-CSRTT, impulsivity rests on an interaction between a lack of functional NK1R and other contributory factor(s). An obvious difference between the two colonies in this study is that NK1R-/- mice from the *Hom* colony were reared by NK1R-/- dams and housed with NK1R-/- littermates only, whereas NK1R-/- mice from the *Het*

colony were reared by NK1R+/- dams and housed with NK1R+/+, NK1R-/- and NK1R+/- littermates. There is extensive evidence that early life environment continues to influence the behaviour of an animal as an adult (see: Meaney, 2010). For instance, neonatal maternal separation has been shown to increase both depression- and anxiety-related behaviours (Plotsky and Meaney 1993; Meaney, 2001; Pryce and Feldon, 2003), as well as impair learning and memory (Huot et al. 2002; Aisa et al. 2007; Wilber et al., 2007). The quality of a pup's maternal care (e.g., in the form of licking and grooming) also affects its reactivity to stressful stimuli later in life (Pedersen et al., 2011).

Numerous environmental factors have been highlighted that increase a person's risk of developing ADHD (see: Elia et al. 2014). Marital conflict, childhood abuse, neglect, maternal stress and exposure to alcohol and nicotine have all been identified as potential risk factors (Nigg et al., 2010). No studies have so far investigated whether maternal care is altered in NK1R-/- dams. It would be interesting to carry out a cross-fostering study of wildtype and NK1R-/- mice to determine the extent to which the quality of maternal care and interaction with littermates impacts performance in the 5-CSRTT. This could help determine why impulsivity is seen in NK1R-/- mice from the *Hom* colony, but not in NK1R-/- mice from the *Het* colony, when tested in the VITI. The possibility that early-life experiences interact with polymorphism(s) of the *TACR1* gene to disrupt behaviours associated with ADHD merits investigation.

With the exception of one report in which repeated cocaine administration was found to decrease DNA methylation of the *TACR3* gene (Barros et al., 2013), there do not appear to be any previous studies investigation the influence of epigenetic mechanism on tachykinin signalling in relation to behaviour and cognition. However, results presented here suggest that epigenetic mechanisms may play a role in certain behavioural abnormalities of NK1R-/- mice, in particular

impulsive behaviour. The role of epigenetic mechanisms (such as DNA methylation) in NK1R-mediated behaviours warrants investigation.

3.4.3. The attentional performance of NK1R-/- mice in the 5-CSRTT

The incidence of omissions did not differ in the two colonies at any stage during either training or testing in the 5-CSRTT. This suggests that non-genetic (e.g., environmental) factors have no bearing on this behaviour. However, a limitation to these findings is that, in this study, a higher incidence of omissions was only evident in NK1R-/- mice tested in the morning session of the LITI. During training, and when tested in the VITI, omissions did not differ in the two genotypes.

There is considerable evidence that attention is associated with the noradrenergic system. In the 5-CSRTT, several studies have shown that attentional performance is disrupted following noradrenergic lesions to the forebrain (Carli et al., 1983; Cole and Robbins, 1992; Milstein et al., 2007). Reports have also found an increase in omissions following treatment with alpha1-, alpha2-, beta1-, and non-selective beta-adrenoceptor agonists (Sirvio et al., 1994; Pattij et al., 2012). The noradrenaline reuptake inhibitors desipramine and atomoxetine have also both been shown to increase the incidence of omissions in this test (Paterson et al., 2011; Pattij et al., 2012). In two recent studies from this laboratory, the selective alpha-2A receptor agonist, guanfacine, increased the incidence of omissions carried out by NK1R-/- mice, although treatment with atomoxetine had no effect on this behaviour (Pillidge et al., 2014a; Pillidge et al., 2014b).

It has been proposed that deficits in attention can be explained by abnormalities in the activity of noradrenergic neurons that project from the locus coeruleus to the prefrontal cortex (‘adaptive gain theory’; Aston-Jones and Cohen, 2005). There is evidence that these noradrenergic

neurons are regulated of NK1R (Ebner and Singewald, 2007). Furthermore, basal noradrenergic efflux is increased two- to four-fold in the prefrontal cortex of anaesthetised NK1R-/- mice (Herpfer et al., 2005; Fisher et al., 2007). Consequently, it would be unsurprising if a deficit in functional NK1R disrupts attentional performance.

However, in this study, the incidence of omissions was only higher in NK1R-/- mice when tested in the morning session of the LITI. This finding differs from a previous study, in which omissions were higher in NK1R-/- mice during training and when tested in both the VITI and LITI tests (morning and afternoon; Yan et al., 2011). However, it is striking that, in this previous study, omissions were similarly dependent on time-of-day: a higher incidence of omissions was evident (in both genotypes) in the morning compared with the afternoon (Yan et al., 2011). These results suggest that the regulation of attentional performance by NK1R is under a circadian rhythm. Indeed, there is extensive evidence for a circadian rhythm in cognitive performance (e.g., Beau, 1992; Weinert and Waterhouse, 1998; Winocur and Hasher, 2004). Furthermore, circadian rhythms are disrupted in patients with ADHD (Chiang et al., 2010; see above). However, in this study, it is not possible to distinguish whether these differences in attention are due to a disruption of circadian influences or an extraneous procedural factor (e.g., feeding schedule). Nonetheless, it is clear that any differences in attentional performance in the morning and afternoon sessions are not due to differences in motivation to carry out the task, as *total trials*, *latency to correct response*, and *latency to collect reward* were all unaffected by time of day.

It remains to be explained why, in the present study, NK1R-/- mice were only inattentive in the morning session in the LITI, and not in the VITI. However, it is now possible to infer that contributory factors such as early life environment have no bearing on the attentional

performance of NK1R-/- mice. It seems that, when genotype differences do occur in this behaviour (as in: Yan et al., 2011), this is a direct consequence of dysfunctional NK1R.

3.4.4. The increased incidence of perseveration seen in NK1R-/- mice is a direct consequence of dysfunctional NK1R

Finally, the incidence of perseveration did not differ in the two colonies at any point during either training or when tested in the VITI or LITI. NK1R-/- mice, from both colonies, displayed a higher incidence of perseverative responding than wildtypes during both training and when tested in the VITI. These findings are consistent with previous reports (Dudley et al. 2013; Yan et al. 2011) and suggest that this behaviour can be attributed directly to a lack of functional NK1R. An influence of early life environment on this behaviour can be ruled out.

Perseveration is the uncontrolled repetition of a specific response, be it a word, phrase or action, despite the cessation of the original stimulus that caused that response. While not a diagnostic criterion, perseveration can be raised in children with ADHD (Houghton et al., 1999). It is possible that perseveration represents a form of 'checking', which is interesting in light of evidence that comorbid obsessive-compulsive symptoms are evident in some ADHD patients (Gillberg et al., 2004). Studies investigating perseverative responding have heavily implicated corticostriatal circuitry (e.g., Schoenbaum et al., 2002; Chudasama and Robbins, 2003; Chudasama et al., 2003; Christakou et al., 2004; Schwabe et al., 2004; Castane et al., 2010). Given the high density of NK1R within the dorsal striatum (Gerfen, 1991), disruption to NK1R would be expected to result in abnormalities in corticostriatal function and consequently likely contributes to this behaviour.

However, in this study, a higher incidence of perseveration was only evident in the VITI test; this behaviour did not differ in the two genotypes in the LITI test. In fact, whilst perseveration is typically increased in NK1R-/- mice in the VITI, this behaviour appears to be more variable in the LITI (c.f., Yan et al., 2011; Dudley et al., 2013). This could suggest that the VITI and LITI recruit different neuronal networks, which affect perseveration in different ways. However, as with both hyperactivity and inattentiveness, when genotype differences in this behaviour do occur, these can also be attributed directly to a lack of functional NK1R.

3.4.5. Concluding remarks

Results from this chapter reveal that the hyperactivity and perseveration of NK1R-/- mice are both a direct consequence of dysfunctional NK1R. The results further suggest that the diurnal regulation of motor activity is disrupted in these mice. NK1R-/- mice did not show inattentiveness in either training or the VITI test in this study, and so interpretation of this behaviour is more difficult. However, the lack of colony differences at any stage of training or testing suggests that, when genotype differences do occur, this behaviour is also a direct consequence of dysfunctional NK1R.

By contrast, the greater impulsivity of NK1R-/- mice appears to be influenced by distinct genetic and non-genetic factors, depending on the stage of the 5-CSRTT procedure and the test conditions. It is possible that differences in early life environment are influencing the behaviour of these mice as adults, and a potential interaction between environmental influences and polymorphisms of the *TACR1* gene in the risk of developing ADHD merits further investigation.

Finally, results from this study demonstrate how the influence of genetic and non-genetic factors on the phenotype of genetically-altered animals can only be assigned following

simultaneous (head-to-head) comparisons of the progeny of homozygous and heterozygous breeding pairs. This is particularly important for research into the aetiology of disorders such as ADHD, for which there is strong evidence for both genetic and environmental influences.

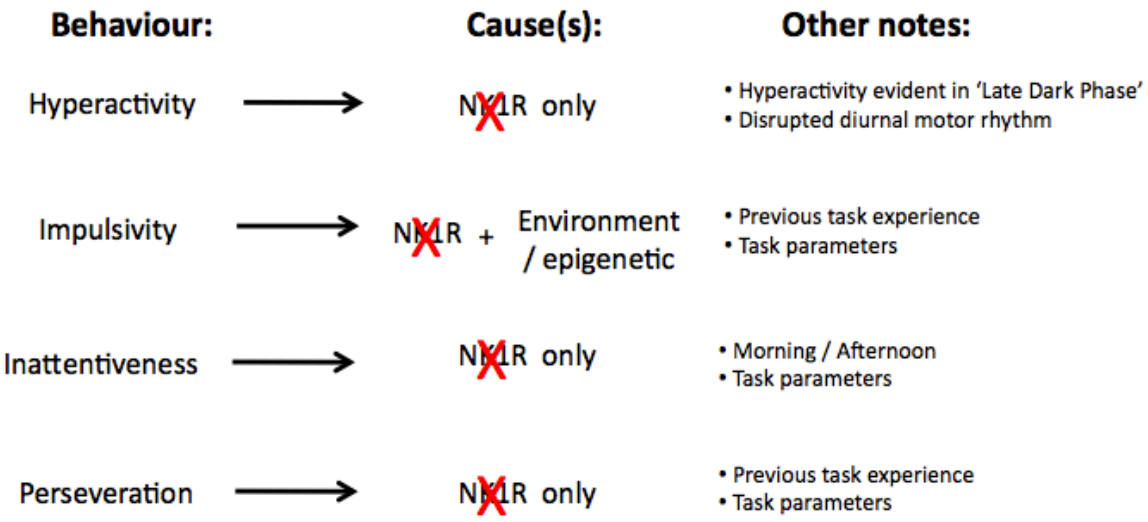

Figure 3.10. Summary of findings

Chapter 4

Hyperactivity in NK1R-/- mice: sex differences and the influence of the brain renin angiotensin system

4.1. Introduction

4.1.1. Sex differences in patients with ADHD

It is common in behavioural neuroscience for experiments to be carried out exclusively on males (Beery and Zucker, 2011). This is partly due to concerns that hormonal fluctuation over the course of the reproductive cycle causes a greater degree of variability in results generated from females (Becker et al., 2005; Meziane et al., 2007; Walf and Frye, 2007). However, males and females differ in many behavioural paradigms. For instance, some studies have shown that locomotor activity is greater in female animals compared with males when tested in the open field (for review, see: Archer, 1975). There are also many reports of male and female animals responding differently to drugs that stimulate locomotor activity (e.g., Robinson, 1984; Camp and Robinson, 1988; Sircar and Kim, 1999; Koenig et al., 2005). For example, in the Spontaneously Hypertensive Rat (SHR), methylphenidate increases locomotor activity to a greater extent in female animals compared with males (Chelaru et al., 2012). This has important implications for any interpretation of the behavioural studies carried out on NK1R-/- mice.

The diagnosis rate of ADHD is higher in males than it is in females, with the ratio being as high as 9:1 in some estimates (Anderson et al., 1987; Bird et al., 1988). However, it has been suggested that ADHD is under-diagnosed in females (Rucklidge, 2010). The typical symptom profile of patients with ADHD also differs in male and female patients: the Predominantly Hyperactive/Impulsive or Combined subtype is more common in males, whereas the Predominantly Inattentive subtype is more common in females (Newcorn et al., 2001; Staller and Faraone, 2006; Polanczyk and Rohde, 2007). All previous studies that have identified behavioural and neurochemical abnormalities in NK1R-/- mice have been carried out in male animals. However, considering the evidence outlined above, it is important to determine whether the behavioural

deficits observed in male NK1R-/- mice are also evident in females. To investigate this possibility, the first part of this chapter compares the locomotor activity of male and female animals using the LDEB.

4.1.2. The brain renin angiotensin system and motor control

The second part of this chapter describes an investigation into the behavioural effects of drugs that target the brain renin angiotensin system (BRAS). As discussed in Section 1.3, the BRAS is thought to play a role in a number of behaviours, including motor control (for review, see: Wright and Harding, 2011). For instance, a number of studies have found that intracerebroventricular administration of angiotensin II (AngII) increases locomotor activity and exploratory behaviour (Braszko et al., 1987, 1988; Georgiev et al., 1985; Georgiev and Kambourova, 1991). As this effect is abolished by co-administration of angiotensin receptor (AT) antagonists, it is likely that this effect of AngII on locomotor activity is due to direct AT receptor activation (Braszko, 2002). It is also striking that both AT receptors and angiotensin converting enzyme (ACE) are highly expressed in corticostriatal circuitry, a neuronal network thought to be central to the regulation of motor function (Chai et al., 1987a; Allen et al., 1991, 1992; Graybiel, 2000).

In this laboratory, interest in the BRAS was prompted by the observation that, *in vitro*, substance P is hydrolysed by ACE (Skidgel et al., 1984; Strittmatter et al., 1985; Thiele et al., 1985). However, it is still uncertain whether ACE metabolises substance P *in vivo* (Mitchell et al., 2013) and this enzyme is also not the only peptidase that metabolises this neuropeptide (Oblin et al., 1988). Nonetheless, given evidence outlined above that the BRAS is involved in the regulation of motor function, a single-dose (10 mg/kg) pilot study was carried out that investigated the effects of the ACE inhibitor, captopril, on the locomotor activity of NK1R-/- mice in the LDEB (Yee et al.,

2008; preliminary communication). Unlike many other ACE inhibitors, captopril penetrates the blood brain barrier in its active form (Geppetti et al., 1987; Ranadive et al., 1992). It was predicted that, if ACE degrades substance P *in vivo*, captopril would reduce the locomotor activity of wildtype mice but leave NK1R-/- mice unaffected. However, contrary to this prediction, captopril reduced the locomotor activity of NK1R-/- mice without affecting the behaviour of wildtype mice (Yee et al., 2008). Nonetheless, as this effect was genotype-specific, this indicates that the BRAS interacts with NK1R, either directly or indirectly, to modulate motor function. In this chapter, this (pilot) finding was extended by carrying out a fully randomised multi-dose study of the effects of captopril on the locomotor activity of NK1R-/- mice in the LDEB.

A potentially important caveat to any study investigating the effects of ACE inhibition on behaviour is that there is considerable evidence for sex differences in ACE activity. In general, ACE activity is higher in men than it is in women (Zapater et al., 2004), and this is thought to be because ACE activity is increased by testosterone but decreased by oestrogen (see: Komukai et al., 2010). As a consequence, any behavioural effect of ACE inhibition may differ in male and female animals. In light of this evidence, it was decided to investigate the effects of captopril in both male and female animals, as part of the above study comparing the locomotor activity of male and female NK1R-/- mice.

Finally, the finding that captopril (in the pilot study) reduced the locomotor activity of NK1R-/- mice indicates that the behavioural response to this drug is not due to changes in substance P. This is because changes to the availability of this peptide would be ineffective in this genotype. However, ACE is also capable of metabolising a number of other peptides, the most well known of which is the conversion of the (presumed) inactive precursor, angiotensin I, to the neurologically active angiotensin II (AngII). Therefore, an obvious possibility is that the response to

captopril is due to a reduction in AngII production. If so, the behavioural response to captopril should be mimicked by treatment with either the angiotensin type 1 (AT_1) receptor antagonist, losartan, and/or the angiotensin type 2 (AT_2) receptor antagonist, PD 123319. To test for this possibility, the final part of this chapter investigates the effects of these drugs on the locomotor activity of wildtype and NK1R-/- mice.

4.1.3. Objectives

The experiments described in this chapter had three main objectives:

1) Determine whether the hyperactivity of male NK1R-/- mice is also evident in females
2) Test the prediction (arising from a pilot study) that the ACE inhibitor, captopril, will prevent the hyperactivity of NK1R-/- mice
3) Test the prediction that the AT_1 receptor antagonist, losartan, and AT_2 receptor antagonist, PD 123319, will also prevent the hyperactivity of NK1R-/- mice

The following experiments have been accepted for publication in *European Neuropsychopharmacology* (Porter et al., 2015b).

4.2. Methods

4.2.1. Animals

All animals were used from the colony described in Section 2.2.1.

4.2.2. Drugs

Drugs were dissolved in 0.9% saline and injected (i.p.) in a volume of 10 mL/kg. Drug doses were chosen based on previous reports confirming their effects in mice or rats (examples provided in Table 4.1). Captopril was purchased from Sigma Aldrich, UK, losartan potassium was purchased from LKT laboratories, UK, and PD 123319 ditrifluoroacetate was purchased from Tocris, UK.

Drug:	**Doses (i.p.):**	**Injection time:** (mins before transfer to LZ)	**Example references:**	**Procedure:**
Captopril	10 and 25 mg/kg	30 min	Raghavendra et al., 2001	inhibitory shock avoidance paradigm
			van den Buuse., 2005	prepulse inhibition
Losartan	10 and 25 mg/kg	60 min	Vijayapandi & Nagappi, 2005	forced swim test
			Marvar et al., 2014	foot-shock fear conditioning paradigm
PD 123319	1 and 3 mg/kg	60 min	Macova et al., 2009	AT receptor binding
			Sanchez et al., 2009	AT receptor binding

Table 4.1. Doses and timings used for each LDEB study

4.2.3. Protocol

The LDEB apparatus and protocol are described fully in Section 2.5.

In all experiments, two LDEBs were placed next to each other and the behaviour of two animals recorded simultaneously: in the captopril study, one male and one female of the same genotype were tested simultaneously; in the losartan and PD 123319 studies, one male wildtype and one male NK1R-/- mouse were tested simultaneously. Animals were brought to the laboratory at 09.30 h in order to allow a minimum of 3 h habituation to the testing room. At 13.00 h, animals were placed into the dark zone of the LDEB apparatus for 90 min habituation. During this time, animals received a randomly (Latin-square) assigned treatment of: no injection ('NI'), vehicle injection (0.9% saline, i.p.), or drug injection (dose 1/dose 2, i.p.). The time of injection depended on the drug being tested (Table 4.1). Following habituation to the dark zone, animals were transferred to the light zone of the LDEB and allowed 30 min free movement across both zones. The behaviour of each animal was recorded using a video camera placed above the apparatus, and subsequently scored 'blind'.

4.2.3.1. Effects of ACE inhibition on locomotor activity

Six male and six female mice were used per group (male wildtypes, female wildtypes, male NK1R-/- mice, and female NK1R-/- mice; age 6 – 11 weeks, weight 20 – 37 g). For each group, animals were selected from nine breeding pairs and chosen to age-match the experimental groups as closely as possible.

Four treatment groups were used in this study: no injection ('NI'), vehicle injection, or captopril (10 or 25 mg/kg) injection. Drug injections were administered 30 min before being placed in the light zone of the LDEB (see: Table 4.1 / Fig. 4.1).

4.2.3.2. Effects of AT_1 and AT_2 receptor antagonism on locomotor activity

Following the observation that hyperactivity was only evident in male NK1R-/- mice, and captopril was ineffective in female mice (see below), it was decided that only male animals would be used in the AT receptor antagonist studies.

In the losartan study, six male mice were used per group (age 6 - 10 weeks, weight 25 – 41 g), with three treatment groups: vehicle injection and losartan (10 and 25 mg/kg) injection. In the PD 123319 study, five male mice were used per group (age 7 – 12 weeks, weight 29 – 40 g), again with three treatment groups: vehicle injection and PD 123319 (1 and 3 mg/kg) injection. Drug injections were administered 60 min before being placed in the light zone of the LDEB (see: Table 4.1 / Fig. 4.1). For both studies, animals from each genotype were selected from a total of eight - nine breeding pairs and chosen to age match the experimental groups as closely as possible.

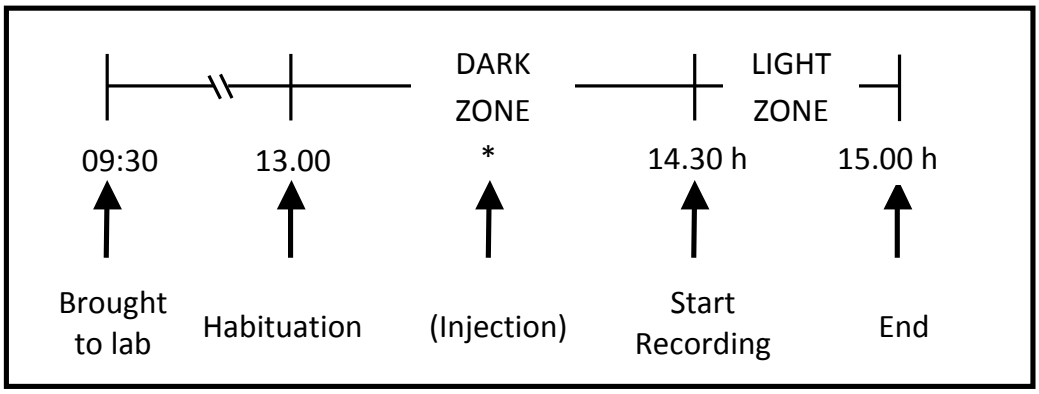

Figure 4.1. Schematic representing the protocol used for each LDEB experiment.
* The timing of injection depended drug treatment.

4.2.3.3. Behavioural scoring

The following behaviours were scored, 'blind', following each experiment (see also Section 2.5):

- *Speed in Light Zone (LZ, lines crossed/time):* a measure of the animals' locomotor activity
- *Time spent in LZ (s):* total time spent in the light zone
- *Latency to leave the LZ (s):* time it takes to leave the LZ following forced entry
- *Latency to first return to LZ (s):* time to return to the LZ after leaving for the first time
- *Number of returns:* number of times the animal enters the LZ

4.2.4. Statistical analysis

Further details of statistical analysis are provided in Section 2.8.

In the captopril study, raw or transformed data were analysed using a multifactorial 3-way single-measures ANOVA with 'Genotype', 'Treatment' and 'Sex' as between-subjects factors, with *post-hoc* (LSD test) pairwise comparisons. In the losartan and PD 123319 studies, data were analysed using a multifactorial 2-way single-measures ANOVA with 'Genotype' and 'Treatment' as between-subjects factors, with *post-hoc* (LSD test) pairwise comparisons.

4.3. Results

4.3.1. Hyperactivity is evident in male NK1R-/- mice, only

In uninjected animals, the effect of Genotype on *locomotor activity* differed in male and female mice ($F(1,20) = 7.41$, $P=0.013$). As in previous reports, the *locomotor activity* of male NK1R-/- mice was higher than male wildtypes (LSD: $P<0.001$; Fig. 4.2a). However, the *locomotor activity* of female NK1R-/- mice did not differ from female wildtypes (LSD: $P=0.074$; Fig. 4.2a). Furthermore, the *locomotor activity* of female NK1R-/- mice was lower than that of their male counterparts (LSD: $P=0.033$; Fig. 4.2a).

Both male and female NK1R-/- mice spent less *time in the light zone* ($F(1,22) = 27.77$, $P<0.001$; Fig. 4.2b) and took longer to *first return to the light zone* ($F(1,22) = 11.22$, $P=0.003$; Fig. 4.2d) compared with wildtypes. However, *latency to first leave the light zone* did not differ in either the two genotypes or in male and female animals (Fig. 4.2c). An overall sex difference in *number of returns to the light zone* just missed the criterion for significance ($F(1,22) = 3.89$; $P=0.061$), with *post hoc* analyses revealing that female wildtypes showed a greater number of returns than both male wildtypes (LSD: $P=0.042$) and female NK1R-/- mice (LSD: $P=0.047$) (Fig. 4.2e).

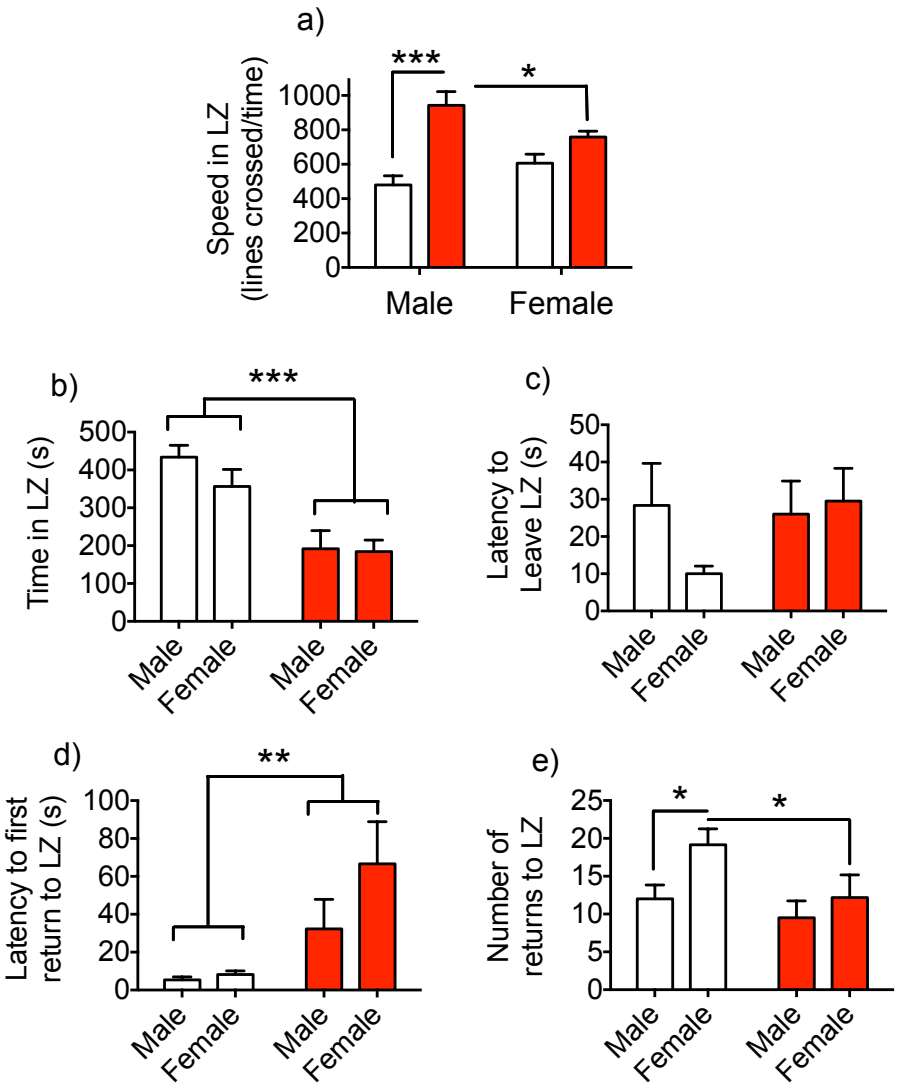

Figure 4.2. Sex differences in locomotor activity and anxiety-like behaviours in the LDEB
Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Genotype / Sex comparison). N = 6 per group.

4.3.2. The hyperactivity of male NK1R-/- mice is prevented by treatment with captopril

As in uninjected mice, the effect of Genotype on *locomotor activity* differed in male and female animals after a vehicle injection ($F(1,60) = 10.75$, $P=0.002$). In males, the *locomotor activity* of vehicle-treated NK1R-/- mice was greater than that of vehicle-treated wildtype mice (LSD: $P<0.001$; Fig. 4.3a). The *locomotor activity* of vehicle-treated male NK1R-/- mice was also greater than their female counterparts (LSD: $P<0.001$; Fig. 4.3a,b). There was no difference in vehicle-injected NK1R-/- mice and uninjected NK1R-/- mice (Fig. 4.2a / 4.3a,b).

An interaction between Sex and Treatment just missed the criterion for significance ($F(2,60) = 2.73$, $P=0.073$). In male animals, both doses of captopril reduced the *locomotor activity* of NK1R-/- mice, abolishing their hyperactivity (LSD (*c.f.*, vehicle): $P=0.05$ (10 mg/kg) and $P=0.006$ (25 mg/kg); Fig. 4.3a). However, captopril did not affect the locomotor activity of male wildtype mice (Fig. 4.3a). Similarly, neither dose of captopril affected the *locomotor activity* of female mice, of either genotype (Fig. 4.3b).

None of the 'anxiety-like' behaviours were affected by treatment with captopril, at either dose. Values for these behaviours are provided in Table 4.2.

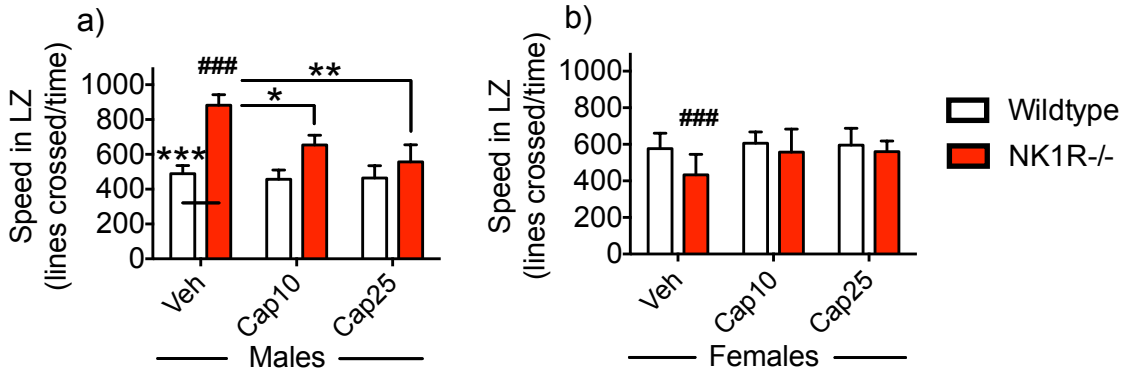

Figure 4.3. Effects of captopril on the locomotor activity of (a) male and (b) female mice in the LDEB. Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. *$P<0.05$, ** $P<0.01$, ***$P<0.001$. (Genotype/Treatment comparison). ### P<0.001. (Sex comparison). N = 6 per group.

4.3.3. The locomotor activity of male NK1R-/- mice is increased by treatment with losartan

Locomotor activity was higher, overall, in NK1R-/- mice ($F(1,30)=66.72$; $P<0.001$). An overall effect of losartan on locomotor activity just missed the criterion for significance ($F(2,30)=2.88$; $P=0.072$). Yet, *post-hoc* analyses revealed that, in contrast to captopril, locomotor activity of male NK1R-/- mice was increased by losartan, at both doses (LSD (*c.f.*, vehicle): $P=0.049$ (10 mg/kg) and $P=0.015$ (25 mg/kg); Fig 4.4a). However, neither dose affected the locomotor activity of wildtype mice (Fig. 4.4a).

None of the 'anxiety-like' behaviours was affected by losartan, at either dose. Values for these behaviours are provided in Table 4.3.

4.3.4. The locomotor activity of male NK1R-/- mice is increased by treatment with PD 123319

Locomotor activity was higher, overall, in NK1R-/- mice ($F(1,24)=29.51$; $P<0.001$). There was also an overall main effect of PD 123319 on activity ($F(2,24)=3.99$; $P=0.032$): both doses of PD 123319 increased the activity of NK1R-/- mice (LSD (*c.f.* vehicle): $P=0.01$ (1 mg/kg) and $P=0.013$ (3 mgkg); Fig. 4.4b) but, as with both captopril and losartan, neither dose affected the locomotor activity of wildtype mice (Fig. 4.4b).

None of the 'anxiety-like' behaviours was affected by treatment with PD 123319, at either dose. Values for these behaviours are provided in Table 4.3.

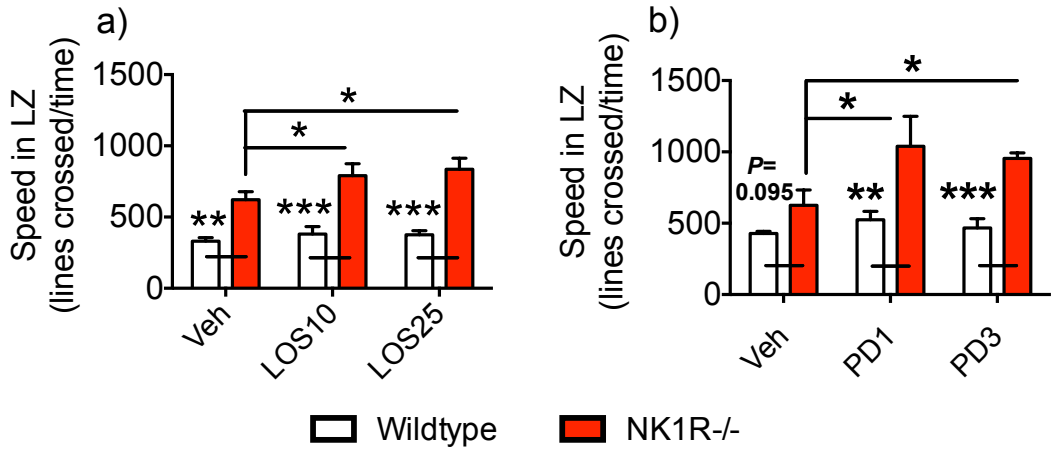

Figure 4.4. Effects of (a) the AT_1 receptor antagonist, losartan, and (b) the AT_2 receptor antagonist, PD 123319, on the locomotor activity of male NK1R-/- and wildtype mice. Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * *P*<0.05, ** *P*<0.01, ****P*<0.001 (Genotype/Treatment comparison). N = 5 – 6 per group

	(a) Effects of captopril - males				(b) Effects of captopril - females			
Behaviour	**Genotype**	**Drug**	**Value ±s.e.m.**	**Effect of drug Treatment**	**Genotype**	**Drug**	**Value ±s.e.m.**	**Effect of drug Treatment**
Time in LZ	Wildtype	Veh	291.3±53.0	F(2,15)=1.13; *P*=0.349	Wildtype	Veh	373.3±59.3	F(2,15)=1.15; *P*=0.3423
		CAP10	316.5±66.2			CAP10	442.0±32.2	
		CAP25	395.5±25.6			CAP25	312.5±79.9	
	NK1R-/-	Veh	152.3±65.5	F(2,15)=0.16; *P*=0.8571	NK1R-/-	Veh	265.3±75.7	F(2,15)=0.15; *P*=0.8634
		CAP10	198.0±66.0			CAP10	263.7±76.7	
		CAP25	158.7±56.0			CAP25	216.2±64.4	
Latency to leave LZ (s)	Wildtype	Veh	16.2±4.6	F(2,15)=1.34; *P*=0.2912	Wildtype	Veh	61.3±39.9	F(2,15)=0.35; *P*=0.7104
		CAP10	17.2±3.7			CAP10	20.3±9.1	
		CAP25	42.5±21.4			CAP25	27.7±13.8	
	NK1R-/-	Veh	36.0±8.0	F(2,15)=0.34; *P*=0.7193	NK1R-/-	Veh	115.8±49.7	F(2,15)=0.79; *P*=0.4698
		CAP10	35.8±12.9			CAP10	97.2±46.4	
		CAP25	31.2±17.4			CAP25	39.8±10.9	
Latency to first return to LZ (s)	Wildtype	Veh	18.2±7.2	F(2,25)=0.31; *P*=0.7354	Wildtype	Veh	7.5±1.6	F(2,15)=0.1; *P*=0.9083
		CAP10	20.8±7.9			CAP10	7.3±1.7	
		CAP25	10.7±2.6			CAP25	18.6±6.8	
	NK1R-/-	Veh	57.8±13.9	F(2,15)=0.54; *P*=0.5931	NK1R-/-	Veh	20.2±9.3	F(2,15)=0.75; *P*=0.4908
		CAP10	75.3±24.9			CAP10	79.7±62.1	
		CAP25	54.7±10.0			CAP25	264.2±237.0	
Number of returns to LZ	Wildtype	Veh	13.8±0.9	F(2,14)=0.34; *P*=0.7194	Wildtype	Veh	13.7±2.1	F(2,15)=2.32; *P*=0.132
		CAP10	11.8±3.0			CAP10	18.3±3.3	
		CAP25	11.2±2.5			CAP25	10.2±2.6	
	NK1R-/-	Veh	5.5±1.9	F(2,15)=0.89; *P*=0.4327	NK1R-/-	Veh	4.5±1.2	F(2,15)=0.98; *P*=0.3991
		CAP10	9.8±3.1			CAP10	7.8±2.3	
		CAP25	7.2±1.7			CAP25	7.3±1.8	

Table 4.2. Treatment with the ACE inhibitor, captopril, did not affect any of the 'anxiety-like' behaviours of either (a) male or (b) female wildtype or NK1R-/- mice in the LDEB. LZ, light zone.

	(a) Effects of losartan - males				(b) Effects of PD 123319 - males			
Behaviour	**Genotype**	**Drug**	**Mean ±s.e.m.**	**Effect of drug Treatment**	**Genotype**	**Drug**	**Mean ±s.e.m.**	**Effect of drug Treatment**
Time in LZ	Wildtype	Veh	465.2±14.5	F(2,15)=0.91; *P*=0.4233	Wildtype	Veh	416.0±34.8	F(2,12)=0.4; *P*=0.677
		LOS10	420.5±38.8			PD1	453.4±30.1	
		LOS25	445.3±18.2			PD3	432.8±28.8	
	NK1R-/-	Veh	264.8±67.0	F(2,15)=0.83; *P*=0.454	NK1R-/-	Veh	189.4±55.0	F(2,12)=0.61; *P*=0.5583
		LOS10	177.2±78.8			PD1	258.6±93.1	
		LOS25	272.8±43.1			PD3	156.8±51.0	
Latency to leave LZ (s)	Wildtype	Veh	62.7±32.8	F(2,15)=0.84; *P*=0.4503	Wildtype	Veh	28.2±12.2	F(2,12)=0.31; *P*=0.738
		LOS10	24.8±4.6			PD1	24.8±10.0	
		LOS25	21.0±10.7			PD3	17.0±10.7	
	NK1R-/-	Veh	38.3±15.2	F(2,15)=0.02; *P*=0.9849	NK1R-/-	Veh	23.6±11.7	F(2,12)=0.18; *P*=0.834
		LOS10	38.2±14.8			PD1	26.2±6.0	
		LOS25	35.3±11.6			PD3	18.8±9.6	
Latency to first return to LZ (s)	Wildtype	Veh	14.7±2.7	F(2,15)=0.11; *P*=0.8929	Wildtype	Veh	12.2±3.9	F(2,12)=0.31; *P*=0.7383
		LOS10	13.7±2.9			PD1	9.0±4.0	
		LOS25	19.2±6.5			PD3	9.4±3.2	
	NK1R-/-	Veh	40.7±25.7	F(2,15)=0.08; *P*=0.923	NK1R-/-	Veh	159.8±80.5	F(2,10)=1.59; *P*=0.2523
		LOS10	26.8±9.6			PD1	55.0±31.5	
		LOS25	21.0±10.6			PD3	40.0±21.1	
Number of returns to LZ	Wildtype	Veh	16.8±2.4	F(2,15)=0.11; *P*=0.8958	Wildtype	Veh	27.8±4.1	F(2,12)=0.11; *P*=0.8991
		LOS10	17.3±3.1			PD1	24.2±5.3	
		LOS25	19.3±3.9			PD3	24.2±11.2	
	NK1R-/-	Veh	16.7±4.5	F(2,15)=0.95; *P*=0.4084	NK1R-/-	Veh	11.4±3.5	F(2,12)=0.25; *P*=0.7806
		LOS10	16.5±11.2			PD1	11.4±5.2	
		LOS25	18.7±4.1			PD3	15.4±6.3	

Table 4.3. Treatment with (a) the AT_1 receptor antagonist, losartan, or (b) the AT_2 receptor antagonist, PD 123319, did not affect any of the 'anxiety-like' behaviours of male wildtype or NK1R-/- mice in the LDEB. LZ, light zone.

4.4. Discussion

4.4.1. The hyperactivity of NK1R-/- mice is only evident in male animals

The first aim of this chapter was to establish whether the hyperactivity of male NK1R-/- mice is also evident in female mice. As found in previous studies, male NK1R-/- mice were hyperactive compared with their wildtype counterparts. However, the locomotor activity of female NK1R-/- mice did not differ from wildtypes. Furthermore, the male NK1R-/- mice were more active than their female counterparts. Combined, these results suggest that the hyperactivity caused by a deficit in functional NK1R is sex-specific.

These results reveal striking parallels between sex differences in the behavioural abnormalities of NK1R-/- mice and the typical symptom profile of male and female patients with ADHD: hyperactivity is a more common symptom in male ADHD patients compared with female ADHD patients (Staller and Faraone, 2006). In general, hypermobility and other externalised disruptive behaviours are more likely to occur in boys than in girls (Abikoff et al., 2002; Gaub and Carlson, 1997; Rucklidge, 2010). Males are also more susceptible to disorders of other excitability and movement, such as Tourette's syndrome, epilepsy or Parkinson's disease (Haaxma et al., 2007; Shulman, 2007; McHugh and Delanty, 2008). Results presented here suggest that impaired *TACR1* function could contribute to these abnormalities.

As discussed in Chapter 3, there is extensive evidence that motor function is governed by corticostriatal circuitry, and these pathways have been strongly implicated in the aetiology of ADHD. These networks are regulated by gonadal hormones during both perinatal development and during puberty (Waddel and McCarthy, 2012), and exposure to these sex hormones during these key stages of development is thought to contribute, at least in part, to the sexual differentiation observed in male and female brains (Stewart and Rajabi, 1994; Kritzer, 1997; Kritzer

and Kohama, 1998; Kritzer et al., 2003; Kritzer and Cruetz, 2008). For example, differences in the density and distribution of dopaminergic D_1 and D_2 receptors in male and female brains have been observed in the dorsal striatum, nucleus accumbens, and prefrontal cortex of juvenile rats, with males experiencing larger increases in receptor expression during puberty than females (Anderson and Teicher, 2000). It has been proposed that this over-expression of dopamine receptors observed in male brains, when combined with other neurochemical (e.g., monoaminergic) abnormalities, contributes to the greater incidence of hyperactivity observed in male patients with ADHD compared with females (Waddell and McCarthy, 2012). This could similarly explain the findings in NK1R-/- mice reported here, which suggest that a functional deficit in NK1R (and, by inference, polymorphisms of the *TACR1* gene) could contribute to sex differences in the incidence of hyperactivity in ADHD patients.

Despite well-established sex differences in a variety of CNS disorders, the underrepresentation of females remains a major issue in biomedical research (Hughes, 2007; Beery and Zucker, 2011). As discussed, this is partly due to the prevailing assumption that the 4-day oestrous cycle results in more variable data in females than in males (Wald and Wu, 2010). However, a recent review of 293 articles found that variability was not significantly greater in females than males across a range of behavioural, morphological, physiological and molecular traits (Prendergast et al., 2014). It was concluded that the use of female mice in neuroscience does not require monitoring of the reproductive cycle in order to generate reliable results, and that studies should incorporate males and females in equal numbers with direct comparisons of the two sexes.

An obvious future experiment on NK1R-/- mice would be to see how the performance of female mice compares with that of males in the 5-CSRTT. Sex differences have previously been

observed in the 5-CSRTT using Long-Evans rats, with a higher incidence of omissions seen in females and a higher incidence of premature responses seen in males (Bayless et al., 2012). As females with ADHD more commonly present as the Predominantly Inattentive Subtype, it would be particularly interesting to see whether female NK1R-/- mice also show a higher incidence of omissions than their male counterparts. Furthermore, in light of evidence for sexual differentiation in male and female brains in neuronal networks implicated in ADHD (outlined above), it would be interesting to carry out *in vivo* microdialysis studies to investigate whether the abnormalities in neurochemistry in male NK1R-/- mice are replicated in females.

4.4.2. The effects of ACE inhibition and AT receptor antagonism on the locomotor activity of NK1R-/- mice

The second aim of the experiments reported in this chapter was to determine the effects of the ACE inhibitor, captopril, on the locomotor activity of NK1R-/- mice. In line with a previous pilot study (Yee et al., 2008), captopril reduced the locomotor activity of male NK1R-/- mice, abolishing the hyperactivity of this genotype. Captopril did not affect any of the 'anxiety-like' behaviours in the LDEB, so the effect of ACE inhibition was specific to locomotor activity. Although it cannot be ruled out that the behavioural effects of captopril in this study are due to changes in blood pressure, the genotype-specific effect points to an interaction between the BRAS and NK1R in the regulation of motor control. Furthermore, the results from this study suggest that hyperactivity, caused by a deficit in functional NK1R, can be ameliorated by treatment with an ACE inhibitor.

This study was prompted by the observation that ACE hydrolyses substance P (Skidgel et al., 1984; Strittmatter et al., 1985; Thiele et al., 1985). Yet, the effects of captopril seen here

cannot be explained by changes in the availability of substance P, as this would be ineffective in NK1R-/- mice. However, ACE is perhaps better known for the conversion of AngI to AngII, and, although findings are somewhat inconsistent, there are a number of reports that AngII stimulates locomotor activity and behavioural arousal (Braszko et al., 1987, 1988; Braszko, 2002; Georgiev et al., 1985). It follows that one alternative explanation is that the behavioural effects of captopril in NK1R-/- mice are due to a deficit in AngII production. If this is the case, then the response to captopril should be mimicked by antagonism at either AT_1 receptors and/or AT_2 receptors.

To test this possibility, the next study went on to investigate the effects of the AT_1 receptor antagonist, losartan, and the AT_2 receptor antagonist, PD 123319, on locomotor activity in these mice. Both of these compounds have previously been shown to influence locomotor activity in rodents, although, as with AngII, the results are somewhat inconsistent (Irvine et al., 1995; Raghavendra et al., 1998a; Braszko, 2002). However, in this study, both losartan and PD 123319 increased (rather than reduced) the locomotor activity of NK1R-/- mice. Again, neither compound affected the locomotor activity of wildtypes. Although it cannot be ruled out that simultaneous antagonism at both receptors is necessary to mimic the effects of captopril, these results suggest that the prevention of hyperactivity seen in captopril-treated NK1R-/- mice is not due to a deficit in AngII production.

The regulation of motor control is widely believed to involve corticostriatal circuitry and the basal ganglia. The classical model of this network proposes that the direct and indirect pathways of the basal ganglia compete for the control of movement, releasing and inhibiting movement respectively (see: Graybiel, 2000). However, this model has since been challenged by a series of studies that suggest that this may be an oversimplification. For instance, around 60% of medium spiny neurons of the direct pathway also send collaterals with terminal fields in the

globus pallidus pars externa (GPe; indirect pathway) (Cazorla et al., 2014). Furthermore, the direct and indirect pathways are now thought to both express D1 and D2 receptors, rather than only D1 receptors (direct) or only D2 receptors (indirect) (Nadjar et al., 2006). There is also evidence that both the direct and indirect pathways experience transient increases in neural activity upon initiation of movement (Cui et al., 2013) and that activation of both pathways causes both excitations and inhibitions of the substantia nigra pars reticulata (Freeze et al., 2013). These studies suggest that the classical direct/indirect model may be an oversimplification. However, it is clear that the basal ganglia play a key role in motor control and so any effect of captopril on motor activity will likely involve this network.

Both ACE and AT receptors are densely expressed within corticostriatal circuits, although with different distributions (Figure 4.5a). Specifically, ACE is found on both the cell bodies and nerve terminals of striatal cholinergic interneurons, within the globus pallidus, and within the substantia nigra pars reticulata, where it is found on GABAergic striatonigral nerve terminals (Strittmatter et al., 1984; Chai et al., 1987a). By contrast, AT receptors are primarily located on nigrostriatal nerve terminals, within the globus pallidus and in the substantia nigra pars compacta, where they are found on dopaminergic cell bodies (Allen et al., 1991, 1992). There is no evidence that AT receptors are expressed within the striatonigral pathway (Strittmatter et al., 1984). In light of this evidence, it is unsurprising that both ACE inhibition and AT receptor antagonism are capable of influencing motor control.

Whilst the evidence from these experiments suggests that neither substance P nor AngII is responsible for the reduction in locomotor activity caused by captopril, there are a number of possible alternative explanations. For instance, although ACE is capable of hydrolysing substance P, more recent evidence suggests that this enzyme is primarily responsible for the metabolism of

substance P metabolic fragments. Michael-Titus et al. (2002) showed that treatment with captopril primarily increases the striatal concentration of the substance P metabolic fragments, substance P(1-7) (SP(1-7)) and substance P(1-4) (SP(1-4)), rather than the parent peptide itself. This is interesting because SP(1-7) is thought to regulate behaviour through an NK1R-independent mechanism (e.g., Hall and Stewart, 1983; Krumins et al., 1993; Kreeger and Larson, 1996). Importantly, this means that any changes in the availability of this peptide could have behavioural effects in NK1R-/- mice. SP(1-7) and SP(1-4) also both regulate striatal dopamine efflux, and there is evidence that this also occurs through an NK1R-independent mechanism (Reid et al., 1990; Khan et al., 1995, 1996, 1998). It follows that captopril could influence the locomotor activity of NK1R-/- mice by increasing the availability of these peptides, which in turn affect dopaminergic regulation of corticostriatal circuitry. This proposal is line with evidence that another ACE inhibitor, perindopril, increases striatal dopamine efflux (Jenkins et al., 1997; Jenkins, 2008).

An alternative explanation focuses on another neuropeptide, neurotensin, which is also metabolised by ACE. There are several reports that neurotensin increases DAergic efflux in the PFC (e.g., Reyneke et al., 1992; Jiang et al., 1994; Prus et al., 2007). As discussed in Chapter 3, the hyperactivity of NK1R-/- mice might be due to reduced dopaminergic efflux in the PFC resulting in disinhibition of glutamatergic projections from the cortex to the striatum (Le Moal and Simon, 1991; Ventura, et al., 2004; see: Yan et al., 2010). It follows that increased neurotensin availability following treatment with captopril could result in increased dopaminergic transmission in the PFC, correcting the dysregulation of striatal function described above (Figure 4.5b). There are several reports that neurotensin attenuates locomotor activity (Meisenberg and Simmons, 1985; Elliott et al., 1986; Vadnie et al., 2014). However, to the best of my knowledge there have no previous studies investigating the effects of captopril on cortical dopaminergic efflux, so this warrants investigation.

ACE is also capable of metabolising bradykinin, which has previously been shown to decrease behavioural arousal in rats (Wisniewski et al., 1974). Furthermore, ACE metabolises both enkephalins and dynorphins, which are expressed in the direct and indirect pathways of the basal ganglia, respectively (see: Steiner and Gerfen, 1998). A number of studies have demonstrated that enkephalins regulate locomotor activity (Michael-Titus et al., 1987, 1989, 1990). Both enkephalins and dynorphins also modulate dopamine transmission within the striatum (Dourmap et al., 1990, 1992; Das et al., 1994). Changes to the availability of any, or all, of these peptide could also contribute to the behavioural effect of captopril in NK1R-/- mice.

It is important to remember that, by inhibiting ACE, captopril will increase the availability of AngI, which can then be metabolised to other angiotensin fragments (von Bohlen und Halbach and Albrecht, 2006). For instance, AngI can be metabolised by prolylendopeptidase (PE-P) to produce Ang(1-7). This peptide is ineffective at AT_1 and AT_2 receptors, instead acting via a separate receptor, the Mas receptor. Therefore, if the behavioural effect of captopril in NK1R-/- mice is due to an increase in Ang(1-7) availability, AT_1 or AT_2 receptor antagonism would not be expected to be mimic this effect. The effects of Ang(1-7) are often functionally opposite to AngII (e.g. Freeman et al., 1996; Ueda et al., 2000). For instance, whereas central administration of AngII increases noradrenergic transmission in the locus coeruleus (Sumners and Phillips, 1983), a number of studies have shown that Ang(1-7) has an inhibitory effect on noradrenergic transmission, decreasing noradrenergic release and synthesis and increasing the expression of the noradrenaline transporter (see: Gironacci et al., 2013). Since an increase in noradrenergic transmission is associated with increased behavioural arousal (de Lecea et al., 2012), a decrease in noradrenergic transmission due to increased Ang(1-7) availability could well underlie the calming effect of captopril seen in NK1R-/- mice.

AngI can also be metabolised by other aminopeptidases to another angiotensin peptide, AngIV, which acts via the AT_4 receptor. Similarly, if the effects of captopril are due to an increase in AngIV availability, this would not be mimicked by either AT_1 or AT_2 receptor antagonism. Furthermore, it has been proposed that many of the behavioural effects originally attributed to AngII are actually caused by AngIV (Brazsko et al., 2006; Wright and Harding, 2011). It would be interesting to investigate the effects of both Ang(1-7) and AngIV receptor antagonism on the locomotor activity of NK1R-/- mice, to test whether this mimics the effects of ACE inhibition.

Whatever the mechanism, results presented in this chapter suggest that any changes in locomotor activity caused by either ACE inhibition or AT receptor antagonism are prevented by functional NK1R, as none of the drugs tested affected the locomotor activity of wildtype mice. In line with this proposal are several reports in which captopril, losartan and PD 123319 did not affect locomotor activity in rodents, including in the Spontaneously Hypertensive Rat (Irvine et al., 1995; Czarnecka et al., 2000; Brazsko, 2002). The presence of NK1R within the striatum, both on cholinergic interneurons and on the nerve terminals of glutamatergic afferents projecting from the cortex and thalamus (Gerfen, 1991; Jakab and Goldman-Rakic, 1996), could be buffering changes to corticostriatal function that occur in response to these drugs. Similarly, any changes in noradrenergic transmission could be buffered by the presence of NK1R within the locus coeruleus (Chen et al., 2000). In order to determine the mechanisms underlying the therapeutic benefit of captopril, it would be interesting to investigate the effects of ACE inhibition on neurotransmission in neuronal networks that are known to be important in the regulation of behavioural arousal and motor control.

4.4.3. Sex differences in the effects of ACE inhibition on locomotor activity

Finally, an interesting finding from this study is that the effects of captopril were only evident in male NK1R-/- mice. Captopril did not affect the locomotor activity of female mice, at either dose. These findings suggest that there are sex differences in the influence of ACE on motor function. Indeed, there is considerable evidence that gonadal steroids regulate ACE activity, which is increased by testosterone but decreased by oestrogen (see: Komukai et al., 2010). In line with this, ACE activity increases in males but decreases in females following puberty (Landazuri et al., 2008), and in healthy young adults ACE activity is higher in men than in women (Zapater et al., 2004). In fact, this is thought to contribute to the lower incidence of cardiovascular disease in (premenopausal) women compared with men of the same age (Kannel et al., 1976), although ACE inhibition appears to be equally effective in male and female hypertensive patients (Turnball et al., 2008). It follows that one possible explanation for the lack of effect of captopril in female animals in this study is low ACE activity at baseline. Furthermore, this could also have important implications for differences in the incidence of hyperactivity seen in male and female patients with ADHD, as well as its potential amelioration by treatment with an ACE inhibitor.

4.4.4. Concluding remarks

In this chapter, the locomotor activity of male and female NK1R-/- mice is compared using the LDEB and the effects of drugs that target the BRAS on motor function are investigated. The hyperactivity of NK1R-/- mice was evident in male animals only, echoing the typical symptom profile of male and female ADHD patients. Treatment with the ACE inhibitor, captopril, prevented the hyperactivity of male NK1R-/- mice, but did not affect the locomotor activity of male wildtype mice, or female mice of either genotype. By contrast, the AT_1 receptor antagonist, losartan, and

AT_2 receptor antagonist, PD 123319, both increased the locomotor activity of male NK1R-/- mice, but also did not affect the locomotor activity of wildtype mice.

The fact that captopril was effective in NK1R-/- mice suggests that this behavioural effect is not due to an increase in substance P availability, as this would be ineffective in this genotype. Furthermore, as the effect of captopril was not mimicked by treatment either an AT_1 or AT_2 receptor antagonist, this effect is also unlikely to be due to a reduction in AngII availability. Instead, the behavioural effects of ACE inhibition could be due to changes in the availability of a range of other neuropeptides. The genotype-specific effects of these drugs point to a functional interaction between the BRAS and NK1R in the regulation of motor function that merits further investigation. Finally, these results suggest that captopril (and possibly other ACE inhibitors) could provide a novel therapeutic target for the treatment of hyperactivity in (male) patients with ADHD, particularly those with polymorphisms of the *TACR1* gene.

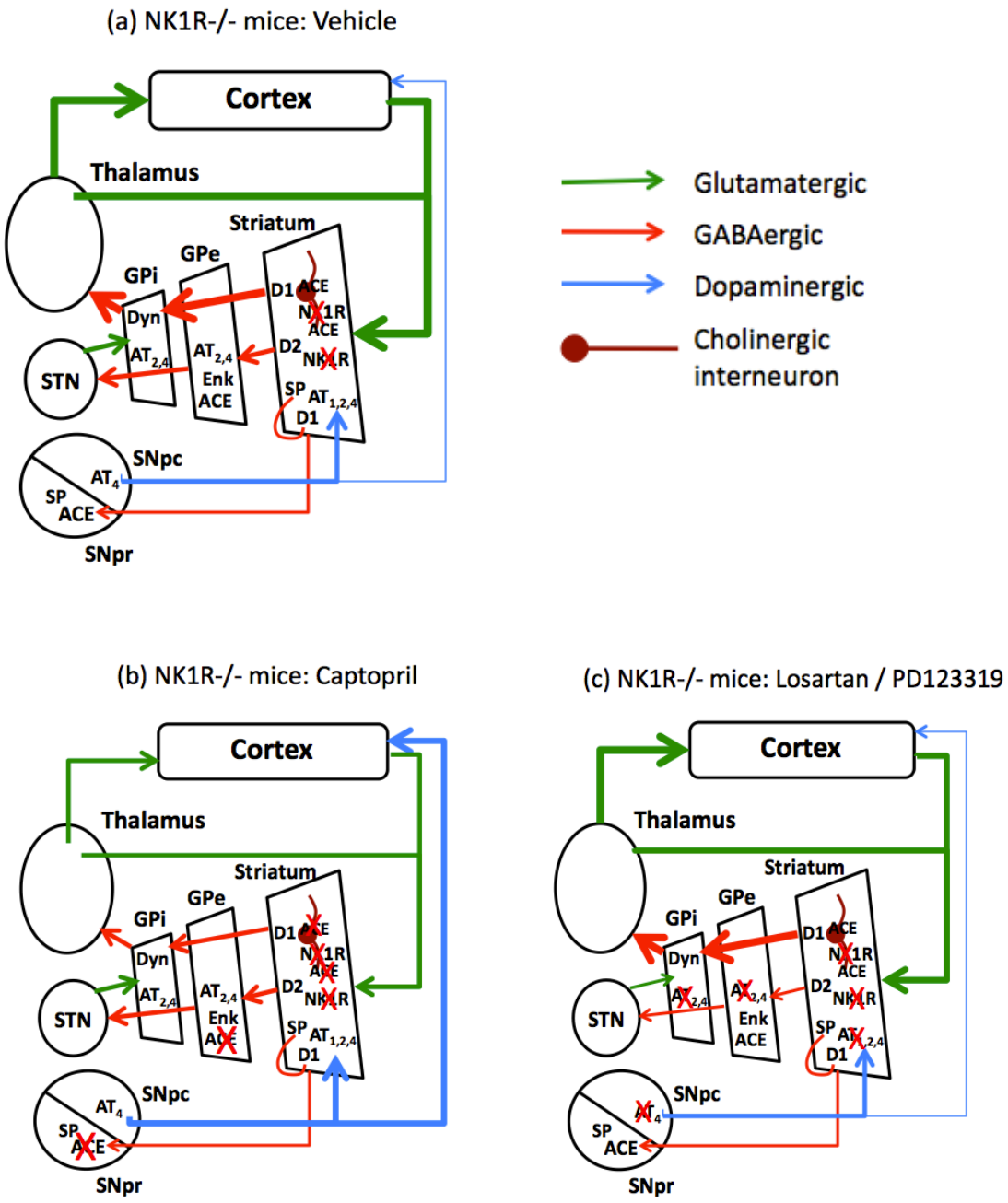

Figure 4.5. Possible changes in corticostriatal circuitry and the basal ganglia in response to (a) vehicle, (b) ACE inhibition and (c) AT receptor antagonism in NK1R-/- mice. The schematics display the localisation of ACE and AT receptors within neuronal circuits known to play a central role in motor control. Changes in arrow thickness represent hypothetical changes in activity in the direct and indirect pathways in response to drug treatment: (a) hyperactivity of the direct pathway relative to the indirect pathway at baseline; (b) the direct and indirect pathways are balanced by ACE inhibition, possibly via increased dopaminergic transmission; (c) hyperactivity of the direct pathway following AT receptor antagonism, which could be exacerbated by a reduction in activity in the indirect pathway. ACE, angiotensin converting enzyme; GPi and GPe, globus pallidus internal and external, respectively; SNpc and SNpr, substantia nigra pars compacta and pars reticula, respectively; SP, substance P

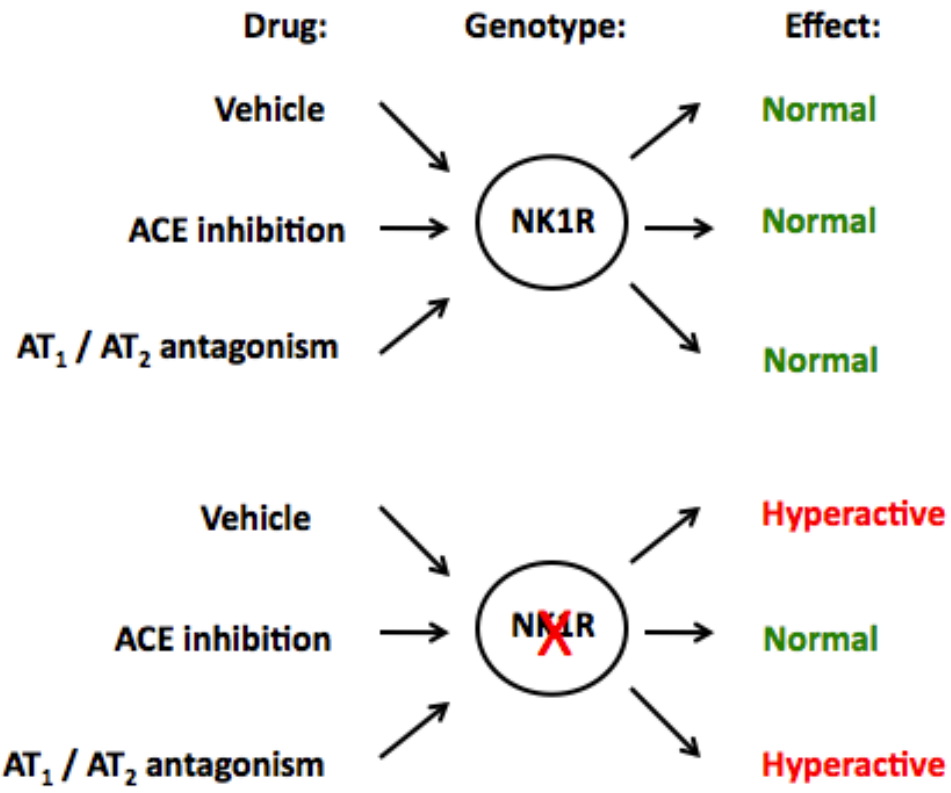

Figure 4.6. Summary of findings. The effects of ACE inhibition and AT receptor antagonism on the locomotor activity of wildtype and NK1R-/- mice. The layout emphasises how the effects of each drug is blocked by functional NK1R.

Chapter 5

The role of the brain renin angiotensin system in attentional performance and response control

5.1. Introduction

5.1.1. The brain renin angiotensin system and cognitive performance

In addition to its role in motor control, there is extensive evidence that the BRAS is involved in cognitive performance. Patients with mild to moderate hypertension treated with captopril have reported improved mental acuity and increased feelings of wellbeing (Croog et al., 1986). Several clinical studies have also demonstrated that the use of ACE inhibitors is associated with a reduction in cognitive decline in patients with dementia (Fogari and Zoppi, 2004; Rozzini et al., 2006; Yasar et al., 2008). However, the facilitatory effects of ACE inhibition are not limited to those with either hypertension or cognitive decline, as captopril also improves short-term memory in healthy male subjects (Currie et al., 1990). Furthermore, a review of clinical trial data found that the improvement of cognitive performance seen in hypertensive patients treated with an ACE inhibitor is independent from their effects on blood pressure (Amenta et al., 2002).

In preclinical studies, ACE inhibitors facilitate learning and memory in a number of behavioural paradigms. The ACE inhibitor, perindopril, ameliorates scopolamine-induced memory deficits in both the Morris water-maze and passive avoidance tests (Tota et al., 2012). It also protects against memory deficits in mouse models of dementia when tested in the Y-maze test (Dong et al., 2011). Captopril facilitates memory retrieval in mice that have received cerebral electroshock treatment (Mondadori and Etienne, 1990) and attenuates memory impairments induced by chronic cerebral hypoperfusion (Kumaran et al., 2008). In the Spontaneously Hypertensive Rat, the most well established rodent model of ADHD, lifetime treatment with captopril attenuated the age-related cognitive impairment normally seen in this model (Wyss et al., 2003).

The ability of captopril to selectively prevent the hyperactivity of NK1R-/- mice, without affecting the locomotor activity of wildtype mice (see Chapter 4), prompted the question of whether ACE inhibition would similarly affect the other behavioural deficits of these mice. At present, no previous studies have specifically tested the effects of ACE inhibition on impulse control. Nevertheless, there is extensive evidence that the BRAS regulates a number of neurotransmitters that have been implicated in impulsive behaviour (Barnes et al., 1989; Brown et al., 1996; Jenkins et al., 1997; Jenkins, 2008; discussed below). Therefore, the experiment described in this chapter investigated the effects of captopril on the performance deficits of NK1R-/- mice in the 5-CSRTT.

5.1.2. Objective

The experiment described in this chapter had the following main objective:

1) Test whether the ACE inhibitor, captopril, improves the cognitive performance and response control of NK1R-/- and wildtype mice in the 5-CSRTT

The following experiments have been accepted for publication in *European Neuropsychopharmacology* (Porter et al., 2015b).

5.2. Methods

5.2.1. Animals

All animals were used from the colony described in Section 2.2.1.

5.2.2. Drugs

Captopril was purchased from Sigma Aldrich, UK, dissolved in 0.9% saline (vehicle) and injected (i.p.) in a volume of 10mL/kg. The doses of captopril were based on results from Chapter 4, as well as previously published reports confirming their effects on behaviour and cognition in mice (Table 5.1)

Drug:	**Doses (i.p.):**	**Injection time:** (mins before testing)	**Example references:**	**Procedure:**
Captopril	5, 10 and 25 mg/kg	30 min	Costall et al., 1990	LDEB
			Czarnecka et al., 2000	hot plate test; tail-flick test
			Raghavendra et al., 2001	active (shock) avoidance task
			Czarnecka and Pietrzak, 2002	ethanol-induced sleep & hyperactivity
			van den Buuse et al., 2005	Prepulse inhibition

Table 5.1. Doses and timing used for the captopril 5-CSRTT study

5.2.3. Protocol: 5-CSRTT

The 5-CSRTT apparatus and protocol are described fully in Section 2.3.

Twelve male wildtype mice (age/weight at the start of testing: 6–7 weeks / 29–33 g) and twelve male NK1R-/- mice (age/weight at the start of testing: 6–7 weeks / 23–32 g) were used for this study. Animals were selected from three breeding-pairs per genotype, and chosen to age-match the experimental groups as closely as possible.

Animals were first trained to stable baseline performance criteria at Stage 6 of training in the 5-CSRTT (100 completed trials, >50 correct trials, >75% accuracy, <25%omissions; see: Section 2.3). Once stable baseline performance had been achieved, treatment-naïve subjects were tested using both the variable intertrial interval (VITI; ITI = 2, 5, 10 and 15 s) and long intertrial interval (LITI; ITI = 10 s) schedules.

The testing schedule for captopril is summarised in Figure 5.1. Each animal underwent a series of once-weekly tests following: no injection ('NI'), vehicle injection (0.9% saline), or captopril injection (5, 10 or 25 mg/kg). These five treatments (NI, vehicle and 3 doses of captopril) were all tested using both the VITI and LITI tests (i.e., a total of 10 testing sessions for each animal). Injections, when applicable, were administered 30 min before the start of the test session. Animals experienced each of these 10 test sessions once, only, in a randomised (pseudo-Latin-square) sequence. Testing was carried out on Fridays, only, with Stage 6 of training carried out on intervening days: animals were required to achieve stable baseline performance criteria for three consecutive days prior to testing in order to experience the next test. The performance variables calculated in the 5-CSRTT are described in Section 2.3.

(Order of testing of each treatment was randomised across subjects)

Variable Intertrial Interval (VITI) (ITI: 2, 5, 10 and 15 s)					**Long Intertrial Interval (LITI)** (ITI: 10 s)				
NI	Veh	Cap5	Cap10	Cap25	NI	Veh	Cap5	Cap10	Cap25

Figure 5.1. Summary of the 10 weeks testing schedule with captopril in the 5-CSRTT
NI, no injection; Veh, vehicle (saline); Cap5, 5 mgkg captopril; Cap10, 10 mgkg captopril; Cap25, 25 mgkg captopril

5.2.4. Statistical analysis

Further details of statistical analysis are provided in Section 2.8.

Raw or transformed data were analysed using a multifactorial 3-way mixed model ANOVA with 'Genotype' and 'Time-of-Day (morning or afternoon session)' as the between subjects factors and 'Treatment' as the within-subjects factor. A significant effect of one of the main factors, or an interaction between them, was used as the criterion for progressing on to 2-way or 1-way ANOVA with *post-hoc* (LSD) comparisons. All animals achieved stable baseline performance criteria for testing in the 5-CSRTT. However, as animals passed training at different rates, some animals did not experience every test condition by the end of the experiment. Consequently, statistical analyses were performed on groups of N = 9 – 12.

5.3. Results

In both the VITI and the LITI, Time-of-Day did not affect any of the behavioural measures, and so was removed as a main factor from all further statistical analyses.

5.3.1. Treatment with captopril reduces the incidence of premature responses in NK1R-/- mice in the VITI test

In the VITI, *premature responses/per 100 trials* were higher, overall, in NK1R-/- mice ($F(1,22) = 18.77$, $P<0.001$; Fig. 5.2a). Treatment with the intermediate dose of captopril (10 mg/kg) reduced the incidence of *premature responses/100 trials* in NK1R-/- mice, only, abolishing the genotype difference in this behaviour (LSD (*c.f.*, vehicle): $P=0.033$). The incidence of *%omissions* did not differ in the two genotypes, and there was no effect of captopril on this behaviour, at any dose (Fig. 5.2b). There was also no genotype difference in *perseveration*, and this behaviour was similarly unaffected by captopril (Fig. 5.2c).

%Accuracy was slightly lower (c. 5%), overall, in NK1R-/- mice ($F(1,22) = 14.75$, $P<0.001$; Fig. 5.3a), whilst *total trials* did not differ in the two genotypes (Fig. 5.3b). Neither of these behaviours was affected by treatment with captopril (Fig. 5.3a,b). *Latency to correct response* also did not differ in the two genotypes, but in wildtype mice was increased slightly (c. 0.05 s) by the intermediate (10 mg/kg) dose of captopril ($F(4,72) = 2.72$, $P=0.036$; LSD: $P=0.042$; Fig. 5.3c). *Latency to collect the reward* was longer, overall, in NK1R-/- mice ($F(1,21) = 8.24$, $P=0.009$; Fig. 5.3d). However, this behaviour was also increased in the wildtypes by captopril (10 mg/kg), abolishing the genotype difference ($F(4,69) = 3.13$, $P=0.02$).

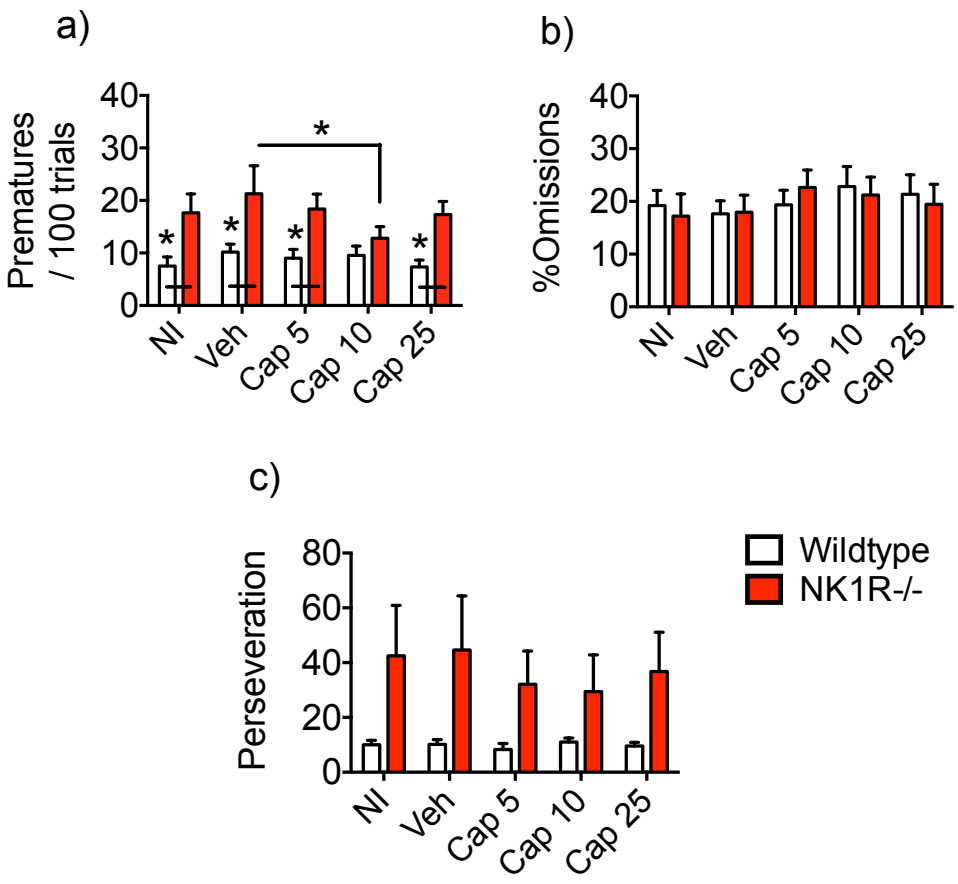

Figure 5.2. Effects of captopril in the VITI: premature responses, omissions and perseveration. Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. N = 9 – 12 per group.

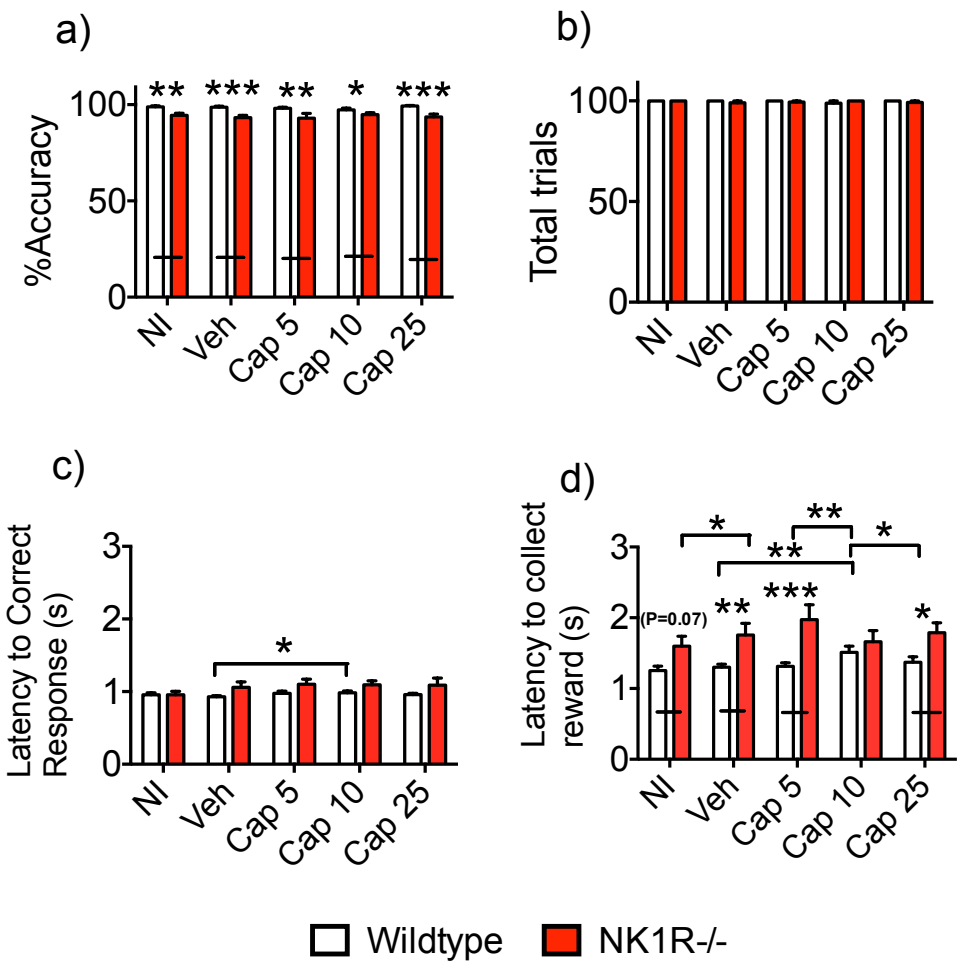

Figure 5.3. Effects of captopril in the VITI: accuracy, total trials and latencies. Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * *P*<0.05, ** *P*<0.01, ***P<0.001. N = 9 – 12 per group.

5.3.2. Treatment with captopril abolishes the genotype difference in the incidence of premature responses and omissions in the LITI test

In the LITI, as in the VITI, *premature responses/per 100 trials* were higher, overall, in NK1R-/- mice compared with wildtypes ($F(1,22) = 10.97$, $P=0.003$; Fig. 5.4a). This genotype difference was abolished in animals treated with the intermediate dose of captopril (10 mg/kg). However, at the higher dose (25 mg/kg), captopril reduced the incidence of this behaviour in wildtype mice (LSD (*c.f.*, vehicle): $P=0.012$; LSD (*c.f.,* 10 mg/kg): $P=0.02$) but did not affect NK1R-/- mice. The incidence of *%omissions* was higher, overall, in NK1R-/- mice ($F(1,22) = 5.8$, $P=0.025$; Fig. 5.4b), and this difference was abolished by treatment with the intermediate dose of captopril (10 mg/kg). As in the VITI, there was no genotype difference in *perseveration* in this test, and this behaviour was also unaffected by captopril (Fig. 5.4c).

%Accuracy was again lower, overall, in NK1R-/- mice ($F(1,22) = 22.08$, $P<0.001$), but in this test was slightly (c. 3%) decreased in NK1R-/- mice by the lowest dose of captopril (5 mg/kg; Fig. 5.5a). There was no genotype difference in either *total trials* or *latency to correct response*, and neither of these behaviours was affected by captopril (Fig. 5.5b & 5.5c). As in the VITI, *latency to collect the reward* was longer, overall, in NK1R-/- mice ($F(1,21) = 8.32$, $P=0.009$), but in this test was unaffected by treatment with captopril (Fig. 5.5d).

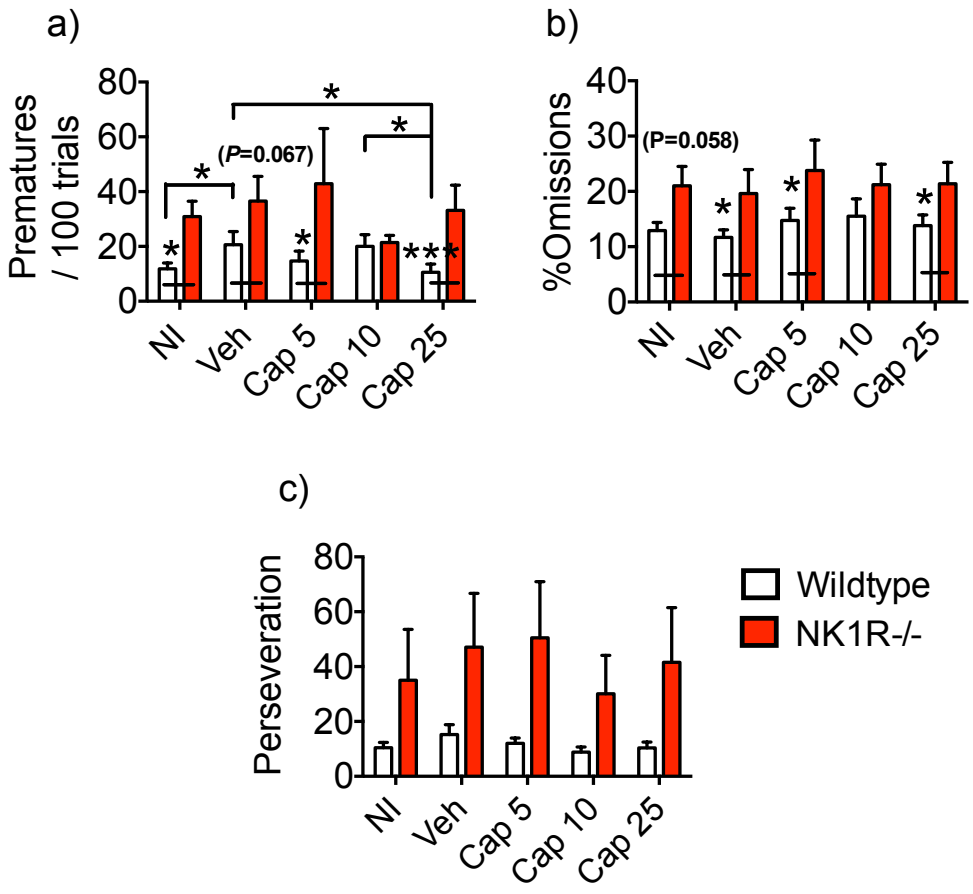

Figure 5.4. Effects of captopril in the LITI: premature responses, omissions and perseveration Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * *P*<0.05, ** *P*<0.01, ***P<0.001. N = 9 – 12 per group.

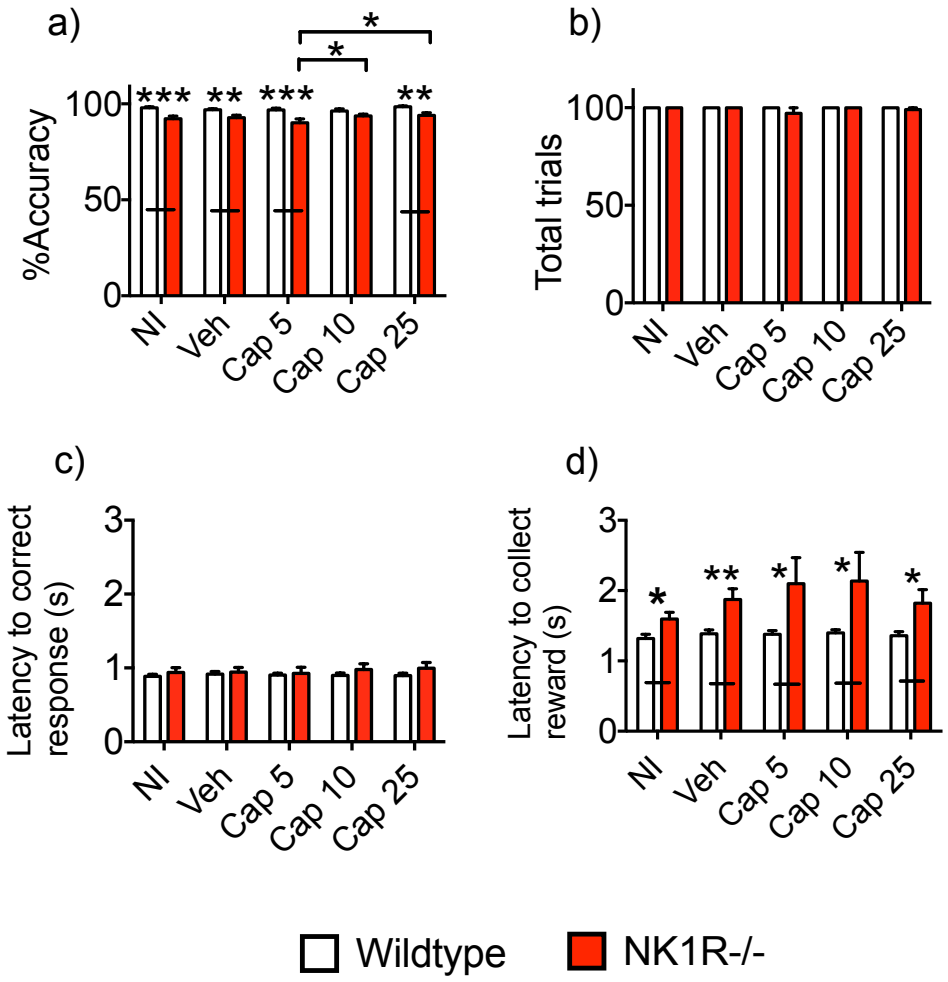

Figure 5.5. Effects of captopril in the LITI: accuracy, total trials and latencies
Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * *P*<0.05, ** *P*<0.01, ****P*<0.001. N = 9 – 12 per group.

5.4. Discussion

The aim of this experiment was to investigate the effects of the ACE inhibitor, captopril, on the cognitive performance and response control of NK1R-/- mice and their wildtype counterparts using the 5-CSRTT. In the VITI and LITI test, captopril dose-dependently abolished the genotype difference in *premature responses*. In the LITI test, captopril also abolished the genotype difference in *omissions*. In NK1R-/- mice, ACE inhibition did not affect *total trials*, *latency to correct response*, or *latency to collect the reward*, and so it is unlikely that the behavioural effects are due to changes in motivation to carry out the task. These results indicate that the impulsivity (and, perhaps, inattentiveness) arising from a deficit in functional NK1R can be ameliorated by treatment with this ACE inhibitor.

There are many reports, from human and preclinical studies, that ACE inhibitors improve cognitive performance (for review, see: Wright and Harding, 2011). However, to the best of my knowledge, this is the first instance of an ACE inhibitor being tested in the 5-CSRTT. There also do not appear to be any previous reports, in either clinical or preclinical research, investigating the effects of ACE inhibitors on impulsivity, in general. Consequently, results from this study provide the first evidence that ACE could be involved in the regulation of impulse control. The mechanisms underlying this are, as yet, unknown. However, as with hyperactivity (described in Chapter 4), the effects of this drug on the behaviours evaluated in this study cannot be explained by an increase in substance P availability, as this would be ineffective in NK1R-/- mice. Nonetheless, there are several possible alternative explanations. The following section explores the mechanisms through which captopril could influence the cognitive performance and response control of NK1R-/- mice.

5.4.1. The effects of ACE inhibition on performance in the 5-CSRTT

One possible explanation is that ACE inhibition improves the performance of NK1R-/- mice by decreasing the availability of AngII. In preclinical research, several studies have shown that administration of AngII, into regions such as the striatum and hippocampus, disrupts cognitive performance (e.g., Morgan and Routtenberg, 1977; Kerr et al., 2005; Bonini et al., 2006). Conversely, AT receptor antagonism, in particular AT_1 receptor antagonism, has cognitive-enhancing effects (e.g., Barnes et al., 1990b). In humans, the AT_1 receptor antagonist, losartan, has received particular interest because it can improve cognitive performance in hypertensive patients (Tedesco et al., 1999). Therefore, the effect of AT_1 receptor antagonism on the cognitive deficits of NK1R-/- mice merits investigation, and this forms the basis of experiments reported in Chapter 6.

However, the role of AngII and AT receptor activation in cognitive performance is not straightforward. A number of studies have, paradoxically, found that intracerebroventricular administration of AngII can also improve cognitive performance (e.g., Georgiev and Yonkov, 1985; Braszko et al., 1987, 1988; Kulakowska et al., 1996). This effect is dependent on AT receptor activation, as it is prevented by intracerebroventricular administration of an AT receptor antagonist (Georgiev and Yonkov, 1985; Kulakowska et al., 1996). One possible explanation for the discrepancy between these studies and those described above is the use of intracerebroventricular administration, since the extent to which the compound travels during testing is not known using this technique.

However, it has also been proposed that the cognitive enhancing effects originally attributed to AngII are actually due to its metabolism to smaller angiotensin peptide fragments, in particular AngIV (Braszko et al., 2006). AT_4 receptor activation has cognitive enhancing effects in a

number of behavioural paradigms (see: von Bohlen und Halbach and Albrecht, 2006; Wright and Harding, 2011) and AngIV administration facilitates long-term potentiation in the hippocampus, a region strongly implicated in learning and memory (Wayner et al., 2001). The AT_4 receptor has also received interest as a target for the treatment of cognitive decline in patients with Alzheimer's disease, and this has led to the development of AngIV analogues that have shown promise as potential new therapeutics in this area (Wright and Harding, 2008). It would also be interesting to test the effects of AT_4 receptor antagonism on the cognitive performance of NK1R-/- mice in the 5-CSRTT.

The two behaviours that were of particular interest in this study were impulsivity, measured as the incidence of *premature responses*, and attentional performance, measured by the incidence of *omissions*. While there do not appear to be any previous reports investigating the effects of ACE inhibition on impulse control, there is considerable evidence that the neuropeptides metabolised by ACE interact with the function of neurotransmitters that influence impulsive behaviour. For example, as discussed in previous chapters, it is widely believed that impulsivity is associated with abnormalities in serotonergic transmission (e.g.: Oades, 2002; Winstanley et al., 2006), and a number of studies have shown that a higher incidence of premature responding in the 5-CSRTT is also associated with abnormalities in serotonergic transmission (e.g., Dalley et al., 2002; Robinson et al., 2008a; Agnoli and Carli, 2012). The AT_1 receptor antagonist, candesartan, given for one week via drinking water increases serotonergic efflux in the PFC (Jenkins, 2008), indicating an inverse relationship between AngII and serotonergic transmission in this brain region.

All neurons of the dorsal raphé nucleus, from which cortical serotonergic neurons project, express receptors for neurotensin (see: Jolas and Aghajanian, 1997). There is extensive evidence that neurotensin stimulates serotonergic release in the PFC (Jolas and Aghajanuan, 1996; Heaulme

et al., 1998; Boules et al., 2001; Petkova-Kirova et al., 2008). It follows that one mechanism through which captopril could influence impulsivity is through changes in the availability of either AngII or neurotensin, which in turn influence serotonergic transmission. The effect of captopril on serotonergic efflux in the PFC merits investigation.

Another neurotransmitter commonly associated with impulsivity is dopamine. As discussed in Section 1.1.5, NK1R-/- mice show a deficit in cortical dopamine efflux, which echoes the presumed 'hypofrontality' seen in patients with ADHD (Zang et al., 2005; Yan et al., 2010). Neurotensin is expressed by dopaminergic neurons projecting from the midbrain to the PFC (Hokfelt et al., 1984; Seroogy et al., 1987). There is a high density of neurotensin receptors within the PFC, where they are found on pyramidal cell bodies, GABAergic interneurons, and dopaminergic nerve terminals (Binder et al., 2001; Petrie et al., 2005). There is extensive evidence that neurotensin stimulates dopaminergic efflux in this brain region (e.g., Hetier et al., 1988; During et al., 1992; Prus et al., 2007). It is possible that treatment with captopril, through an increase in the availability of neurotensin, increases release of dopamine in the PFC, which would correct the reduction in dopamine efflux in the PFC of NK1R-/- mice (Yan et al., 2010). If so, this effect would mimic the effects of dopamine-releasing agents currently used in ADHD treatment. Again, the effect of captopril on dopaminergic transmission in the PFC merits investigation.

There is extensive evidence that the incidence of *omissions* in the 5-CSRTT is influenced by noradrenergic transmission (Carli et al., 1983; Cole and Robbins, 1992; Milstein et al., 2007), and deficits in attentional performance have been linked with noradrenergic neurons that project from the locus coeruleus to the PFC (Aston-Jones and Cohen, 2005). There is evidence that AngII stimulates noradrenergic transmission in a number of brain regions, including the locus coeruleus (Sumners and Phillips, 1983; Qadri et al., 1991; Stadler et al., 1992). Moreover, captopril inhibits

stimulation-evoked noradrenaline release in both the medulla oblongata and in the hypothalamus (Tsuda et al., 1995): i.e., central ACE inhibition reduces noradrenergic transmission. It follows that captopril could influence attentional performance in the 5-CSRTT via regulation of the noradrenergic system.

Finally, it is well known that disruption to cholinergic transmission interferes with cognitive performance (see: Klinkenberg and Blokland, 2010). Within the striatum (a region strongly implicated in action selection/inhibition), NK1R are densely expressed on cholinergic interneurons, which regulate the function of medium spiny GABAergic efferents (Gerfen, 1991). As discussed in Chapter 3, there is evidence that the number of choline acetyltransferase (ChAT)-expressing neurons in the dorsal striatum is lower in NK1R-/- mice than wildtypes (Dudley, 2013). This suggests that striatal function is impaired in NK1R-/- mice, which could contribute to the deficits in response control and cognitive performance seen in these animals (Galarraga et al., 1999; Ding et al., 2010; Jupp and Dalley, 2014). There is evidence that AngII inhibits cholinergic release (Barnes et al., 1989), and it has been proposed that the improvement in cognitive performance seen following treatment with ACE inhibitors may be due to their ability to remove this inhibitory influence on acetylcholine (Barnes et al., 1990b). This is supported by preclinical evidence that ACE inhibitors prevent the impairment in cognitive performance caused by treatment with the muscarinic receptor antagonist, scopolamine (Costall et al., 1989). It is therefore possible that treatment with captopril, by reducing AngII availability, could increase striatal cholinergic efflux in NK1R-/- mice and offset the reduction in cholinergic function seen in these mice.

Whilst the mechanisms underlying the effects of captopril in this study have yet to be determined, it seems that any changes in task performance caused by ACE inhibition is blunted by

functional NK1R. This could be due to NK1R offsetting changes in neurotransmitter function that occur in response to ACE inhibition. However, it is also possible that the lack of effect in wildtype mice is due to a floor effect. Nonetheless, results from this study suggest that ACE and NK1R interact in the regulation of behaviours assessed in the 5-CSRTT. As mentioned, there do not appear to be any previous reports investigating the role of the BRAS in relation to impulsive behaviour. Consequently, work presented in this chapter opens up a wide, previously unstudied, and potentially important field of research that could provide a new way of tackling cognitive impairment in the future.

5.4.2. Concluding remarks

The experiment reported in this chapter tested the effects of the ACE inhibitor, captopril, on the performance of NK1R-/- mice in the 5-CSRTT. The results provide the first evidence that ACE could be involved in the regulation of impulsive behaviour. As with locomotor activity, it cannot be ruled out that the effects seen in this study are secondary to changes in blood pressure, although, as mentioned, a review of clinical data indicated that ACE inhibitors have cognitive enhancing effects that can be dissociated from their antihypertensive action (Amenta et al., 2002). The mechanisms underlying the effects of captopril in this test remain unknown, but the genotype-specific effect of captopril indicates that there is an interaction between the BRAS and NK1R in behaviours tested in the 5-CSRTT, and there is extensive evidence to suggest that the neuropeptides metabolised by ACE regulate several neurotransmitters implicated in both impulsivity and attentional performance. Finally, findings from this chapter suggest that ACE inhibitors could provide a novel therapeutic target for the treatment of ADHD, particularly in patients with polymorphisms of the *TACR1* gene.

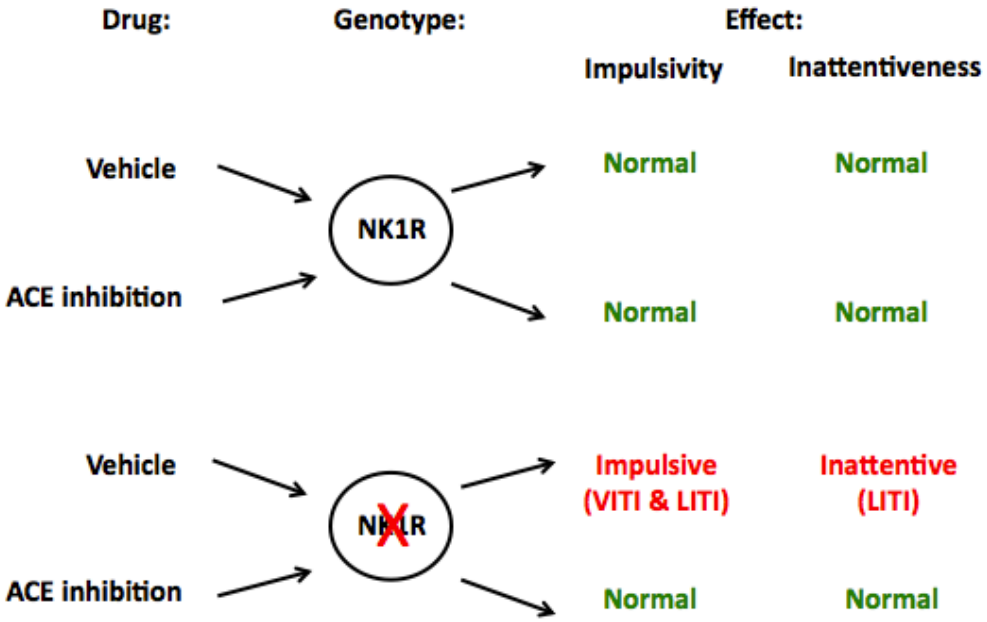

Figure 5.6. Summary of findings. The effects of ACE inhibition on the impulsivity and attentional performance of wildtype and NK1R-/- mice. The layout emphasises how the effects of drug treatment are blocked by functional NK1R.

Chapter 6

The performance of NK1R-/- mice in the 5-Choice Continuous Performance Test and Novel Object Tests: effects of AT_1 receptor antagonism

6.1. Introduction

6.1.1. The 5-Choice Continuous Performance Test

A Continuous Performance Test (CPT) is an umbrella term that describes a range of tests used in human research to assess sustained attention and impulse control (see: Riccio et al., 2002). In all of these tests, the subject must learn to respond to a 'Go' signal and withhold a response to a 'No-go' signal. Consequently, a range of response outcomes are possible, where the subject may: respond correctly to a Go signal (Hit); fail to respond correctly to a Go signal (Miss); correctly withhold a response to a No-Go signal (Correct Rejection); or incorrectly respond to a No-Go signal (False Alarm). The use of CPTs is common in the assessment of ADHD (Huang-Pollock et al., 2012) and patients with the disorder demonstrate poorer performance in terms of both impulsivity and attentional performance compared with healthy controls (Epstein et al., 2003; Klein et al., 2006).

It has been proposed that the 5-CSRTT is a preclinical equivalent of a human CPT (Jones and Higgins, 1995; Day et al., 2008). Whilst this test is a useful measure of both sustained attention and response control, there is a problem with this comparison: the 5-CSRTT does not include any 'No-go' signals. Although it has been suggested that an incorrect response in the 5-CSRTT is analogous to a 'False Alarm' in a CPT (Day et al., 2008), there is no explanation for what might constitute a 'Correct Rejection'. The use of false alarms in a No-go trial, as opposed to premature responding, is important when investigating the impulsivity of animal models of ADHD as patients with the disorder demonstrate increased false alarm rates when performing human versions of the task (Kuntsi et al., 2006; Crosbie et al., 2008; Groman et al., 2009). Furthermore, there are several different types of impulsivity (Evenden, 1999b; Winstanley et al., 2006; Chamberlain and Sahakian, 2007; Robinson et al., 2008b), and the use of premature responses in the 5-CSRTT captures only one aspect of these. There is also evidence from human research that

'Go' and 'No-Go' trials activate different brain regions (Fallgatter, 2001; McKenna et al., 2013). These observations limit the 5-CSRTT in its translational relevance to the human CPT and make it difficult to compare preclinical and clinical data on impulsivity and attention in these two tests.

In order to overcome this problem, Young and colleagues developed the 5-Choice Continuous Performance Test (5-CCPT), an adapted version of the 5-CSRTT (Young et al., 2009). In the 5-CCPT there are two different types of trials. In the first, a light stimulus appears in one of the five apertures and the animal has to respond by nose-poking into this hole in order to receive a food reward, as in the 5-CSRTT ('Go' trials). In the second, all five apertures light up at the same time and the animal must withhold from responding in order to receive a reward ('No-go' trials). In the 'Go' trials, the possible outcomes are equivalent to the 5-CSRTT: correct, incorrect, premature or omission. In the 'No-go' trials, the animal can either correctly withhold a response ('Correct Rejection'), or incorrectly respond to the light stimuli by nose-poking in one of the five apertures ('False Alarm'). Thus, the 5-CCPT enables the measurement of two different forms of impulsivity simultaneously: *premature responses* ('motor impulsivity') and *probability of false alarm* ('behavioural disinhibition'). Furthermore, the 5-CCPT provides two additional performance variables that are not available in the 5-CSRTT: the *sensitivity index* (SI), which indicates an animal's ability to discriminate between the 'Go' and 'No-go' trials, and is an alternative measure of attentional performance or 'vigilance'; and the *responsivity index* (RI), which indicates an animal's 'tendency to respond' (conservative or liberal). Therefore, in order to further investigate the phenotype of the NK1R-/- mouse model of ADHD, the first aim of the experiments reported in this chapter was to compare baseline (uninjected) performance of these mice and wildtypes during training and testing in the 5-CCPT.

6.1.2. The effects of AT receptor antagonism in the 5-CCPT

In experiments reported in Chapter 4, the AT_1 receptor antagonist, losartan, and the AT_2 receptor antagonist, PD 123319, both increased the locomotor activity of NK1R-/- mice, whilst leaving that of wildtype mice unaffected. This effect was opposite to that of the ACE inhibitor, captopril, which reduced the locomotor activity of NK1R-/- mice, but also did not affect the locomotor activity of wildtypes. Collectively, these results suggest that ACE and AT receptors both interact with NK1R to influence motor control, but in different ways. In the experiment described in Chapter 5, captopril also abolished the genotype difference in premature responses and, possibly, omissions in the 5-CSRTT. These results indicate that ACE could also interact with NK1R in the regulation of impulse control and attentional performance. As with motor control, this effect of ACE inhibition could be due to a number of different mechanisms (discussed in Chapter 5).

One possibility is that ACE influences these behaviours by reducing the availability of AngII, which would blunt activation of AT receptors. To test this possibility, the experiment reported in the second part of this chapter investigated the effects of AT receptor antagonism on performance in the 5-CCPT. Whilst both AT_1 and AT_2 receptors have shown efficacy in behavioural screens of cognitive performance, the literature is more extensive for that of the AT_1 receptor subtype (see: Saavedra et al., 2005; De Bundel et al., 2008; Ciobica et al., 2009). AT_1 receptors, in particular, also influence long-term potentiation (LTP), a specific form of synaptic plasticity that is thought to represent a cellular correlate of learning and memory (von Bohlen und Halbach and Albrecht, 2006; Tchekalarova and Albrecht, 2007; De Bundel et al., 2008). Moreover, the AT_1 receptor antagonist, losartan, has cognitive-enhancing effects in both hypertensive patients (Tedesco et al., 1999; Fogari et al., 2003) and normotensive young adults (Mechaeil et al., 2011). In light of all of this evidence, the second aim of the experiments reported in this chapter was to investigate the effects of the AT_1 receptor antagonist, losartan, on the performance of NK1R-/- mice in the 5-CCPT.

6.1.3. The effects of AT receptor antagonism in novel object tests

Finally, there are a number of reports that patients with ADHD suffer from deficits in memory (e.g., Castellanos and Tannock, 2002; Frazier et al., 2004; Westerberg et al., 2004). It has even been proposed that this could contribute to the core symptoms of the disorder (Barkley, 2006). There is evidence that NK1R are involved in the processes underlying learning and memory, since treatment with an NK1R antagonist has memory-enhancing effects in rats (e.g., Kart et al., 2004; Kart-Teke et al., 2007). Furthermore, neurogenesis and brain-derived neurotrophic factor are both increased in the hippocampus of NK1R-/- mice (Morcuende et al., 2003); it has been proposed by other groups that these processes influence cognition (e.g., Hall et al., 2000; Saxe et al., 2006; Kitamura and Inokuchi, 2014). Despite this, NK1R-/- mice do not differ from wildtypes in several tests of hippocampal-dependent learning, such as the Morris water maze and contextual fear conditioning (Morcuende et al., 2003).

In preclinical research, a common way of measuring memory is by using tests of novel object recognition (NOR) and novel object location (NOL). These tests are based on an animal's exploratory behaviour of two objects: one that has been seen previously and one that has an element of novelty to it, either because it is in a different position (NOL) or because it is a different object entirely (NOR). AngII and AT_1 receptor antagonists both influence spatial and object-recognition memory (Braszko, 1996; Conner et al., 2010; De Bundel et al., 2010; Paris et al., 2013). Therefore, the third and final aim of the experiments reported in this chapter is to determine the effects of AT_1 receptor antagonism on the performance of NK1R-/- mice in two tests of memory, the NOR and NOL tests.

6.1.4. Objectives

1) Compare the cognitive performance and response control of wildtype and NK1R-/- mice in the 5-CCPT
2) Determine the effects of the AT_1 receptor antagonist, losartan, on performance in the 5-CCPT
3) Determine the effects of the AT_1 receptor antagonist, losartan, on performance in novel object tests.

6.2. Methods

6.2.1. Animals

All animals were used from the colony described in Section 2.2. The 5-CCPT, NOL and NOR studies were each performed on a separate batch of animals.

6.2.2. Drugs

Losartan potassium was purchased from LKT laboratories, UK, dissolved in 0.9% saline and injected (i.p.) in a volume of 10 mL/kg. Doses of losartan were based on published reports confirming their effects on behaviour and cognition in rodents (Table 6.1.) and also on the study described in Chapter 4, in which the higher two doses were effective in the LDEB. The same batch of losartan was used in all three studies (5-CCPT, NOL and NOR).

Drug:	**Doses (i.p.):**	**Injection time:** (mins before testing)	**Example references:**	**Procedure:**
Losartan	5, 10 and 25 mg/kg	60 min	Wayner et al., 1994	open field test; ethanol-intoxication (e.g., righting reflex)
			Lee et al., 1995	shock avoidance paradigm
			Gard et al., 2001	LDEB; EPM
			Raghavendra et al., 2001	shock avoidance paradigm
			Marvar et al., 2014	foot-shock fear conditioning paradigm

Table 6.1. Doses and timing used for these studies

6.2.3. Protocol: 5-CCPT

Further details of the 5-CCPT apparatus and protocol are described fully in Section 2.4.

Twelve male wildtype mice (aged 6 – 7 weeks; weight 30 – 34 g) and twelve male NK1R-/- mice (aged 6 — 7 weeks; weight 29 – 31 g) were used. Animals were selected from a total of three separate breeding pairs per genotype and were age matched across groups as closely as possible.

Animals were first trained to baseline performance criteria for testing in the 5-CCPT. During Stage 1 (of training), only 'Go' trials were used, delivered on a fixed ITI schedule (ITI = 5 s). During Stage 2, again only 'Go' trials were used but delivered on a VITI schedule (ITI = 3, 4, 5, 6, 7 s). During Stage 3, 'Go' and 'No-Go' trials were used, delivered in a 2:1 ratio and on a VITI schedule. During Stage 4, 'Go' and 'No-Go' trials were again used, delivered in a 5:1 ratio and again on a VITI schedule. The task parameters used for each stage of training are also summarised in Table 2.3. Once stable baseline had been achieved at Stage 4 (sensitivity index > 0), uninjected, treatment-naïve animals were tested, once, using the extended test session of the 5-CCPT (250 trials, 5:1 ratio, VITI schedule (ITI = 7, 8, 9, 10, 11 s); see Section 2.4).

After testing once at (uninjected) baseline, the effects of methylphenidate on task performance were investigated. Animals experienced five (once-weekly) test conditions: no injection, vehicle injection (0.9% saline, i.p.), and three doses of methylphenidate (3, 10 and 30 mg/kg, i.p.). The results from this section of the experiment are reported elsewhere. All animals experienced the same number of tests, and were required to re-establish stable baseline performance criteria before the effects of losartan were investigated.

The testing schedule for losartan is summarised in Figure 6.1. Each animal experienced a series of once-weekly test conditions: no injection 'NI', vehicle injection (0.9% saline, i.p.), and three doses of losartan (5, 10 and 25 mg/kg, i.p.). When applicable, animals were injected 60 min before testing. Animals experienced each of these test conditions, once, in a randomised (pseudo-Latin Square) sequence. Testing was carried out on Fridays, only, with Stage 4 of training carried out on intervening days. Animals were required to achieve stable baseline performance criteria for three consecutive days prior to testing in order to experience the next test.

6.2.3.1. Additional performance variables in the 5-CCPT

The performance variables calculated in the 5-CCPT include those calculated in the 5-CSRTT, plus: the *probably of false alarm* (pFA); the *sensitivity index* (SI), and *responsivity index* (RI). The calculation of these behaviours and an explanation of their meaning are provided in Section 2.4.

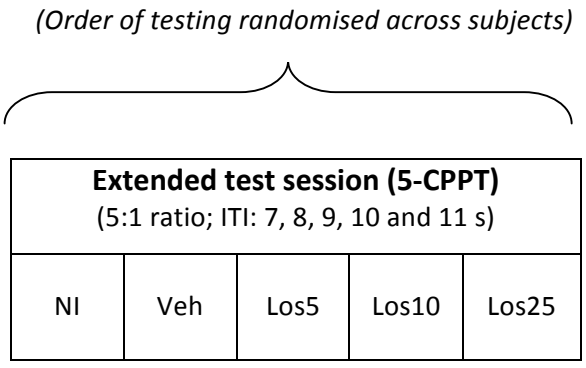

Figure 6.1. Summary of the 5 weeks testing schedule with losartan in the 5-CCPT.
NI, no injection; Veh, vehicle (saline); Los5, 5 mgkg losartan; Los10, 10 mgkg losartan; Los25, 25 mgkg losartan

6.2.4. Protocol: Novel Object Location / Novel Object Recognition tests

Full details of the NOL and NOR apparatus and protocol are provided in Section 2.7. The NOL and NOR studies were carried in parallel with the 5-CCPT study (on separate batches of animals), and spanned the duration of training / testing in the 5-CCPT. Importantly, this means that the NOL and NOR studies serve as active controls for results from the 5-CCPT study (discussed below).

6.2.4.1. NOL study

Sixteen male wildtype mice (age 6 – 14 weeks / weight 29 - 40 g) and sixteen male NK1R-/- mice (aged 6 – 11 weeks / weight 23 – 38 g) were used. Animals were selected from a total of 5 - 6 breeding pairs per genotype, so as to age-match the groups as closely as possible. Animals were randomly (Latin square) assigned to receive either: no injection ('NI'), vehicle injection (0.9% saline, i.p.), or a losartan injection (10 or 25 mg/kg, i.p) (N=4 per group). When applicable, this injection was made 60 min prior to trial 1 on day 2 (see below).

The NOL protocol was carried out over two days (see also: Figure 2.9). On both days, animals were brought to the laboratory at 09.30 h and allowed a minimum of 3 h habituation to the testing room. On day 1, animals were placed in the testing apparatus at 13.00 h for 30 min habituation. During this time, the testing apparatus remained empty. On day 2, animals experienced two trials. During trial 1 (13.00 h), animals were placed into the testing apparatus, which contained two identical objects, for 10 min. After this, they were placed back into their home-cages. During trial 2 (14.00 h), animals were placed back into the testing apparatus for 10 min; this time, one of the objects had been moved to a different location. The moved object was referred to as 'moved', whereas the object that had not been moved was referred to as

'stationary'. The behaviour of each animal was recorded using a video camera placed above the apparatus. The time spent exploring the moved and stationary objects during trial 2 was subsequently scored, blind. Further details of behavioural scoring are provided in Section 2.7.4.

6.2.4.2. NOR study

Sixteen male wildtype mice (aged 6 – 14 g / weight 26 – 40 g) and sixteen male NK1R-/- mice (aged 6 - 12 weeks / weight 25 – 38 g) were used. Animals were selected from a total of 5 – 6 breeding pairs per genotype and chosen so as to age-match the groups as closely as possible. Animals were randomly (Latin square) assigned to receive either: no injection ('NI'), vehicle injection (0.9% saline, i.p.), or a losartan injection (10 or 25 mg/kg, i.p) (N=4 per group). When applicable, this injection was made 60 min prior to trial 1 on day 2 (see below).

The protocol was similar to that used in the NOL study, except in trial 2 of the NOR study one of the objects was replaced by a different object (see also: Figure 2.8). The replaced object was treated as 'novel', whereas the object that had not been replaced was treated as 'familiar'. The position of the objects did not differ in trials 1 and 2.

6.2.5. Statistical analysis

Further details of statistical analyses are described in Section 2.8.

Training in the 5-CCPT: Each Stage of the training data was analysed separately. Data from the fixed ITI Stage (Stage 1) were analysed using mixed model ANOVA with 'Stimulus Duration' as the within-subjects factor and 'Genotype' and 'Time-of-Day (morning or afternoon session)' as the

between-subjects factors. Data from the VITI, 2to1 and 5to1 Stages (Stages 2 – 4) of training were each analysed using single-measures ANOVA with 'Genotype' and 'Time-of-Day' as the between-subjects factors.

Testing in the 5-CCPT: Data from the (uninjected) baseline extended test session were analysed using single-measures ANOVA with 'Genotype' and 'Time-of-Day' as the between subjects factors. Data from the losartan-testing phase were analysed using mixed model ANOVA with 'Treatment' as the within-subjects factor and 'Genotype' and 'Time-of-Day' as the between-subjects factors.

Within-session performance: In order to determine how performance changed within the uninjected test session, the data were also split into bins of trials (50 trials/bin). These were analysed using mixed model ANOVA with 'Trial-Bin' as the within-subjects factor and 'Genotype' and 'Time-of-Day' as the between-subjects factors.

Novel object tests: Data from the novel object tests were analysed using mixed model ANOVA with 'Genotype' and 'Treatment' as the between-subjects factors and 'Novelty' (NOL: Moved vs. Stationary; NOR: Novel vs. Familiar object) as the within-subjects factor.

6.3. Results

6.3.1. 5-CCPT (training): NK1R-/- mice express a higher incidence of premature responding than wildtype mice during the early stages of training, but the incidence of false alarms, omissions and sensitivity index does not differ in the two genotypes

NK1R-/- mice needed fewer *total number of sessions* to pass the first Stage of training (fixed ITI) compared with wildtype mice, overall ($F(1,22)=4.99$; $P=0.036$; Fig. 6.2a). However, in subsequent stages (VITI, 2to1, 5to1), there were no genotype differences in the *total number of sessions* required to pass (Fig. 6.2b-d). All animals were capable of reaching stable baseline performance criteria for testing in the 5C-CPT.

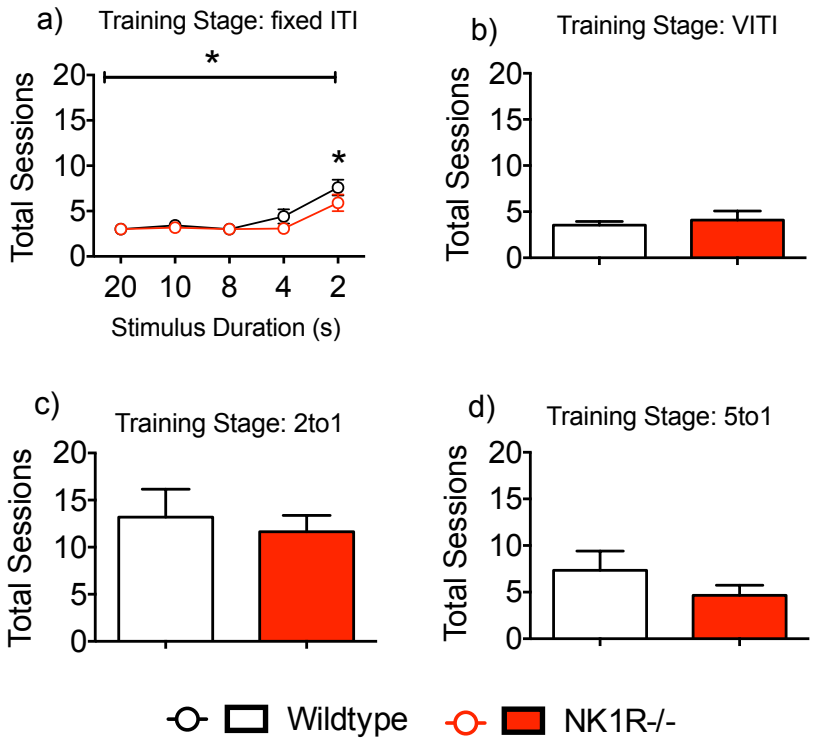

Figure 6.2. Training in the 5-CCPT: total sessions required to pass each stage. Circles / bars show mean±s.e.m. (a) Line above graph indicates a statistically significant difference between the two genotypes across the fixed ITI Stage of training. * $P<0.05$. N = 12 per group

The incidence of *%premature responses* was higher in NK1R-/- mice across the first Stage of training (fixed ITI), compared with wildtype mice ($F(1,22)=7.32$; $P=0.013$; Fig. 6.3a). This was particularly evident during early exposure to the task (LSD: $P<0.001$). However, there were no genotype differences in *%premature responses* during later Stages of the training period (VITI, 2to1, 5to1; Fig. 6.3b-d). *%Omissions* did not differ in the two genotypes during any stage of the training procedure (Fig. 6.3e-h). However, *perseveration* was greater in NK1R-/- mice, throughout training (fixed ITI: $F(1,22)=8.25$; $P=0.009$; VITI: $F(1,22)=40.34$; $P<0.001$; 2to1: $F(1,22)=5.78$; $P=0.025$; 5to1: $F(1,22)=11.5$; $P=0.003$; Fig. 6.3i-l).

During the first Stage of training (fixed ITI), *%accuracy* was lower in NK1R-/- mice than in wildtype mice ($F(1,22)=6.09$; $P=0.022$; Fig. 6.4a). However, once animals were trained on a 2 s stimulus duration, there were no longer any genotype differences. Furthermore, there were no genotype differences in *%accuracy* during the later stages of training, either (VITI, 2to1, 5to1; Fig. 6.4b-d).

During both the fixed ITI and 5to1 Stages of training, *total trials* were higher in NK1R-/- mice than in wildtype mice (fixed ITI: $F(1,22) = 13.14$; $P=0.001$; 5to1: $F(1,22)=5.43$; $P=0.029$; Fig. 6.4e,h). During the other Stages of training (VITI, 2to1), there were no genotype differences in *total trials* (Fig. 6.4f-g). Furthermore, there were no genotype differences in *latency to correct response* at any point during training (Fig. 6.4i-l). *Latency to reward* was greater in NK1R-/- mice during the VITI, 2to1 and 5to1 Stages of training (VITI: $F(1,22)=16.1$; $P<0.001$; 2to1: $F(1,22)=6.04$; $P=0.022$; 5to1: $F(1,22)=5.08$; $P=0.0344$ Fig. 6.4n-p), but not during the fixed ITI Stage of training (Fig. 6.4m).

Finally, the two genotypes did not differ in the *probability of false alarm*, *sensitivity index*, or *responsivity index*, during either the 2to1 or 5to1 stage of training (6.5a-f).

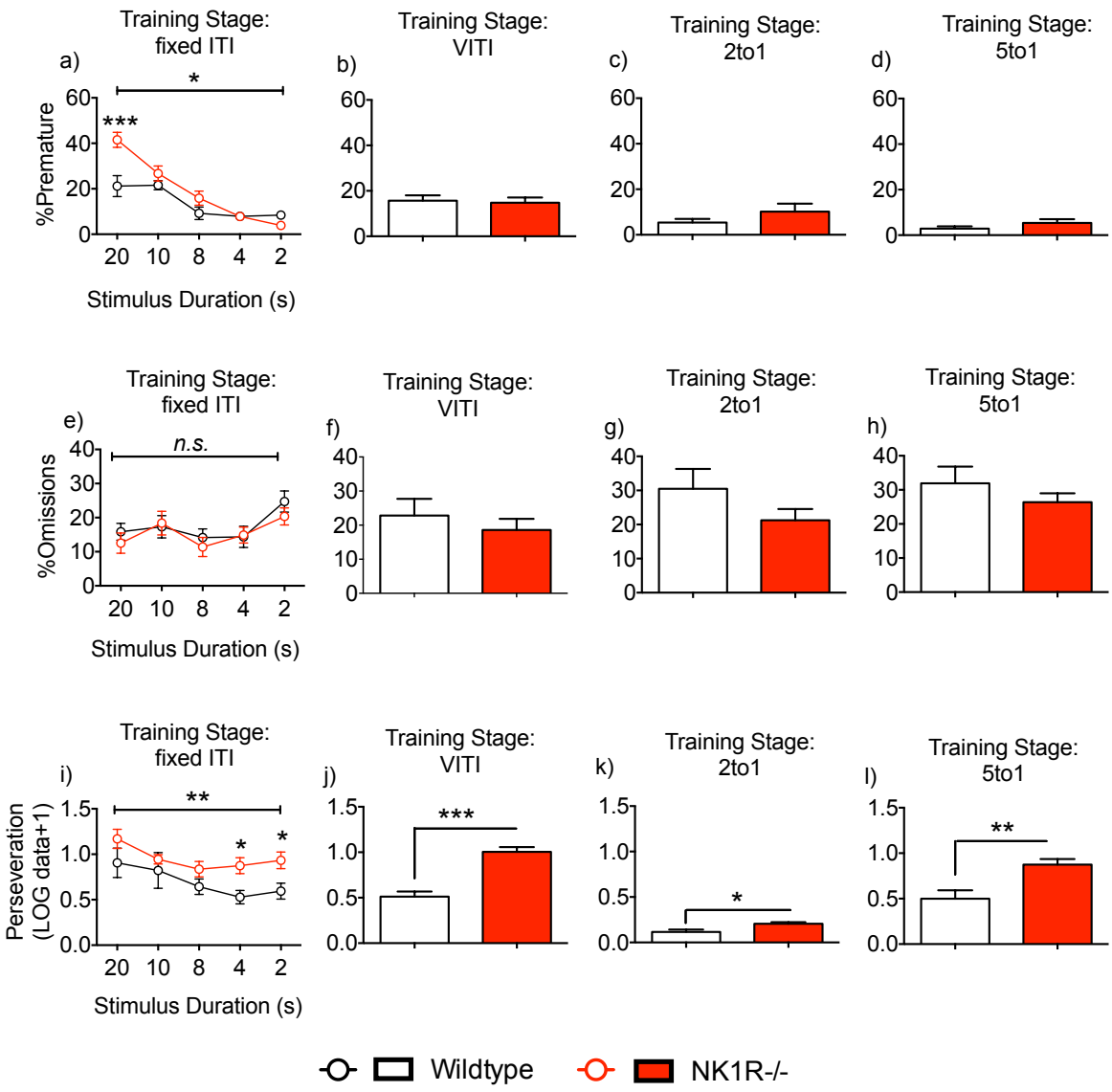

Figure 6.3. Training in the 5-CCPT: premature responses, omissions and perseveration. Circles / bars show mean±s.e.m. (a,e,i) Lines above graphs indicate statistically significant differences between the two genotypes across the fixed ITI Stage of training. Numbers underneath graphs indicate the stimulus duration (gradually reduced from 20 to 2 s as animals achieved progression criteria). (j,k,l) Lines indicate statistically significant differences between the two genotypes. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. *n.s.,* non-significant. N = 12 per group.

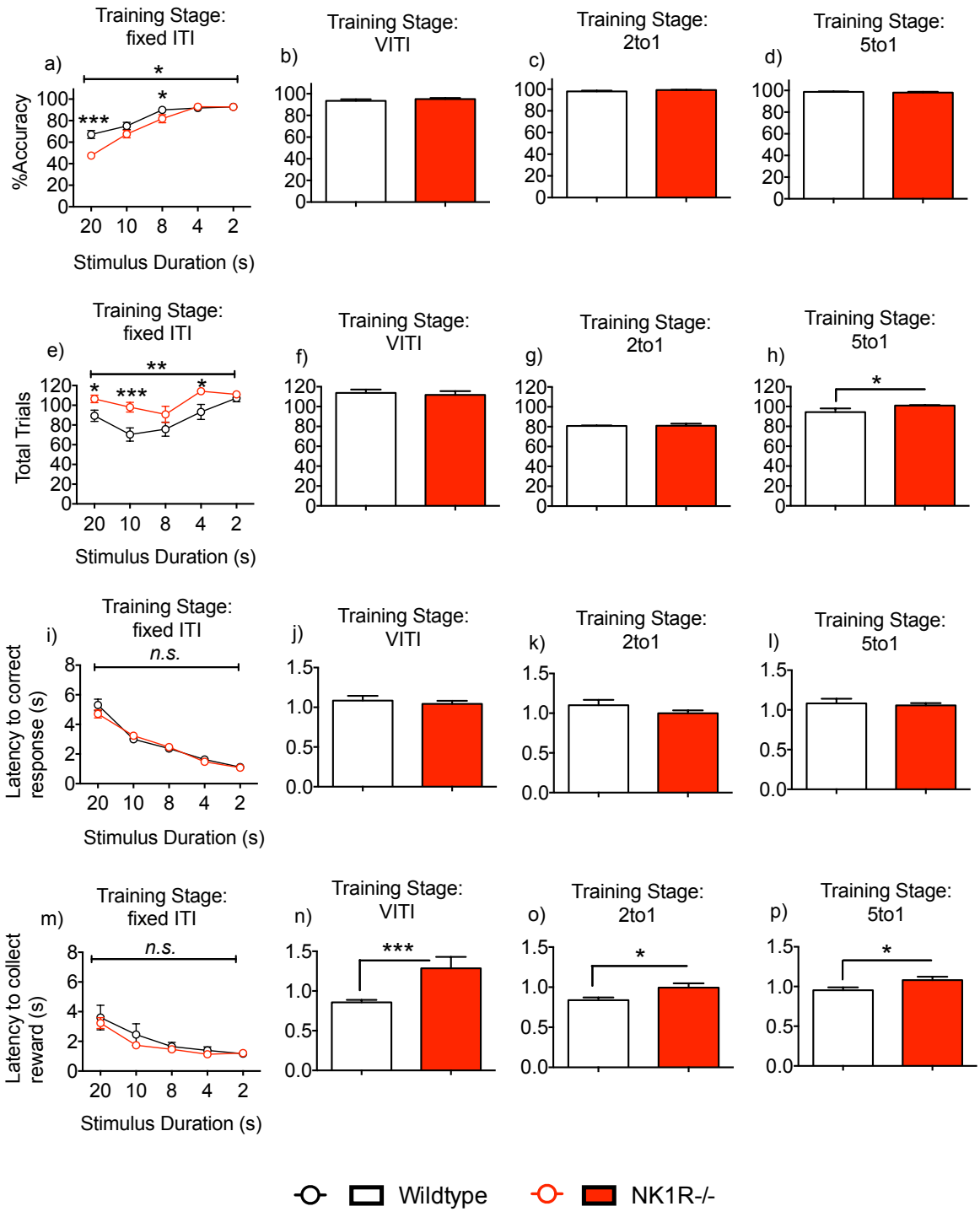

Figure 6.4. Training in the 5-CCPT: accuracy, total trials and latencies.

Circles/bars show mean±s.e.m. (a,e,i,m) Lines above graphs indicate statistically significant differences between the two genotypes across the fixed ITI Stage of training. (h,n,o,p) Lines indicate statistically significant difference between the two genotypes.

* *P*<0.05, ** *P*<0.01, *** *P*<0.001. *n.s.,* non-significant. N = 12 per group.

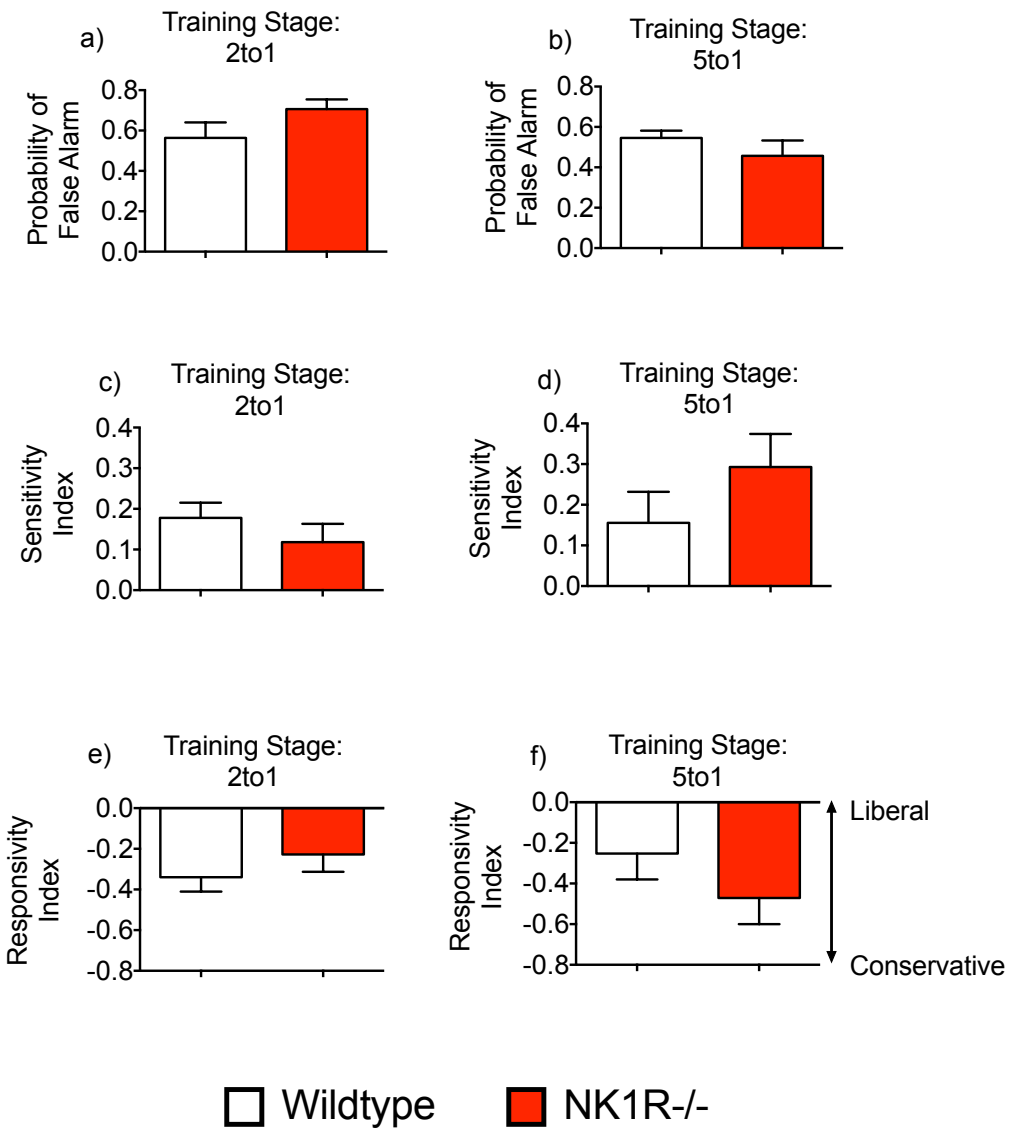

Figure 6.5. Training in the 5-CCPT: false alarms, sensitivity index and responsivity index.
Bars show mean±s.e.m. N = 12 per group.

6.3.2. 5-CCPT (extended 5to1 VITI test session): uninjected NK1R-/- mice do not display a higher incidence of premature responding, false alarms or omissions in the extended test session

Genotype differences in *%premature responses* depended on whether animals were tested in the morning or afternoon ($F(1,20)=5.47$; $P=0.03$; Fig. 6.6a): in the morning, wildtype mice approached

significance for carrying out a greater number of *premature responses* than NK1R-/- mice (*P*=0.072), whilst in the afternoon there was no genotype difference in *premature responses*. Wildtypes tested in the morning also carried out more *premature responses* than wildtypes tested in the afternoon (LSD: *P*=0.042). As in training, there was no genotype difference in *%omissions* (Fig. 6.6b), but *perseveration* was greater in NK1R-/- mice compared with wildtype mice (F(1,22)=27.65; *P*<0.001; Fig. 6.6c).

There was no genotype difference in *%accuracy* (Fig. 6.6d). However, *total trials* was greater in NK1R-/- mice compared with wildtypes (F(1,22)=6.8; *P*=0.016; Fig. 6.6e). Similarly, both *latency to correct response* (F(1,22)=8.38; *P*=0.0084; Fig. 6.6f) and *latency to reward* (F(1,22)=5.95; *P*=0.0232; Fig. 6.6g) were greater in NK1R-/- mice, but for both the effect size was small (c. 0.1 s).

The *probability of a false alarm* was lower in NK1R-/- mice compared with wildtypes (F(1,22)=5.31; *P*=0.031; Fig. 6.7a). By contrast, the *sensitivity index* was greater in NK1R-/- mice compared with wildtypes (F(1,22)=4.46; *P*=0.046; Fig. 6.7b). A genotype difference in the *responsivity index* just missed the criterion for significance (F(1,22)=3.72; *P*=0.067; Fig. 6.7c), with NK1R-/- mice tending towards a more 'conservative' response strategy than wildtypes.

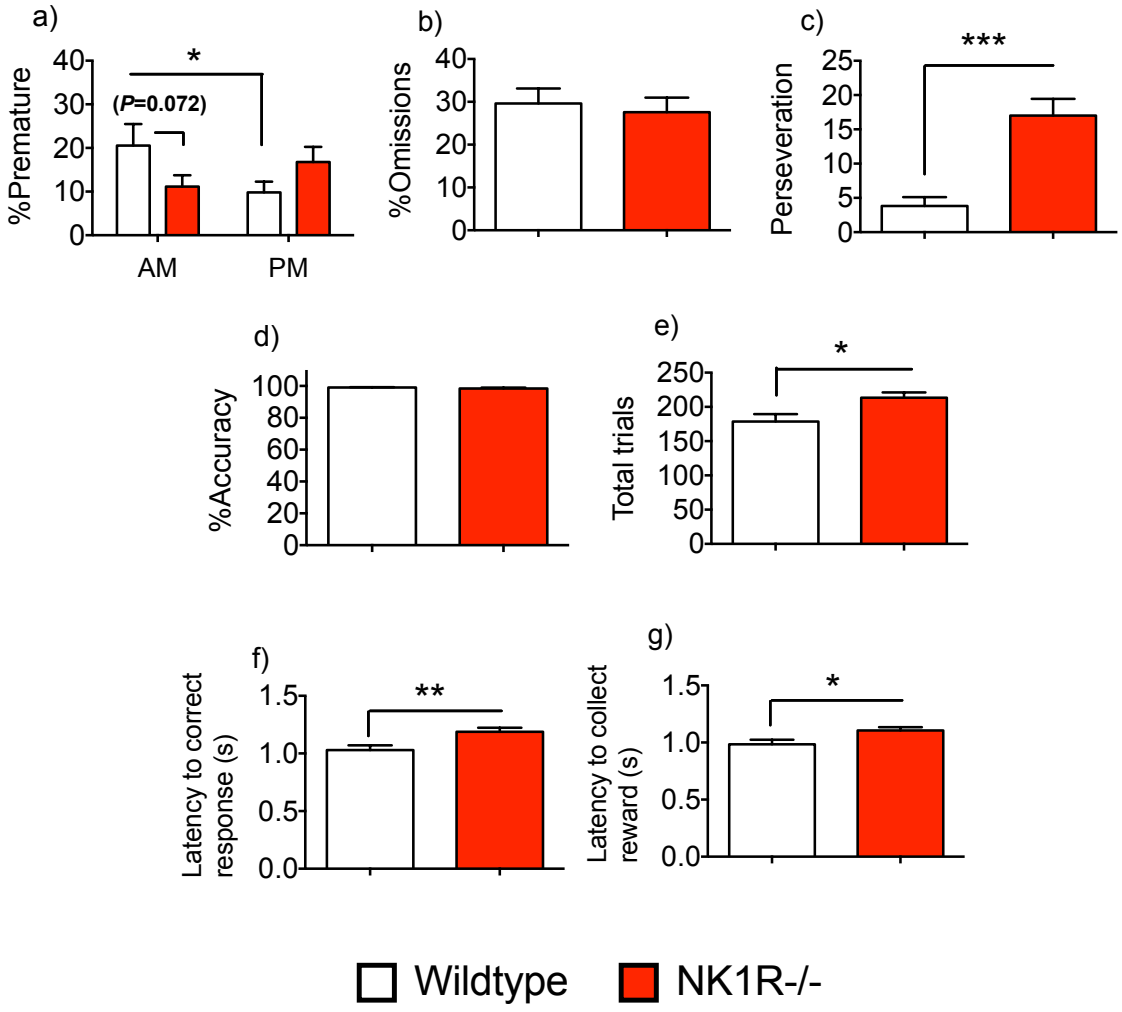

Figure 6.6. Testing in the 5-CCPT - baseline (uninjected) performance: premature responses, omissions, perseveration, accuracy, total trials and latencies.
Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * *P*<0.05, ** *P*<0.01, *** *P*<0.001. N = 12 per group.

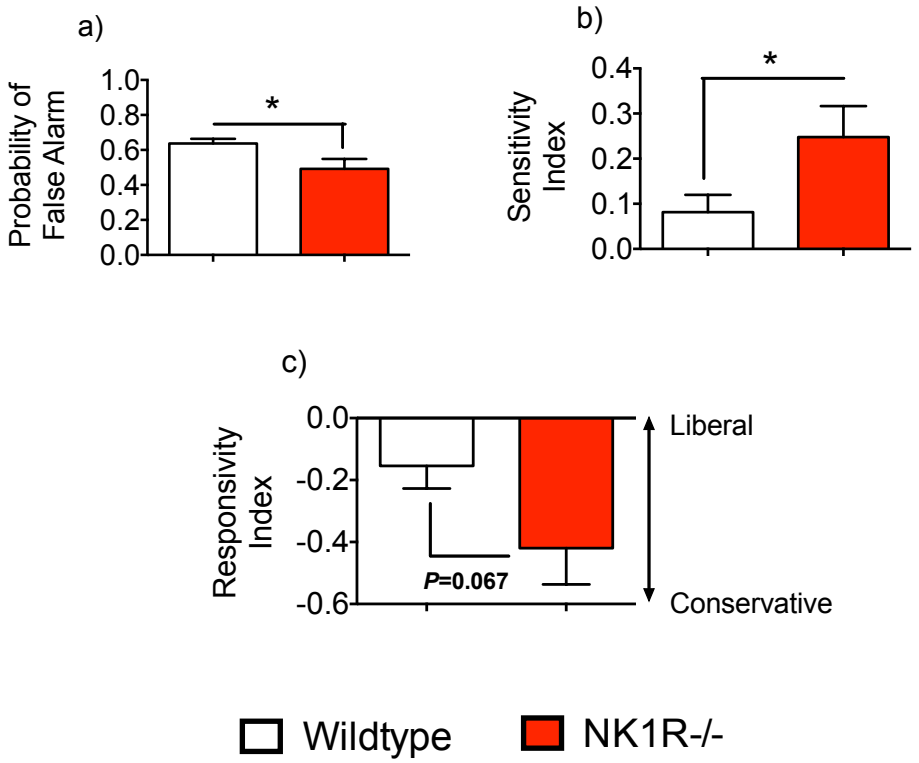

Figure 6.7. Testing in the 5-CCPT - baseline (uninjected) performance: false alarms, sensitivity index and responsivity index.
Bars show mean±s.e.m. Lines indicate statistically significant differences between groups. * *P*<0.05, ** *P*<0.01, *** *P*<0.001. N = 12 per group.

6.3.3. 5-CCPT (within-session performance)

The incidence of *%premature responses* decreased over the course of the test session, in both genotypes (F(4,64)=30.72; *P*<0.001). However, as above, genotype differences depended on whether animals were tested in the morning or afternoon (F(1,20)=5.21; *P*=0.034): in the morning, *%premature responses* were greater in wildtype mice than NK1R-/- mice, during the first bin (LSD: *P*=0.015; Fig 6.8a); in the afternoon, *%premature responses* were greater in NK1R-/- mice compared with wildtype mice, during the second bin (LSD: *P*=0.023; Fig 6.8b).

%Omissions also changed over the course of the test session (F(4,72)=2.63; *P*=0.041), and this differed in wildtype and NK1R-/- mice (F(4,72)=4.78; *P*=0.002; Fig 6.8c). For the majority of the

test session, *%omissions* did not differ in the two genotypes, but in the final bin *%omissions* were higher in wildtype mice (LSD: *P*<0.001). Despite a Genotype*TrialBin interaction for *%accuracy* (F(4,71)=2.91; *P*=0.028), *post-hoc* (LSD) tests for this behaviour only reached trends to significance (Fig. 6.8d).

Finally, the *probability of false alarm* decreased over the course of the test session, in both genotypes (F(4,71)=4.26; *P*=0.004; 6.8e). An overall genotype difference just missed the criterion for significance (F(1,22)=3.44; *P*=0.077), with a trend towards wildtypes displaying a higher *probability of false alarm* during the earlier bins but not later bins.

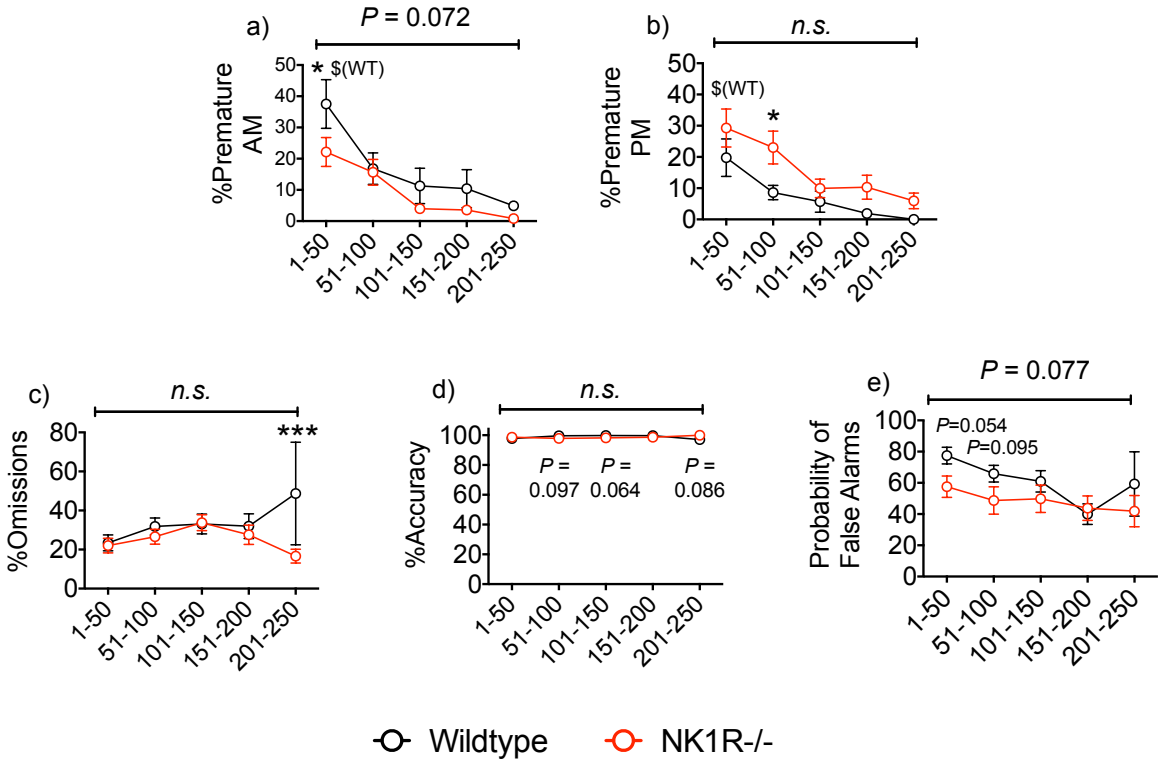

Figure 6.8. Within-session performance: testing
Circles show mean±s.e.m. Numbers underneath graph indicate bins of trials.
* *P*<0.05 (Genotype comparison). $ *P* <0.05 (Time-of-Day comparison)

6.3.4. 5-CCPT (effects of losartan): AT_1 receptor antagonism does not influence the performance of NK1R-/- mice in the extended test session

There was no effect of losartan on either *%premature responses* (F(4,83)=0.57; *P*=0.683; Fig. 6.9a) or *%omissions* (F(4,83)=0.78; *P*=0.5405; Fig. 6.9b) when animals were tested in the extended 5to1 test session. An overall increase in *%premature responses* in NK1R-/- mice just missed the criterion for significance (F(1,21)=3.04; *P*=0.0958), but there was no genotype differences in *%omissions* (F(1,21)=0.76; *P*=0.3941). Losartan also did not affect *perseveration* (F(4,83)=0.107; *P*=0.3762; Fig. 6.9c). However, as in training, *perseveration* was greater in NK1R-/- mice, overall (F(1,21)=6.82; *P*=0.0163).

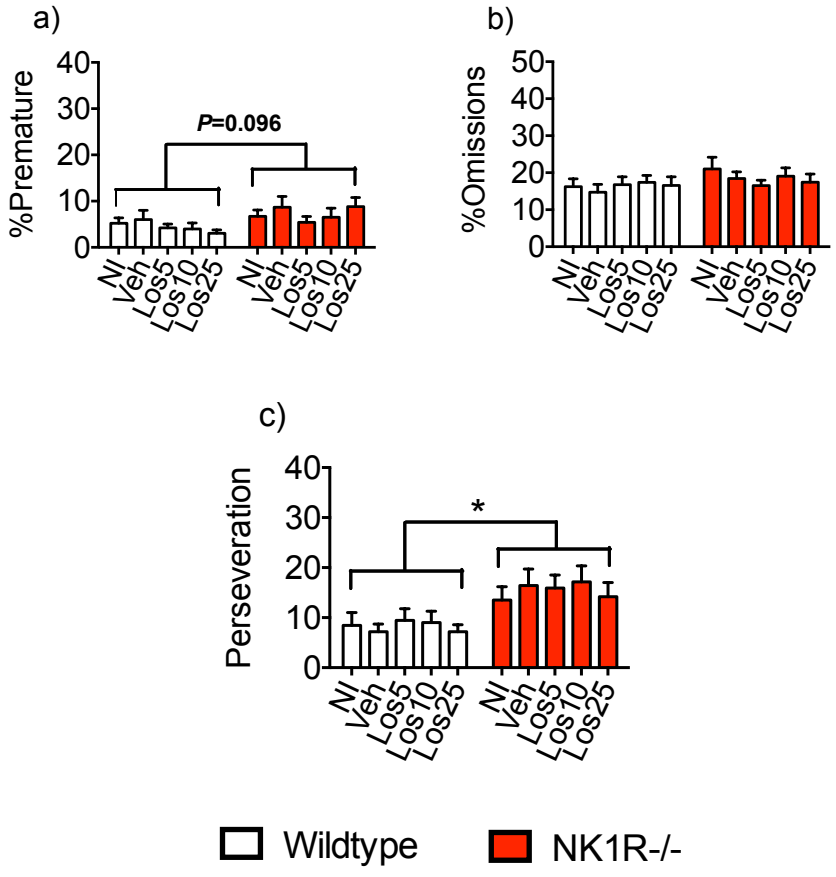

Figure 6.9. Testing in the 5-CCPT – effects of losartan: premature responses, omissions and perseveration.
Bars show±s.e.m. **P*<0.05 N = 11 – 12 per group.

%Accuracy and *total trials* were similarly unaffected by losartan (F(4,83)=0.81; *P*=0.5203 and F(4,83)=1.33; *P*=0.2652, respectively; Fig. 6.10a,b). *%Accuracy* also did not differ in the two genotypes (F(1,21)=2.3; *P*=0.1443), but *total trials* were greater in NK1R-/- mice, overall, compared with wildtypes (F(1,21)=12.38; *P*=0.002). Both *latency to correct response* and *latency to reward* were also unaffected by losartan (F(4,83)=0.72; *P*=0.5781 and F(4,83)=0.55; *P*=0.6969, respectively; Fig. 6.10c,d), but both were greater in NK1R-/- mice, overall (F(1,21)=13.12; *P*=0.0016 and F(1,21)=9.44; *P*=0.0058, respectively).

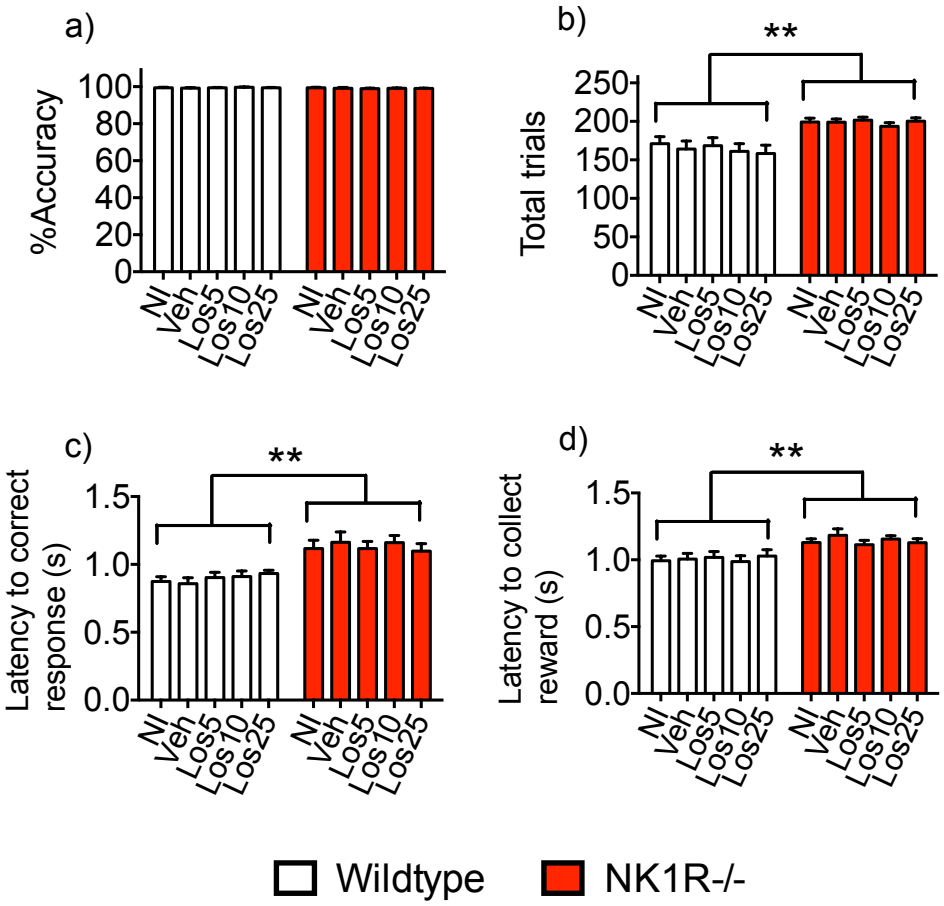

Figure 6.10. Testing in the 5-CCPT – effects of losartan: accuracy, total trials and latencies

Bars show±s.e.m. **P<0.01 N = 11 – 12 per group.

Despite a genotype*drug interaction, there was no clear effect of losartan on either the *probability of false alarm* (F(4,83)=3.18; *P*=0.0177; Fig. 6.11a) or the *sensitivity index* (F(4,83)=2.64; *P*=0.0394 Fig. 6.11b): the *probability of false alarm* was greater in wildtype mice compared with NK1R-/- mice when treated with 5 mg/kg losartan (LSD: 0.0154) but not other doses, whilst the *sensitivity index* was greater in wildtype mice treated with 25 mg/kg losartan compared with wildtype mice treated with 5 mg/kg losartan (LSD: 0.048). There was no effect of losartan on the *responsivity index* (Fig. 6.11c).

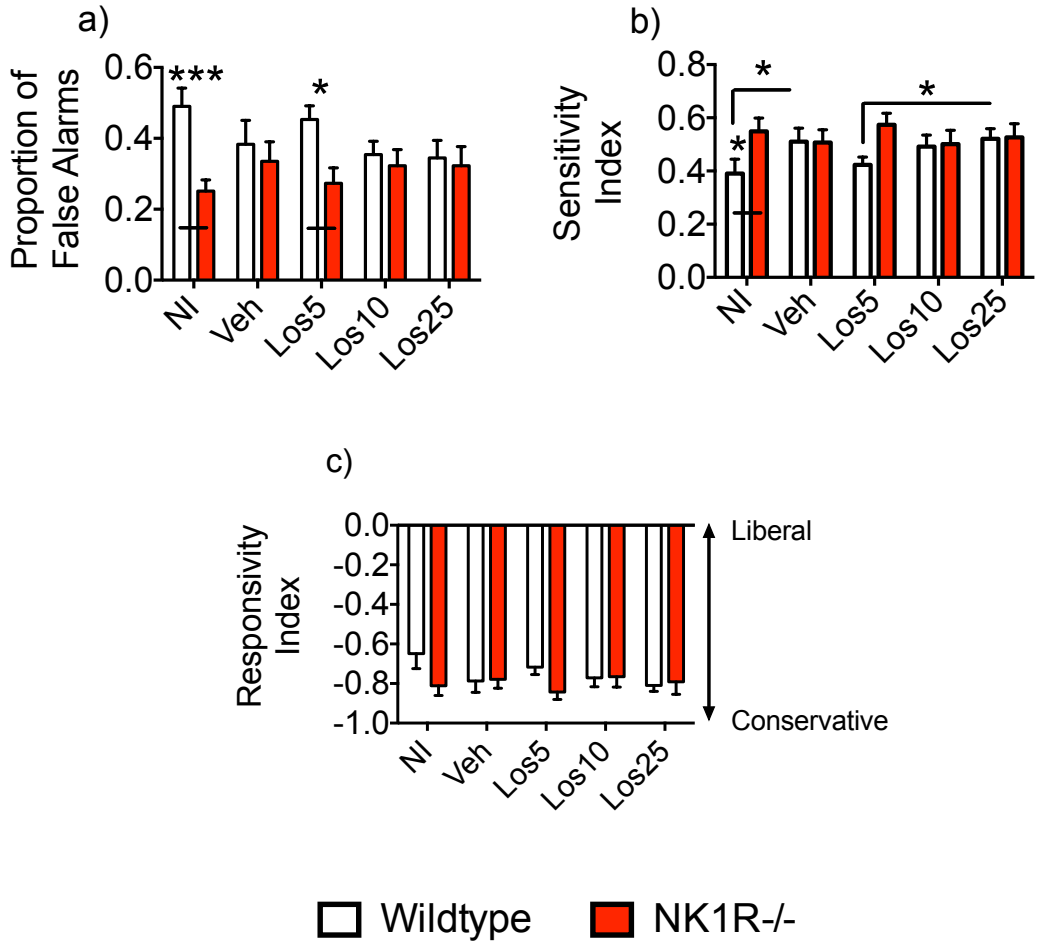

Figure 6.11. Testing in the 5-CCPT – effects of losartan: false alarms, sensitivity index and responsivity index.
Bars show±s.e.m. **P*<0.05, ****P*<0.001. N = 11 – 12 per group.

6.3.5. NOL / NOR tests

6.3.5.1. AT_1 receptor antagonism disrupts the performance of both genotypes in the NOL test

Overall, both genotypes spent more time exploring the moved object compared with the stationary object (main effect of Novelty: F(1,24)=45.43; *P*<0.001; Fig. 6.12a). This difference in time spent exploring the moved and stationary objects did not differ in the two genotypes. Treatment with losartan decreased the amount of time both genotypes spent exploring the moved object so that there was no longer any difference between the moved and stationary objects (Treatment*Novelty interaction: F(3,24)=7.55; *P*=0.001; LSD (*c.f.*, vehicle): *P*=0.001 (10mg/kg), *P*=0.002 (25 mg/kg); Fig. 6.12a). Similarly, in both genotypes, treatment with losartan reduced the discrimination index for time exploring the two objects (main effect of Treatment: F(3,28)=5.05; *P*=0.006; LSD (*c.f.*, vehicle): *P*=0.002 (10 mg/kg); *P*=0.027 (25 mg/kg); Fig. 6.12b).

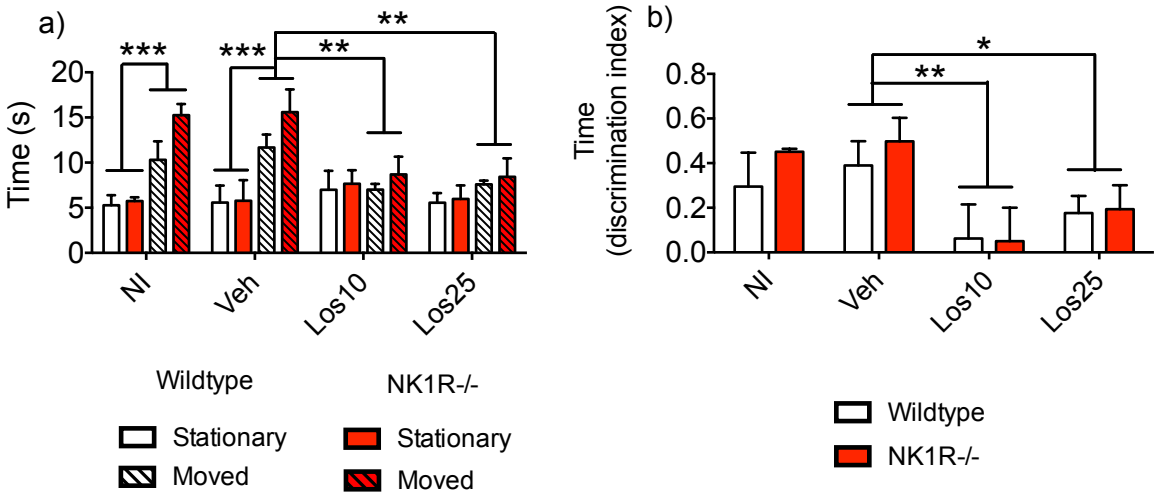

Figure 6.12. Novel Object Location test: (a) Time spent exploring each object; and (b) discrimination index. Bars show±s.e.m. N = 4 per group. Lines indicate statistically significant differences between groups. **P*=0.05, ***P*=0.01, ****P*=0.001; *n.s.*, non-significant; NI, no injection; Veh, vehicle (saline); Los10, losartan 10 mg/kg; Los25, losartan 25 mg/kg.

6.3.5.2. AT_1 receptor antagonism does not affect the performance of either genotype in the NOR test

Again, both genotypes spent more time exploring the novel object compared with the familiar object (main effect of Novelty: F(1,30)=27.56; *P*<0.001; Fig. 6.13a). However, NK1R-/- mice spent more time exploring objects, overall (main effect of Genotype: F(1,30)=5.55; *P*=0.025; Fig. 6.13a), particularly time spent exploring the novel object (LSD: *P*=0.01). Losartan did not affect performance in this test, in either genotype (main effect of Treatment: F(3,24)=0.24; *P*=0.869). In line with this, losartan did not affect the discrimination index, in either genotype (main effect of Treatment: F(3,24)=0.35; *P*=0.786; Fig. 6.13b).

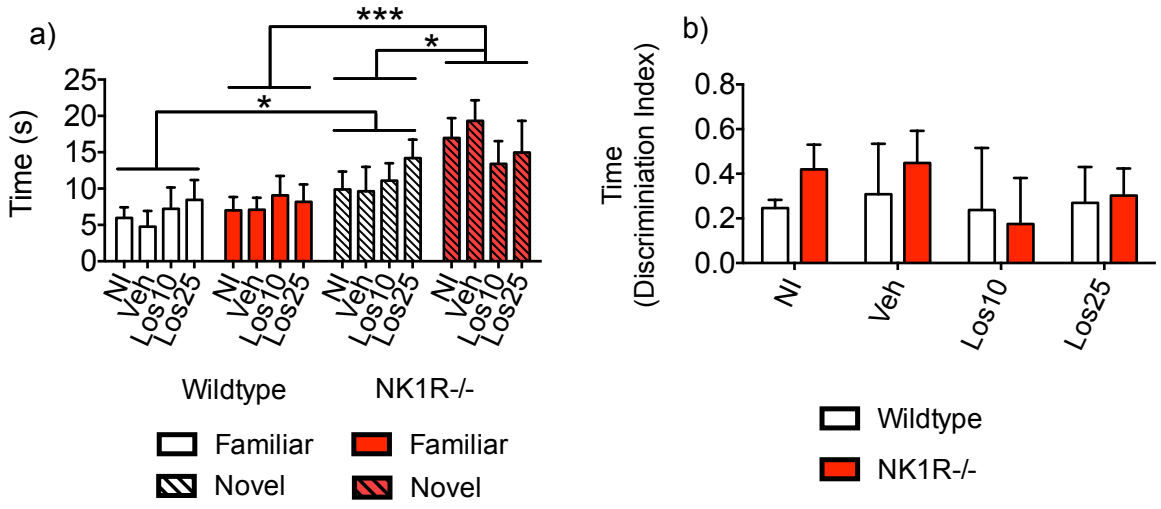

Figure 6.13. Novel Object Recognition test: (a) Time spent exploring each object; and (b) discrimination index. Bars show±s.e.m. N = 4 per group. Lines indicate statistically significant differences between groups. **P*=0.05, ****P*=0.001; *n.s.*, non-significant; NI, no injection; Veh, vehicle (saline); Los10, losartan 10 mg/kg; Los25, losartan 25 mg/kg.

6.4. Discussion

6.4.1. Baseline (uninjected) performance of NK1R-/- mice in the 5-Choice Continuous Performance Test

This is the first study in which NK1R-/- mice have been trained and tested using the 5-CCPT, a modified version of the 5-CSRTT that is argued to be more analogous to human CPTs used in the evaluation of ADHD symptoms (Young et al., 2009). By using both 'Go' and 'No-go' trials, this test provides a number of additional performance variables that are not measured in the 5-CSRTT. These include the *probability of false alarm* (an index of 'behavioural disinhibition', an alternative measure of impulsivity), the *sensitivity index* (an index of 'vigilance', an alternative measure of attentional performance), and *response bias* (an index of an animal's 'tendency to respond').

The first aim was to determine the baseline (uninjected) performance of these animals in this test. NK1R-/- mice displayed a higher incidence of *premature responses* only during the early stage of the training procedure, when the ITI was fixed. During subsequent stages of training, and when tested in the extended test session, there was either no genotype difference or this behaviour was greater in wildtype mice. Similarly, the *probability of false alarm* did not differ in the two genotypes throughout training, but was greater in wildtype mice in the extended test session. Therefore, overall, NK1R-/- mice did not display an impulsive phenotype in the 5-CCPT.

It is widely argued that 'impulsivity' is not a unitary construct, but instead encompasses a variety of behaviours with distinct underlying neurobiological mechanisms (Evenden, 1999; Moeller et al., 2001; see: Winstanley et al., 2006). In the 5-CCPT, premature responses and false alarms represent different types of impulsivity (motor impulsivity and behavioural disinhibition, respectively; Young et al., 2009) and there is evidence that these two behaviours are regulated by different neuronal networks. For instance, mice with reduced dopamine D4 receptor expression ('DRD4+/-') exhibit a higher incidence of false alarms, but not premature responses, compared

with wildtype controls; by contrast, administration of a $5HT_{2C}$ antagonist increases the incidence of premature responding but not false alarms (Young et al., 2011). Also, in High Impulsive rats, treatment with methylphenidate does not affect false alarms but reduces premature responses, whereas treatment with atomoxetine improves both of these measures (Tomlinson et al., 2014). Functional MRI data from a reverse-translated human version of the 5-CCPT also suggest that 'Go' and 'No-go' trials activate distinct brain regions: in Go trials, areas such as the basal ganglia, thalamus, inferior parietal lobe, and premotor cortex are activated; in No-go trials, areas such as the inferior frontal cortex, inferior parietal lobe, pre-supplementary motor area, and premotor cortex are activated (McKenna et al., 2013).

The fact that NK1R-/- mice did not display a higher incidence of false alarms in this test suggests that 'behavioural disinhibition' is not exacerbated by a lack of functional NK1R. Reports from both human and animal studies indicate that different measures of impulsivity do not correlate within individuals (e.g., McDonald et al., 2003; Winstanley et al., 2004), and so it is possible that polymorphisms of the *TACR1* gene contribute to some forms of impulsivity (e.g., motor impulsivity, as in the 5-CSRTT) but not others. However, this interpretation is limited by the observation that, in this study, NK1R-/- mice did not display a higher incidence of premature responses, either, making it difficult to suggest that a deficit in functional NK1R contributes to 'motor impulsivity' but not 'behavioural disinhibition'.

An obvious explanation is that there are procedural differences in the 5-CCPT that result in the loss of impulsivity in this genotype; such differences could also underlie the lack of impulsivity seen in NK1R-/- mice in terms of false alarms. One notable difference between the 5-CSRTT and 5-CCPT is the duration of the test session. Testing in the 5-CCPT involves extending the task to 60 min (or 250 trials). This is double the length of time used in the VITI of the 5-CSRTT, which is 30 min (or 100 trials). It is striking that *total trials* were higher in NK1R-/- mice compared wildtype

mice in this test because, in the 5-CSRTT, this behaviour does not differ in the two genotypes (Yan et al., 2011; Dudley et al., 2013). It is possible that wildtypes are less motivated to carry out the task for the entire duration of the test session than NK1R-/- mice. This could, for example, be due to differences in satiety in the two genotypes in response to the high volume of liquid milk delivered over the 250 trials which, in turn, would mask genotype differences in other performance variables during earlier trials of the test session. However, this possibility was investigated by splitting the test session into bins of trials and this revealed that NK1R-/- mice were not impulsive at the earlier stages of the test session, either.

A further difference between the two tests is that animals in the 5-CSRTT are trained on a fixed ITI, whereas animals in the 5-CCPT are trained on a variable ITI. In other words, animals in the 5-CCPT are repeatedly exposed to a VITI schedule before they are tested. This is important because, in a previous study from this laboratory, repeated exposure to a VITI schedule reduced the incidence of premature responses in NK1R-/- mice and abolished the genotype difference in this behaviour (Weir et al., 2014). Similar findings have also been reported in other strains, with repeated exposure to the VITI reducing the incidence of premature responding in C57BL/6J mice (Walker et al. 2011). A possible explanation for this phenomenon is that repeated exposure to the VITI schedule shifts the variable nature of the test condition from “unexpected uncertainty” (as in the 5-CSRTT) to “expected uncertainty” (as in the 5-CCPT). There is considerable evidence that these two conditions recruit different neuronal processes, which are broadly thought to involve noradrenaline and acetylcholine, respectively (see: Yu and Dayan, 2005; Sarter et al., 2014). It is possible that the impulsivity of NK1R-/- mice is dependent on the “unexpected uncertainty” of the task parameters. To investigate this possibility, it would be interesting to train NK1R-/- mice in the 5-CCPT using a fixed ITI schedule, to see whether the higher incidence of premature responses

typically seen in this genotype is reinstated during the first exposure to the VITI extended test session.

Another interesting finding from this study is that, when tested, NK1R-/- mice demonstrated a higher *sensitivity index* than wildtype mice. In CPTs, this indicates a subject's ability to distinguish between different types of stimuli and is considered an index of 'vigilance', or an alternative measure of attentional performance (Riccio et al., 2002; Young et al., 2009). Patients with ADHD have lower scores for this behaviour compared with healthy controls (Epstein et al., 2003), but this is improved by treatment with both *d*-amphetamine and methylphenidate (Sostek et al., 1980; Weingartner et al., 1980; Klorman et al., 1988). In preclinical research, several studies have investigated the effects of pharmacological manipulation on sensitivity index scores, and implicated the glutamatergic (Barnes et al., 2012a), dopaminergic (Barnes et al., 2012b) and cholinergic systems (Young et al., 2013). Results from this study suggest that a deficit in functional NK1R does not disrupt this behaviour, but rather may improve it. This further suggests that any deficits in this performance measure in ADHD patients are not due to polymorphisms of the *TACR1* gene.

Finally, the *responsivity index* refers to an animal's overall 'tendency to respond'. During training, this behaviour did not differ in the two genotypes. During testing, a genotype difference just missed the criterion for significance, with NK1R-/- mice tending towards more 'conservative' responding compared with wildtypes. Given that these animals show an impulsive phenotype (in the 5-CSRTT, at least), this result could be considered counterintuitive. Nevertheless, it is striking that a 'conservative' response strategy is (possibly) evident in a genotype that also frequently exhibit a higher incidence of perseverative responding (Yan et al., 2011; Dudley et al., 2012; Weir et al., 2014), which, as described previously, may represent a form of 'checking' behaviour.

6.4.2. Effects of AT_1 receptor antagonism on performance in the 5-Choice Continuous Performance Test

The second aim of the experiments reported in this chapter was to determine the effects of the AT_1 receptor antagonist, losartan, on performance in the 5-CCPT. This was prompted by the observation that the ACE inhibitor, captopril, improved the performance of NK1R-/- mice in the 5-CSRTT. There is also evidence that AT_1 receptor antagonism has cognitive-enhancing effects that can be dissociated from its effects on blood pressure (Tedesco et al., 1999; Fogari et al., 2003; Mechaeil et al., 2011). However, treatment with losartan was largely ineffective in the 5-CCPT. Losartan did not affect *%premature responses*, *%omissions*, *perseveration* or the *responsivity index*. Furthermore, there was no clear effect of losartan on either the *probability of false alarm* or the *sensitivity index*. Overall, these results indicate that AT_1 receptors do not play a role in any of the behaviours measured in the 5-CCPT.

Losartan has shown cognitive-enhancing effects in a number of preclinical behavioural tests. These include, for example, a passive avoidance step-down task (Raghavendra et al., 1998b) and a radial arm maze task (Srinivasan et al., 2005). To the best of my knowledge, this is the first time that the effects of an AT_1 receptor antagonist have been investigated using the 5-CCPT, or other related tests. The fact that losartan did not affect either *%omissions* or the *sensitivity index* suggests that AT_1 receptors do not play a role in attentional performance, or 'vigilance'. However, *%omissions* also did not differ in the two genotypes, at any point during either training or when tested in the extended test session. It is possible that any beneficial effect of AT_1 receptor antagonism on this behaviour relies on a deficit in performance at baseline.

Treatment with losartan also did not affect *%premature responses*, and there was no clear effect of drug treatment on the *probability of false alarm*. There do not appear to be any previous reports investigating the effects of AT_1 receptor antagonism on impulse control, but results

presented here suggest that these receptors do not influence impulsive behaviour. There is some evidence that AT_1 receptors may be involved in the regulation of other neurotransmitters implicated in impulsivity: for instance, treatment with another AT_1 receptor antagonist, candesartan, for one week via drinking water increased serotonin concentration in the PFC (Jenkins, 2008). This could indicate that chronic treatment is necessary for AT_1 receptor antagonism to produce an effect on impulsive behaviour. As with attentional performance, it is also possible that any effect of AT_1 receptor antagonism on impulsivity relies on a deficit in performance at baseline.

The possibility that it is this particular batch of losartan that is ineffective can be ruled out. This is because the same batch was also used in the NOL study, which spanned the duration of the training / testing procedure of the 5-CCPT, and treatment with losartan influenced performance in the NOL test (discussed below). Overall, results from this study reveal that AT_1 receptor antagonism does not influence performance in the 5-CCPT. If so, this would suggest that the improvement seen in NK1R-/- mice treated with captopril is not due to a reduction in AT_1 receptor activation. As discussed in Chapter 5, a number of other possible explanations for the effects of captopril are possible, especially changes in the availability of other angiotensin peptide fragments, or effects on other neuropeptides that are targeting by ACE. Nonetheless, it would be interesting to investigate the effects of losartan on the performance of NK1R-/- mice in the 5-CSRTT, to see whether AT_1 receptor antagonism has any effect on the performance of these animals when they demonstrate a deficit at baseline.

6.4.3. The effects of AT_1 receptor antagonism on the performance of NK1R-/- mice in tests of memory

The final aim of the experiments reported in this chapter was to determine the effects of losartan on the performance of NK1R-/- mice in two tests of memory: the novel object recognition (NOR) and novel object location (NOL) tests. This was prompted by evidence for memory deficits in ADHD patients (Castellanos and Tannock, 2002; Frazier et al., 2004; Westerberg et al., 2004), and the fact that AT_1 receptors in particular have been implicated in the process of long-term potentiation (LTP) (see: von Bohlen und Halbach and Albrecht, 2006). In both tests, wildtype and NK1R-/- mice spent more time at the novel/moved object compared with the familiar/stationary object. In the NOR test, performance was unaffected by treatment with losartan. However, in the NOL test, losartan reduced the amount of time both genotypes spent at the moved (but not stationary) object. Combined, these results show that a deficit in NK1R does not disrupt performance in either of these two tests, and that AT_1 receptors may be involved in the processes underlying spatial memory.

Memory can be broadly divided into two categories: declarative and non-declarative. The NOR and NOL tests both fall within the former, which is thought to depend on an interconnected network within the medial temporal lobe (Cohen and Stackman, 2014). However, the relative contribution of different brain structures within this network is thought to differ in these two tests. Specifically, the mechanisms underlying “object-place associations”, as occurs in the NOL test, are highly dependent on the hippocampus (Mumby et al., 2002; Gilbert and Kesner, 2004; Winters et al., 2004), whereas “object recognition”, as occurs in the NOR test, is thought to be primarily dependent on the perirhinal cortex (Zhu et al., 1995; Brown and Xiang, 1998; Winters et al., 2004; Winters and Bussey, 2005; see: Dere et al., 2005). The fact that NK1R-/- mice did not display performance deficits in either of these two tests suggests that NK1R do not play a role in either of

these types of memory. This proposal in line with a previous report in which NK1R-/- mice did not show a deficit in several other tests of hippocampal-dependent learning, such as contextual fear conditioning or the Morris water maze (Morcuende et al., 2003).

In the NOL test, treatment with losartan reduced the amount of time both genotypes spent exploring the moved object relative to the stationary object. This indicates that activation of AT_1 receptors may be involved in the mechanisms underlying spatial memory. There are several previous reports that intracerebroventricular administration of AngII has memory-enhancing effects, including in tests of spatial memory (Georgiev and Yonkov, 1985; Braszko et al., 1987, Braszko et al., 1988). There is also evidence that AT_1 receptors are expressed within the hippocampus (Tonelli et al., 2000; von Bohlen und Halbach and Albrecht, 1998, 2006), and that the cognitive-enhancing effects of AngII administration can be prevented by AT_1 receptor antagonism (Braszko, 1996; Kulakowska et al., 1996).

The precise role of both AngII and AT_1 receptors in learning and memory remains poorly understood, and previous reports are somewhat inconsistent. For instance, unlike the above examples, other studies have reported that AngII administration to the hippocampus disrupts, rather than improves, cognitive performance in tests of memory (Lee et al., 1995; Kerr et al., 2005). Another report has found that AngII-deficient mice do not display spatial memory deficits (Walther et al., 1999). Furthermore, AT_1 receptor antagonism has previously been shown to either have no effect (Chalas and Conway, 1996; Shepherd et al., 1996) or to actually improve (Sharma and Singh, 2012) performance in spatial memory tasks. Whatever the case, because losartan affected both genotypes, any such effect evidently occurs through an NK1R-independent mechanism.

One possible explanation for these discrepancies is that the cognitive-enhancing effects originally attributed to AngII are actually due to its conversion to other neurologically active peptides, in particular AngIV (Brazsko et al., 2006). This peptide has cognitive-enhancing effects in

several preclinical behavioural tests (see: Gard, 2008). For example, intracerebroventricular administration of AngIV improves performance in the plus-maze spontaneous alternation task, a test of working spatial memory (De Bundel et al., 2010). Interestingly, this effect was prevented by treatment with an AT_1 receptor antagonist, suggesting that AngIV improves spatial memory through an AT_1 receptor-mediated mechanism. Therefore, it is possible that the disruption to task performance following treatment with losartan in the NOL test is due to a reduction in activation of AT_1 receptor by AngIV, rather than AngII.

It is widely believed that the cellular mechanisms underlying learning and memory involve a process called long-term potentiation (LTP), a specific form of synaptic plasticity. A number of studies have demonstrated that AngII and its related peptides influence LTP (see: Wright et al., 2002; von Bohlen und Halbach and Albrecht, 2006). For example, administration of AngII inhibits LTP in both the hippocampus (Denny et al., 1991) and the amygdala (von Bohlen und Halbach and Albrecht, 1998b), both of which are prevented by AT_1 receptor antagonism (Armstrong et al., 1996; von Bohlen und Halbach and Albrecht, 1998b). AngII also inhibits a related process, long-term depression (LTD); this, again, is prevented by AT_1 receptor activation (Tchekalarova and Albrecht, 2007). By contrast, AngIV is thought to facilitate LTP, which, interestingly, is also prevented by AT_1 receptor antagonism (Wayner et al., 2001). Combined, these results strongly indicate that AngII and its related peptides regulate synaptic plasticity through an AT_1 receptor-mediated mechanism in neuronal networks implicated in spatial memory.

Finally, in contrast to the NOL test, there was no effect of losartan in the NOR test, in either genotype. This indicates that AT_1 receptors do not play a role in "object recognition" memory, which, as described above, is thought to be primarily mediated by the perirhinal cortex (Zhu et al., 1995; Winters et al., 2004; Winters and Bussey, 2005; see: Antunes and Biala, 2012; Kim et al., 2014). Published reports investigating the role of AT_1 receptors in the perirhinal cortex

are limited, although there is some evidence that AT_1 receptors are highly expressed in the perirhinal cortex during postnatal development but that receptor expression decreases into adulthood (Tonelli et al., 2000). There is also evidence that deficits in performance in the NOR test, induced by whole-brain irradiation, are prevented by both ACE inhibition (Lee et al., 2012) and AT_1 receptor antagonism (Conner et al., 2010). However, results from this study suggest that AT_1 receptors do not play a role in perirhinal cortex-dependent learning.

Overall, results from this study demonstrate that a deficit in NK1R does not disrupt performance in either the NOR or NOL test. This suggests that polymorphisms of the *TACR1* gene may not contribute to memory-deficits in ADHD patients (Castellanos and Tannock, 2002; Frazier et al., 2004; Westerberg et al., 2004). Results from this study also indicate that AT_1 receptors may be involved in the processes underlying certain types of memory (spatial) but not others (object-recognition). Although precise mechanisms have yet to be determined, this reveals that the NOR and NOL tests recruit different neuronal networks and suggests that AT_1 receptors play a role in one network but not the other. Therefore, the role of the BRAS and AT_1 receptors in different type of memory processes warrants further investigation.

6.4.4. Concluding remarks

In experiments reported in this chapter, the performance of NK1R-/- mice in the 5-CCPT was evaluated for the first time. The results indicate that, at baseline, NK1R-/- mice do not display either an impulsive or inattentive phenotype in this test. This could be due to any of a range of factors but differences in elements of the protocol used here and that used in the 5-CSRTT are evidently key. Results presented in this chapter also indicate that NK1R-/- mice do not display deficits in either the NOR or NOL test, suggesting that memory-deficits that have been reported in patients with ADHD are unlikely to involve polymorphisms of the *TACR1* gene. Finally, results

described in this chapter indicate that AT_1 receptors do not influence performance in the 5-CCPT, but that these receptors may play a role in the mechanisms underlying spatial memory.

Chapter 7

General Discussion

7.1. Background

Attention Deficit Hyperactivity Disorder (ADHD) is a common neuropsychiatric disorder with an estimated worldwide prevalence of 8 – 12% (Faraone et al., 2003). Two classification systems exist for the diagnosis of ADHD (Diagnostic and Statistical Manual of Mental Disorders and International Classification of Diseases), but in both, the disorder is characterised by three core behavioural symptoms: hyperactivity, impulsivity, and inattentiveness. Despite considerable advances in recent decades, the precise aetiology of ADHD remains unknown. Psychostimulants remain the first-line choice of treatment for the disorder, despite being ineffective in approximately 10 – 30% of patients and the fact that these drugs are associated with a number of adverse side effects (Banaschesk et al., 2004; Fone and Nutt, 2005). There remains an urgent need for a better understanding of the pathophysiology of ADHD and the development of therapeutic alternatives.

In neuroscience, a common approach for investigating the underlying cause(s) of a disorder is through the use of animal models. In particular, genetically modified animals are an extremely useful tool for investigating the role of specific proteins in health and disease. This thesis reports findings from a series of experiments that explored the behavioural and cognitive abnormalities of NK1R-/- mice, a relatively recently proposed murine model of ADHD (Yan et al., 2009). Previous reports have identified that these mice are typically hyperactive, inattentive, and impulsive compared with their wildtype controls ('face validity'; Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010, 2011; Dudley et al., 2013). Furthermore, these behavioural abnormalities can be ameliorated by drugs that are used to treat ADHD patients ('predictive validity'; Yan et al., 2010; Pillidge et al., 2014a; Pillidge et al., 2014b). Finally, these mice exhibit abnormalities in dopaminergic, noradrenergic and serotonergic function, echoing the disruption to monoaminergic function evident in ADHD patients ('construct validity'; Froger et al., 2001; Herpfer et al., 2005; Fisher et al., 2007; Guiard et al., 2007; Yan et al., 2010).

The first major theme of this thesis has been to further phenotype the NK1R-/- mouse model of ADHD by investigating the behavioural abnormalities of these mice, with particular reference to environmental influences, sex differences and their performance in previously untested behavioural paradigms. The second major theme has been to test the behavioural effects of drugs that target the brain renin angiotensin system (BRAS), following preliminary evidence that NK1R and the BRAS interact in the regulation of locomotor activity. The following section summarises the main findings of these studies and discusses their overall conclusions.

7.2. The behavioural phenotype of NK1R-/- mice: environmental influences, sex differences and performance in previously untested paradigms

There is extensive evidence that an individual's early life environment influences their risk of developing psychopathologies later in life. This includes ADHD, which is now widely believed to result from complex interactions between an individual's genetic background and environmental factors (Elia et al., 2014). All previous reports of behavioural abnormities in NK1R-/- mice have been carried on wildtype and NK1R-/- mice bred from separate, homozygous breeding pairs. Whilst such a breeding method has the ethical benefit of reducing over-breeding, it is possible that differences in the early life environment of the two genotypes (e.g., the quality of maternal care or interaction with littermates) could be influencing the behavioural phenotype of this model. To check this possibility, the first study in this thesis compared the behaviour of NK1R-/- mice bred from homozygous breeding pairs (*Hom* colony, as above) with that of NK1R-/- mice bred from heterozygous breeding pairs (*Het* colony) (see: Chapter 3).

The first experiment used home-cage activity sensors to monitor the locomotor activity of the animals across the entire 24 h cycle, and found that NK1R-/- mice, from both colonies, were hyperactive compared with their respective wildtypes. This suggests that this behaviour is a direct

consequence of dysfunctional NK1R, and is not influenced by environmental factors. It is also interesting that hyperactivity was evident during the 'late' dark phase of the 24 h cycle, echoing the delayed sleep onset pattern commonly found in ADHD patients (van Veen et al. 2010; Kooij and Bijlenga, 2013).

The second experiment went on to investigate the impulsivity, attentional performance and perseveration of the two colonies using the 5-CSRTT. Although NK1R-/- mice from both colonies were impulsive during training, only NK1R-/- mice from the *Hom* colony displayed a higher incidence of premature responses when tested in the VITI. This suggests that, unlike hyperactivity, the impulsivity of NK1R-/- mice results from an interaction between a lack of functional NK1R with other contributory factor(s). Neither colony of NK1R-/- mice displayed an inattentive phenotype in this study, so an interpretation of the role of environmental factors on this behaviour is more difficult. Nevertheless, the lack of colony differences at any time during either training or testing indicates that environmental influences have no bearing on this behaviour. Finally, perseveration did not differ in the two colonies at any stage of the training or testing procedure; NK1R-/- mice, from both colonies, were more perseverative than their respective wildtypes during both training and the VITI test. This suggests that, as with hyperactivity, this behaviour is also a direct consequence of a lack of functional NK1R.

This is the first study that has investigated the role of environmental influences on the behavioural phenotype of NK1R-/- mice. It is interesting that some behavioural abnormalities of this genotype are a direct consequence of dysfunctional NK1R (hyperactivity, perseveration and, possibly, inattentiveness) but that at least one (impulsivity) is the result of an interaction with other contributory factor(s). ADHD is a complex, multifaceted disorder, and it is highly likely that both genetic and environmental factors play a role in its aetiology (Elia et al., 2014). Results presented here highlight how genes and the environment may influence the various behavioural

symptoms of the disorder in different ways. Specifically, they suggest that polymorphisms of the *TACR1* gene, alone, can result in hyperactivity, whereas impulsivity relies on the contribution of other factors. This could have important implications for the search for biomarkers for ADHD, which could differ for each behavioural aspect of the disorder. Future studies could extend these findings by incorporating both male and female animals, to determine whether environmental influences affect male and female NK1R-/- mice in different ways.

The next experiment went on to compare the behaviour of male and female NK1R-/- mice (see: Chapter 4). This was prompted by the observation that male and female animals perform differently in a number of behavioural paradigms (see: Archer, 1975), and the fact that there are clear sex differences in the typical symptom profile of ADHD patients (Waddell and McCarthy, 2012). In light of this evidence, the locomotor activity of male and female wildtype and NK1R-/- mice was compared using the light/dark exploration box (LDEB).

Whilst male NK1R-/- mice were hyperactive compared with their wildtype counterparts, female NK1R-/- mice were not. Furthermore, the locomotor activity of male NK1R-/- mice was greater than that of their female counterparts. These results suggest that the influence of NK1R on locomotor activity differs in male and female animals, and, by inference, suggest that the effects of polymorphisms of the *TACR1* gene could similarly be sex-specific. As mentioned, typical symptom profiles differ in male and female ADHD patients, with males more commonly presenting as the Predominantly Hyperactive/Impulsive subtype and females more commonly presenting with the Predominantly Inattentive subtype (Waddell and McCarthy, 2012). Results from this study suggest that polymorphisms of the *TACR1* gene could contribute to these differences. In light of this evidence, it would be interesting to investigate the performance of female

NK1R-/- mice in the 5-CSRTT, in particular to determine whether female NK1R-/- mice display an inattentive phenotype in this test.

In a subsequent experiment reported in this thesis, NK1R-/- mice were trained and tested in the 5-CCPT, an adapted version of the 5-CSRTT that is argued to be more analogous to human CPTs used in the evaluation of ADHD symptoms (Young et al., 2009; Huang-Pollock et al., 2012) (see: Chapter 6). Through the use both 'Go' and 'No-go' trials, this test captures multiple aspects of impulsivity simultaneously (premature responses and false alarms) as well as other performance variables not measured in the 5-CSRTT (sensitivity index and responsivity index). Overall, NK1R-/- mice did not display either an impulsive or inattentive phenotype in this test and, perhaps counter-intuitively, displayed both an increased sensitivity index and more 'conservative' response strategy than wildtype mice.

These results could indicate that polymorphisms of the *TACR1* gene contribute to some forms of impulsivity (e.g., 'motor impulsivity') but not others (e.g., 'behavioural disinhibition'). This is in line with evidence that different types of impulsivity do not correlate within individuals (e.g., McDonald et al., 2003; Winstanley et al., 2004) and the proposal that each type of impulsivity has different underlying neurobiological mechanisms (Evenden, 1999; Moeller et al., 2001; see: Winstanley et al., 2006). However, since NK1R-/- mice did not display a higher incidence of premature responses in this test, either, it is also possible that differences in the protocol of the 5-CCPT (compared with the 5-CSRTT) resulted in the loss of impulsivity in this genotype. In particular, the use of a variable ITI schedule, during training, repeatedly exposed animals to an unpredictable stimulus before they were tested, and it is possible that this caused the loss of impulsivity in this genotype. Future studies could investigate this possibility by training NK1R-/- mice in the 5-CCPT using a fixed ITI schedule, so that their first exposure to an

unpredictable stimulus occurs during the test session. However, the impulsivity of NK1R-/- mice is not invariable in the 5-CSRTT, either (Yan et al., 2011; Dudley et al., 2013; Pillidge et al., 2014a) and dissipates on repeated exposure to the VITI (Weir et al., 2014). In any case, ADHD is associated with high levels of intra-individual variability in various neuropsychological tests (e.g., de Zeeuw et al., 2008; Christiansen and Oades, 2009). Consequently, replication of this study, using the current 5-CCPT protocol, would also be advisable.

Finally, memory deficits are evident in some ADHD patients (see: Castellanos and Tannock, 2002; Frazier et al., 2004). For example, patients perform poorly in tests of verbal (Gau and Chiang, 2013) or visuo-spatial (Westerberg et al., 2004) working memory. Prompted by these observations, NK1R-/- mice and their wildtype counterparts were tested in two tests of memory: the novel object location (NOL) and novel object recognition (NOR) tests (see: Chapter 6). These studies provided no evidence for either spatial or object-recognition memory deficits in NK1R-/- mice, since their performance did not differ from wildtype mice in either test (see: Chapter 6). Consequently, this suggests that the memory deficits observed in some ADHD patients are not caused by polymorphisms of the *TACR1* gene. Results from this study are in line with a previous report in which NK1R-/- mice did not display memory deficits in several other tests of hippocampal-dependent learning (Morcuende et al., 2003). Nonetheless, since different types of memory are thought to be mediated by different neuronal networks (e.g., Gilbert and Kesner, 2004; Winters et al., 2004; Winters and Bussey, 2005), it would be interesting to extend these findings by investigating the role of NK1R in other tests of memory, such as a radial arm maze or a conditioned avoidance response test.

7.3. The effects of ACE inhibition and AT receptor antagonism on the behavioural abnormalities of NK1R-/- mice: implications for the treatment of ADHD

The brain renin angiotensin system (BRAS) has received considerable interest in recent years in relation to both motor control and cognitive performance (Gard, 2002; Wright et al., 2008). In this laboratory, interest in the BRAS was initially prompted by the report that, *in vitro*, substance P is hydrolysed by angiotensin converting enzyme (ACE; Skidgel et al., 1984; Strittmatter et al., 1985; Thiele et al., 1985). This led to a single-dose pilot study investigating the effects of the ACE inhibitor, captopril, on the locomotor activity of NK1R-/- mice. Contrary to the prediction that captopril would reduce the activity of wildtype mice, ACE inhibition selectively prevented the hyperactivity of NK1R-/- mice (Yee et al., 2008). Based on this observation, the second major theme of this thesis has been to investigate the effects of drugs that target the BRAS on the behavioural abnormalities of NK1R-/- mice.

The first experiment carried out a full study of the effects of the ACE inhibitor, captopril, and the AT receptor antagonists, losartan and PD 123319, on the locomotor activity of NK1R-/- mice in the LDEB (see: Chapter 4). In light of evidence that there are sex differences in ACE activity (Komukai et al., 2010), the effects of captopril were tested in both male and female animals. Treatment with captopril again prevented the hyperactivity of male NK1R-/- mice, but did not affect the locomotor activity of male wildtypes. Moreover, captopril was ineffective in female mice, of both genotypes. By contrast, treatment with the AT_1 receptor antagonist, losartan, and the AT_2 receptor antagonist, PD 123319, both exacerbated the hyperactivity of (male) NK1R-/- mice. Like captopril, these drugs did not affect the behaviour of wildtype mice.

Combined, these results strongly indicate that NK1R interact with the BRAS to regulate motor control. More specifically, they suggest that the hyperactivity caused by a lack of functional NK1R can be ameliorated by ACE inhibition. Yet, because this effect was evident in male animals

only, there are clearly sex differences in the influence of ACE on locomotor activity. This could be due to the effects of gonadal hormones on ACE activity, which is increased by testosterone but decreased by oestrogen (Komukai et al., 2010). There is also evidence that ACE activity increases in males and decreases in females during adolescence (Landazuri et al., 2008). Understanding the influence of ACE on locomotor activity could help to explain sex differences in the hyperactivity of male and female patients with ADHD patients. In particular, the effect of ACE inhibition on the hyperactivity of ADHD patients with polymorphism of the *TACR1* gene merits investigation.

These studies did not determine the mechanism underlying the effects of these drugs on locomotor activity. However, the fact that captopril was effective in NK1R-/- mice rules out the involvement of substance P, as this neuropeptide would be ineffective in this genotype. Moreover, since neither AT receptor antagonist mimicked the effects of captopril, a reduction in AngII availability is also unlikely to be responsible for the effects of ACE inhibition. Nonetheless, ACE is responsible for the metabolism of a number of other neuropeptides that regulate neuronal circuitry implicated in motor control (e.g., Reid et al., 1990; Steiner and Gerfen, 1998; Binder et al., 2001). Future studies should determine the effects of ACE inhibition and AT receptor antagonism on neurotransmission in neuronal circuitry implicated in motor control, such as the dorsal striatum and other corticostriatal circuitry.

The next study went on to investigate the effects of captopril on the other behavioural abnormalities of NK1R-/- mice (see: Chapter 5). There are many reports, both clinically and in preclinical research, of ACE inhibitors improving cognitive function (e.g., Currie et al., 1990; Wyss et al., 2003; Fogari and Zoppi, 2004; Yasar et al., 2008; Tota et al., 2012). There is also evidence that the beneficial effect of ACE inhibition on cognitive performance is independent of any effects on blood pressure (Amenta et al., 2002). However, to the best of my knowledge, this is the first instance of an ACE inhibitor being tested in the 5-CSRTT, or related tasks.

Overall, treatment with captopril improved the performance of NK1R-/- mice in this test. In the VITI, captopril reduced the incidence of premature responses in NK1R-/- mice, leaving wildtype mice unaffected. In the LITI, captopril abolished the genotype difference in both premature responses and omissions. Combined, these results indicate that ACE is involved in the regulation of both motor impulsivity and attentional performance. Further, they suggest that treatment with an ACE inhibitor can ameliorate the impulsivity and, perhaps, inattentiveness, caused by a deficit in functional NK1R.

As with locomotor activity, the mechanisms underlying the effects of captopril in the 5-CSRTT were not determined. However, impulsivity and attentional performance in the 5-CSRTT have been associated with abnormalities in serotonergic, dopaminergic and noradrenergic function (e.g., Dalley et al., 2002; Robbins, 2002; Milstein et al., 2007; Robinson et al., 2008a). Although there is limited direct evidence that captopril modifies the function of these neurotransmitters, several of the neuropeptides that are targeted by ACE regulate monoaminergic function (Barnes et al., 1989; Jiang et al., 1994; Prus et al., 2007; Petkova-Kirova et al., 2008), and so an effect of captopril on these neurotransmitters is plausible. It would be interesting to perform *in vivo* microdialysis studies to investigate this possibility. Overall, and in combination with evidence from Chapter 4, results presented in this thesis indicate that ACE inhibitors such as captopril could be useful in the treatment of (male) patients with ADHD, particularly of the Predominantly Hyperactive/Impulsive Subtype.

The fact that ACE inhibition and AT receptor antagonism had opposite effects on the locomotor activity of NK1R-/- mice indicates that ACE and AT receptors have distinct effects on motor control. Following the observation that captopril also improves the performance of NK1R-/- mice in the 5-CSRTT, interest turned to the effects of AT receptor antagonism on the other

behavioural abnormalities of these mice. Accordingly, the following experiment went on to investigate the effects of the AT_1 receptor antagonist, losartan, on the performance of NK1R-/- mice in the 5-CCPT (see: Chapter 6).

However, on the whole, treatment with losartan was ineffective in the 5-CCPT. There was no clear effect of losartan on premature responses, false alarms, omissions or the sensitivity index, suggesting that AT_1 receptors do not influence either form of impulsivity or attentional performance. However, as discussed previously, it is possible that any effect of AT_1 receptor antagonism on these behaviours relies on a deficit in baseline at performance. Again, to the best of my knowledge, this is the first instance of an AT receptor antagonist being tested in the 5-CCPT or other related tasks. It would be interesting to investigate the effects of losartan on the performance of NK1R-/- mice in the 5-CSRTT, to determine whether this drug has any effect when these animals do show a deficit in performance at baseline.

The final experiment in this thesis studied the effects of losartan on performance in the NOR and NOL tests (see: Chapter 6). As discussed above, the performance of NK1R-/- mice did not differ from wildtype mice in either of these two behavioural paradigms. However, treatment with losartan disrupted the performance of both genotypes in the NOL test, indicating that activation of AT_1 receptors may play a role in spatial memories, and that this occurs through an NK1R-independent mechanism. These findings are in line with a number of studies in which AngII administration has improved cognitive performance, including in spatial memory tasks (Georgiev and Yonkov, 1985; Braszko et al., 1987, Braszko et al., 1988). However, previous reports on this role of AngII and AT_1 receptors are somewhat inconsistent (Lee et al., 1995; Kerr et al., 2005; Sharma and Singh, 2012), and it is possible that other angiotensin peptide fragments are

responsible for the beneficial effects of AT_1 receptor activation on spatial memory, in particular AngIV (Brazsko et al., 2006; De Bundel et al., 2010).

Overall, results from this thesis provide compelling evidence that both ACE and AT_1 receptors are involved in certain behaviours disrupted in ADHD. It is striking that AT_1 receptor antagonism exacerbates locomotor activity and disrupts performance in tests of memory, yet has no effect on either impulsivity or attentional performance. This suggests that these behaviours are governed by distinct neuronal networks and that the influence of the BRAS differs in each of these networks. The possibility that disruption to the BRAS contributes, at least in part, to some of the behavioural abnormalities and cognitive deficits in ADHD patients merits further investigation.

7.4. Final conclusions

This thesis further explores the NK1R-/- mouse model of ADHD. The results suggest that the hyperactivity and perseveration of this model are both a direct consequence of dysfunctional NK1R, but that their impulsivity is influenced by a lack of functional NK1R in combination with other contributory (e.g., environmental and/or epigenetic) factors. However, this behaviour may also be dependent on others factors, such as previous test experience or specific task parameters. In order to further determine the role of NK1R in impulsive control and attentional performance, NK1R-/- mice could be tested in other behavioural paradigms, such as a delay-discounting or a stop-signal task, for which there is evidence of performance deficits in ADHD patients (Barkley et al., 2001; Aron and Poldrack, 2005; Scheres et al., 2006).

In addition, this thesis explores the behavioural effects of several drugs that target the brain renin angiotensin system. The results reveal that both ACE and AT receptors influence several behavioural and cognitive deficits that are associated with ADHD, and that these, at times,

interact with the function of NK1R. Consequently, the role of the BRAS in these behaviours, as well its interaction with NK1R, merits further investigation. In particular, results from this thesis reveal the exciting possibility that hyperactivity, impulsivity and, possibly, inattentiveness can be alleviated by treatment with an ACE inhibitor, and the effects of these drugs on patients with these symptoms warrants further investigation.

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Appendix I – Genotyping protocol

Step 1 – Ear punching

Ear samples are taken using a 2 mm ear punch and stored in a 0.5 mL eppendorf tube over ice or in a -20°C freezer until required.

Step 2 – Tissue Digestion

Once the sample has defrosted, 75 µL of alkaline lysis reagent (Table A1) is added. Samples are then heated at 95°C for 30 min using a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA), and subsequently allowed to cool to 4°C. To finish, 75 µL of neutralising reagent (Table A2) is added and samples are then stored over ice or in a -20°C freezer until required.

Alkaline lysis reagent
25 mM NaOH
0.2 mM Na_2 EDTA
Ultrapure water (18MΩ)

Table A1. Contents of alkaline lysis reagent

Neutralising reagent
40 mM Tris-HCl
Ultrapure water (18MΩ)

Table A2. Contents of neutralising reagent

Step 3 – Polymerase Chain Reaction – DNA amplification

The sample is allowed to defrost and 6 µL placed in a separate eppendorf tube. To this, 19 µL of a PCR Master Mix is added (Table A3). As well as the samples being run, three controls are run that contain samples from a wildtype mouse, an NK1R-/- mouse, and ultrapure water. The tube is spun

in a centrifuge, in order to draw all of the contents to the bottom. Tubes are then placed in a thermocycler and run through a series of temperatures for DNA amplification (Table A4).

Component	**Amount**
Thermophilic DNA Polymerase 10 Reaction Buffer, Mg-free (Promega)	43.86 µL
dNTP mix	9.418 µL
25 mM MgCl	28.322 µL
NeoF: 5'- GCAGCGATCGCCTTCTATC-3'	23.375 µL
NK1-F: 5'-CTGTGGACTCTAATCTCTTCC-3'	23.375 µL
NK1-R: 5'-ACAGCTGTCATGGAGTAGATAC-3'	23.375 µL
Ultrapure water (18MΩ)	169.388 µL
Taq DNA polymerase	1.887 µL

Table A3. The Polymerase Chain Reaction Master Mix

Temperature (°C)	Duration	
95	5 min	
60	30 s	Cycle x 35
72	30 s	
94	30 s	
60	30 s	
72	5 min	
Allow to cool to 4 °C		

Table A4. Series of temperatures used for DNA amplification

Step 4 – Electrophoresis

A 2% agarose gel is made by adding 2 g of agarose to 100 mL of running buffer (Table A5) and heating in a microwave. To this, 8 µL ethidium bromide is added, and the solution is poured into a gel tray. Combs are added and the solution is allowed to set. 2 µL of ethidium bromide is then added to 500 mL running buffer and this is poured into the gel tray.

4 µL of DNA loading buffer (Table A6) is added to each sample, and then mixed. 15 µL of each sample is then loaded into the wells of the gel, with 5 µL of DNA ladder added to the first well. The gel is run at 100-120 mV for approximately 1 h, and then visualized and photographed using an ultraviolet transilluminator plate. A band is seen at 350 bases for wildtype mice, 260 bases for NK1R-/- mice, and both 350 and 260 bases for NK1R+/- mice.

Running buffer
30 mL 10x Tris-borate-EDTA
570 mL distilled H_2O

Table A5. Contents of running buffer

DNA loading buffer
0.25% bromophenol blue
0.25% xylene cyanol FF
30% glycerol

Table A6. Contents of DNA loading buffer