The role of the NK1 receptor in impulsive and perseverative behaviour in the mouse

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I, Ruth Kathryn Weir, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

The neurokinin-1 receptor (NK1) knockout mouse (NK1R-/-) has a hyperactive phenotype that is attenuated by psychostimulant treatment. This finding led to the NK1R-/- mouse being proposed as a model for Attention Deficit Hyperactivity Disorder (ADHD). The three core diagnostic symptoms of ADHD, hyperactivity, inattention and impulsivity have been investigated in the NK1R-/- mice alongside wildtype controls. Circular corridor apparatus was used to monitor activity over a 48-hour period, during which there was no overall difference in activity, but NK1R-/- mice displayed significantly higher levels of activity in the hour after lights out than wildtype mice. The 5-choice serial reaction time-task (5-CSRTT) is used to measure levels of attention and impulsivity (the other core features of ADHD) in wildtype and NK1R-/- mice. Both genotypes were able to learn the task to the pre-determined baseline level, but when the difficulty of the task was increased, the NK1R-/- mice displayed impulsivity and deficits in attention. Interestingly, the NK1R-/- mice consistently made more perseverative responses compared to wildtype controls. This experiment was extended to test whether the phenotype of the NK1R-/- mice could be replicated with NK1 receptor antagonist treatment in wildtype animals. The NK1 receptor antagonist RP 67580 increased premature responding in wildtype animals, but did not effect omissions or perseveration. Because of the robust nature of the perseverative phenotype of the NK1R-/- at all stages of the 5-CSRTT, marble burying and burrowing tests were used as alternative behavioural paradigms to measure perseveration. In these 'species-typical' behavioural tests no robust difference in behaviour was noted between wildtype and NK1R-/- animals. These findings highlight the multifarious nature of perseverative behaviour and provide evidence for differential neural circuitry for perseverative behaviour in a cued task versus repetitive motor movement. Using an atlas of the distribution of the NK1 receptor and its preferred ligand substance P, this thesis concludes with explanations of the underlying neurobiology that may be responsible for these observations.

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List of abbreviations

A list of abbreviations of brain regions can be found on page 78.

5-CSRTT	5-choice serial reaction time task
5-HT	5-hydroxytryptamine; serotonin
ABC	Avidin-biotin-peroxidase complex
ACh	Acetylcholine
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
$[Ca^{2+}]_I$	Intracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
CO_2	Carbon dioxide
cM	centimorgan
CPA	Conditioned place avoidance
CPP	Conditioned place preference
CREB	cAMP-response element (CRE)-binding protein
DA	Dopamine
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
d-amp	d-amphetamine
DAT	Dopamine transporter
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual of mental disorders, 4_{th} revision
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
ES	Embryonic stem (cells)
EtBr	Ethidium bromide
FITC	Fluorescein isothiocyanate
GABA	Gamma-amino butyric acid
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
i.p.	Intraperitoneal / intraperitoneally
IP_3	Inositol 1,4,5-triphosphate
IRES	Internal ribosome entry site
ITI	Inter-trial interval
K^+	Potassium
LDEB	Light-dark exploration box
LH	Limited hold
LITI	Long inter-trial interval
mAChR	Muscarinic acetylcholine receptor
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neuron
NA	Noradrenaline
nAChR	Nicotinic acetylcholine receptor
NI	No-injection

NK1	Neurokinin 1
NK1R-/-	NK1 receptor knockout
NK2	Neurokinin 2
NK3	Neurokinin 3
NKA	Neurokinin A
NKB	Neurokinin B
NPK	Neuropeptide K
NPγ	Neuropeptide γ
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PPT-A	Preprotachykinin-A
PPT-B	Preprotachykinin-B
RNA	Ribonucleic acid
SEM	Standard error of the mean
SD	Stimulus duration
SHR	Spontaneously hypertensive rat
SNP	Single nucleotide polymorphism
SP	Substance P
SSRI	Selective serotonin reuptake inhibitor
TACR1	Tachykinin receptor 1
TBE	Tris-borate-EDTA (Tris base, boric acid, EDTA)
TO	Time out
TSA	Tyramide signal amplification
tTA	Tetracycline transactivator
TTBS	Tris-Triton-buffered saline
UP H_2O	Ultrapure water (18 MΩ/cm)
VITI	Variable inter-trial interval
WKY	Wistar-Kyoto rat

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1 Introduction

1.1 Substance P and the NK1 Receptor (NK1R)

The year 2011 marked the 80th anniversary of the discovery 'Substance P' by Ulf Von Euler and John Gaddum. Today, the field of neuroscience would be almost unrecognisable to these two scientists, but with Substance P research now in its ninth decade, there are still many more questions that require answers. This thesis investigates the role that SP and its preferred receptor, the neurokinin1 (NK1) receptor, play in impulsive and perseverative behaviour in mice, using the NK1 receptor knockout mouse and pharmacological blockade of the NK1 receptor.

1.1.1 History of Substance P and NK1R research

Figure 1.1 provides a timeline of key-findings in the long history of research into the NK1 receptor and its preferred ligand Substance P.

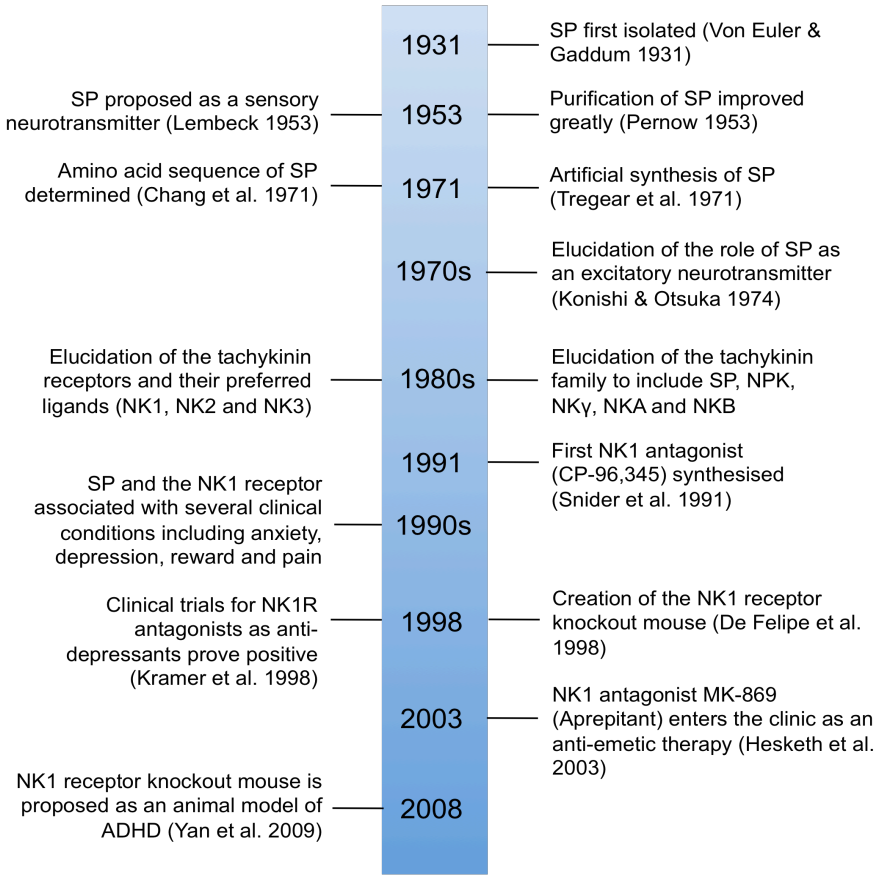


Figure 1.1Timeline outlining key-findings in the history of SP and NK1 receptor research

1.1.1.1 Substance P

Substance P (SP) was first isolated in 1931, as a crude extract from equine brain and gut tissue. It was found to have potent hypotensive and smooth muscle contractile properties (V Euler and Gaddum, 1931). The newly discovered "factor" was named three years later, with P referring to the powder obtained during the extraction process (Gaddum and Schild, 1934). In the 1950s Fred Lembeck noted the high distribution of substance P in the dorsal root of the spinal cord and proposed that substance P was involved in sensory neurotransmission (Lembeck, 1953). In parallel to Lembeck, members of Von Euler's laboratory were still working on SP, namely it's purification, distribution and biological action (Pernow, 1953). Great advances were made in the isolation procedure for substance P, increasing the yield from a crude extract from 15 to 3000 units per mg (Pernow, 1953; Lembeck, 2008).

In 1971, Chang et al. identified the amino acid sequence of substance P, an undecapeptide, as H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Chang et al., 1971). This discovery paved the way for the production of synthetic substance P, thereby facilitating all subsequent research into substance P (Tregear et al., 1971). Artificial synthesis of substance P gave activity of 200,000 units per mg (1 unit is 5ng) (Lembeck, 2008), which enabled researchers to elucidate the role of SP. In a series of studies, researchers demonstrated greater levels of SP in dorsal versus ventral roots from bovine spinal cord (Takahashi et al., 1974), and excitatory action of SP in frog and rat spinal cord with potency more than 200-fold that of glutamate (Konishi and Otsuka, 1974a, 1974b; Otsuka et al., 1975). These findings confirmed SP as an excitatory transmitter of primary afferent fibres, thereby validating Lembeck's hypothesis more than two decades later.

Substance P became part of a group of related polypeptides characterised by their speed of the contractions on smooth muscle preparations, they were called 'tachykinins', in opposition to the slow-acting kinins or 'bradykinins'. Gradually, other mammalian peptides were discovered with similar contractile properties, leading to their inclusion in the tachykinin family. All mammalian tachykinins, including these newly characterised peptides, now known as neurokinin A (NKA) and neurokinin B (NKB) share a common carboxy-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X is Phe or Val (Otsuka and Yoshioka, 1993).

Substance P is synthesised *in vivo* by alternative splicing of the preprotachykinin (PPT-A) gene, which produces three mRNA transcripts, α-, β-, and γ-PPT-A. All three PPT-A mRNAs encode for the substance P precursor sequence, whilst β-PPT-A, also contains precursor sequences for neurokinin A (NKA) and neuropeptide K (NPK). γ-PPT-A contains sequences for NKA, and neuropeptide γ (NPγ) (Figure 1.2). Neurokinin B is synthesised from a separate gene, preprotachykinin-B (PPT-B) (Nawa et al., 1983, 1984; Tatemoto et al., 1985; Kawaguchi et al., 1986; Carter and Krause, 1990).

Figure 1.2 Schematic representation of the biosynthesis of substance P (SP) and other tachykinins via alternative splicing of the PPT-A gene. The numbers in the boxes represent the exons of the PPT-A gene. NPK, neuropeptide K; NKA, neurokinin A; NPγ, neuropeptide γ. Figure modified from (Otsuka and Yoshioka, 1993).

1.1.1.2 NK1 Receptor

The identified tachykinins had differential contractile effects on smooth muscle preparations indicating the presence of distinct receptor subtypes (Iversen et al., 1982; Lee et al., 1982). In a series of studies, three types of neurokinin receptors (NK1, NK2 and NK3) were identified (Buck et al., 1984; Hunter and Maggio, 1984; Maggi et al., 1987; Regoli et al., 1987). Substance P, NKA and NKB have some binding affinity for all neurokinin receptors, although they preferably bind to NK1, NK2 and NK3 respectively (Table 1.1) (Saffroy et al., 1988; Regoli et al., 1989; Hökfelt et al., 2001).

Molecular cloning of rat and bovine neurokinin receptors identified them as members of the G-protein coupled receptor superfamily, comprising a rhodopsin-like structure with seven hydrophobic transmembrane domains connected by intra- and extracellular loops (Masu et al., 1987; Yokota et al., 1989; Hershey and Krause, 1990; Shigemoto et al., 1990; Nakanishi, 1991). The human NK1 receptor was cloned in 1991 and was shown to have 94.5% homology with the rat NK1 receptor (Gerard et al., 1991; Takeda et al., 1991).

Tachykinin Receptor	Relative binding affinities of tachykinins
NK1	SP>NKA>NKB
NK2	NKA>NKB>SP
NK3	NKB>NKA>SP

Table 1.1 Relative binding affinities of three tachykinins and the three neurokinin receptors

1.1.2 SP binding and activation of signaling pathways

SP binds strongly to the NK1 receptor at the extracellular ends of the transmembrane helices between the second and seventh extracellular loops of the receptor, with a reported Kd value of 0.16nM (Takeda et al., 1992; Gether et al., 1993; Huang et al., 1994). When the SP/NK1R complex is formed, the associated G-proteins are activated ($G_{q/11}$, $G\alpha_s$ and $G\alpha_o$ in humans (Roush and Kwatra, 1998)), leading to the activation of three separate secondary messenger systems (Harrison and Geppetti, 2001). Activation of phospholipase $C\beta$ leads to the cleavage of phosphotidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which subsequently binds to calcium channels in the endoplasmic reticulum leading to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Phospholipase A_2 activation leads to production of arachidonic acid, while adenylyl cyclase increases intracellular levels of cyclic adenosine monophosphate (cAMP) (Takeda et al., 1992; Garcia et al., 1994; Khawaja and Rogers, 1996; Quartara and Maggi, 1997). The 5' untranslated region of the NK1 receptor contains a sequence for cAMP response element binding protein (CREB)/calcium response element sequence, meaning that activation of the NK1 receptor can lead to enhanced gene transcription in a feed forward loop (Regoli et al., 1994). Finally the SP/NK1R complex is internalised into acidic endosomes, where the complex dissociates. Substance P is then degraded and the NK1 receptor is recycled back into the cell membrane (Grady et al., 1995; Garland et al., 1996).

1.1.3 Distribution

The distribution of the NK1 receptor and it's preferred ligand substance P, has been studied using a variety of techniques in a wide range of mammalian species (but not mouse) and is generally well conserved through evolution. NK1 receptors are located on cell bodies and dendrites, normally with SP-positive fibres nearby. In brief, the strongest staining for the NK1 receptor in rat brain is seen in cortical amygdaloid nucleus, locus coeruleus, caudate-putamen, nucleus accumbens, olfactory tubercle, and

raphe nuclei (Nakaya et al., 1994). However staining of receptor and ligand do not always correlate. The substantia nigra is abundant with substance P, as seen by intense staining with the SP antibody, yet NK1 receptor staining differs little from background levels in both rat and human tissue (Kowall et al., 1993; Nakaya et al., 1994). Previous studies of NK1 receptor and SP distribution are discussed in depth in Chapter 3, which investigates the staining patterns of the NK1 receptor and SP throughout the mouse brain.

1.2 Roles of SP-NK1R system

On the basis of their anatomical localisation, substance P and the NK1 receptor have been implicated in the pathophysiology of a diverse range of conditions including asthma, inflammatory bowel disease, pain, psoriasis, migraine, movement disorders, schizophrenia, depression, anxiety and emesis (Quartara and Maggi, 1997; Rupniak and Kramer, 1999). The main avenues of research have concerned the role of the NK1 receptor in pain and affective disorders such as depression and anxiety. This section covers research from cell ablation studies and receptor blockade, while the work using the NK1 receptor knockout mouse is covered in section 1.4.3.

1.2.1 Affective disorders

The umbrella term of 'affective disorders' covers conditions with deficits in emotional processing such as depression and anxiety. The NK1 receptor has been implicated in many of these disorders, given their prevalence in brain regions associated with fear-processing (including the amygdala, septum, hippocampus, hypothalamus and periaqueductal grey).

1.2.1.1 Anxiety and stress

The role of SP in anxiety and stress related responses is comprehensively reviewed by (Ebner and Singewald, 2006), while the key features are highlighted below. Stress has many connotations and can be measured in several ways e.g. physical (restraint paradigms), emotional (maternal separation) and painful (foot shock) stressors. Activation of the hypothalamic-pituitary-adrenal (HPA) axis releases a cascade of 'stress' hormones including corticotropin releasing hormone, adrenocorticotropic hormone and cortisol. Concentrations of neuropeptides NPY and SP also change after

stress. Early studies measured changes in SP concentration in various brain regions associated with stress (foot-shock or restraint) but some groups reported increases in SP levels (Siegel et al., 1984; Rosén et al., 1992; Brodin et al., 1994), while others reported decreases (Lisoprawski et al., 1981; Takayama et al., 1986; Siegel et al., 1987). There is a plethora of studies into the pharmacological manipulation of NK1 receptor activity and response to stress. Intracerebral injections of SP and other NK1 receptor agonists have been reported to elicit anxiogenic-like effects, whereas NK1 receptor antagonists tend to evoke an anxiolytic effect, however the data varies depending on behavioural paradigm, dose and brain region. For instance, microinjections of SP into the dorsal periaqueductal gray, lateral septal nucleus and medial amygdala induced anxiogenic action (Aguiar and Brandão, 1994, 1996; De Araújo et al., 1999; Gavioli et al., 1999), whereas injection in the nucleus basalis magnocellularis exerted anxiolytic-like effects in the elevated plus maze (EPM) (Hasenöhrl, Jentjens, et al., 1998; Nikolaus et al., 1999). In contrast to the above, NK1 receptor antagonism tends to elicit anxiolytic effects. For example, the NK1 receptor antagonists FK-888, NKP-608 and RP 67580 all induced an anxiolytic-like profile in the elevated plus maze (Teixeira et al., 1996; Santarelli et al., 2001; Vendruscolo et al., 2003), while MK-869 and L-733,060 reduced isolation-induced vocalisations in guinea pigs (Kramer et al., 1998; Rupniak et al., 2000). However ablation of NK1 receptor containing neurons in the amygdala decreased time spent in the open arms of the EPM, indicative of an anxiogenic profile (Gadd et al., 2003), thereby creating a very complex picture for the regulation of stress and anxiety responses by the SP/NK1R system. For a comprehensive list of NK1 receptor related anxiety and stress studies see Ebner and Singewald, (2006).

1.2.1.2 Depression

Given that the symptoms of anxiety and depression somewhat overlap, NK1 receptor antagonists were also explored as a potential treatment for depression. Early work produced promising results with the NK1 receptor antagonists L-760,735 and L-733,060 (but not their respective inactive enantiomers) reducing isolation-induced vocalisation in guinea pigs to the same extent as traditional anti-depressants and anxiolytic drugs such as fluoxetine (a selective serotonin reuptake inhibitor (SSRI)) and diazepam (a benzodiazepine) (Kramer et al., 1998). Also, NK1 receptor antagonism reduced attack behaviour in the resident intruder test (Rupniak et al., 2001). In phase II clinical trials, symptoms of depression in patients diagnosed with major depressive

disorder were improved by the NK1 receptor antagonist MK-869 (Aprepitant) to the same extent as in patients given the classic anti-depressant paroxetine (an SSRI), but with fewer detrimental side effects (Kramer et al., 1998). The field of NK1 receptor antagonism as an anti-depressant treatment was further bolstered when another NK1 receptor antagonist, L-759,274, also produced positive results in another phase II clinical trial in patients with depression (Kramer et al., 2004). Although NK1 receptor antagonism appeared to work well as an antidepressant therapy, the mechanism of action was largely unknown. The most obvious connection was to the monoaminergic systems, given the mode of action of the already established anti-depressant treatments. Systemic administration of NK1 receptor antagonists has been shown to enhance the firing rate of dopaminergic, noradrenergic and serotonergic neurons (Adell, 2004). Firstly, NK1 receptor antagonists CP-96,345 and WIN 51,708 reduced the inhibitory effect of clonidine (an α2-adrenoceptor agonist) on the firing activity of serotonergic neurons in the rat dorsal raphé nucleus and noradrenergic neurons of the locus coeruleus (Haddjeri and Blier, 2000), while sustained administration of CP-96,345 to dorsal raphé serotonergic neurons increased spontaneous firing (Haddjeri and Blier, 2001). Dopaminergic systems are also effected by NK1 receptor antagonism. In rats, administration of GR 205,171 increased firing of dopaminergic neurons in the parabrachial nucleus of the ventral tegmental area resulting in increased dopamine efflux in the frontal cortex (Lejeune et al., 2002).

1.2.2 Reward and addiction

Substance P and the NK1 receptor are also found in brain regions that are associated with motivation and reward such as the amygdala, globus pallidus, and nucleus accumbens (Everitt and Robbins, 2005). As outlined in the previous section, injection of substance P into various brain regions heightens symptoms of stress and anxiety, so it is surprising to learn that application of SP to the reward pathway has a reinforcing effect. This has mainly been tested using the conditioned place preference paradigm. This test consists of two chambers with very different distinguishing features (e.g. stripy wall vs. a spotty wall and/or different floor textures), which the animals are free to travel between. The mice are administered a drug and immediately placed in one of the chambers, while the other chamber is associated with a saline injection. This is repeated over a number of tests until a final probe test is conducted with no drug administration,

where the animal is free to move between the chambers. An animal will spend more time in the chamber associated with a rewarding drug or tend to avoid a chamber associated with an aversive drug (conditioned place preference (CPP) and avoidance (CPA), respectively). Injection of SP into the globus pallidus, central nucleus of the amygdala and nucleus basalis magnocellularis all resulted in conditioned place preference for SP treatment over controls (Holzhäuer-Oitzl et al., 1988; Hasenöhrl, Frisch, et al., 1998; Kertes et al., 2009, 2010). Ablation of NK1 receptor containing neurons in the amygdala, but not the striatum or nucleus accumbens, blunted morphine reward behaviour in mice in the CPP test (Gadd et al., 2003). The SP/NK1R pathway is not solely responsible for the modulation of reward as there are known interactions with the opioid and dopaminergic systems. Pre-treatment with naloxone, an opioid receptor antagonist, blocked the reinforcing effects of SP treatment (Hasenöhrl et al., 1991), while dopamine efflux increased in the nucleus accumbens after systemic SP administration (Huston et al., 1993). Given the strong evidence for the NK1 receptor playing a role in reward, it is not surprising that it is also implicated in addiction (Heilig et al., 2010). NK1 receptor antagonism decreases alcohol consumption in free choice and operant self-administration tests and suppresses alcohol seeking behaviour (Steensland et al., 2010; Thorsell et al., 2010; Schank et al., 2011). Furthermore NK1 antagonist treatment has shown promising results in clinical trials in reducing alcohol cravings (George et al., 2008), while polymorphisms in the TACR1 gene (the human equivalent of the NK1 receptor gene) have been associated with alcohol dependence (Seneviratne et al., 2009).

1.2.3 Other roles

The most widely researched role of the SP/NK1R pathway, aside from affective disorders, is that of the regulation of pain and nociception focused on the dorsal horn of the spinal cord. In rats, depletion of SP in the spinal cord results in thermal analgesia (Yaksh et al., 1979) while intrathecal injection of SP causes hyperalgesia to thermal stimuli (Moochhala and Sawynok, 1984), providing evidence for the SP/NK1R system having a key role in the modulation of pain. Ablation of NK1 receptor positive neurons in the spinal cord resulted in prolonged reduction in thermal hyperalgesia, which lead to the suggestion that NK1 receptor antagonist therapy could be used for the treatment of chronic neuropathic pain (Nichols et al., 1999). Pre-clinical trials in rats using a model

of neuropathic pain (ligation of the sciatic nerve) were promising (Cumberbatch et al., 1998), but subsequent clinical trials were unsuccessful (Hill, 2000). SP also plays a role in inflammation, by promoting angiogenesis at sites of neurogenic pain, as shown by increased endothelial cell proliferation (Seegers et al., 2003).

Finally the SP/NK1R pathway is prevalent in the brainstem nuclei that regulate emesis. The NK1 receptor antagonist CP-99,994 successfully prevented emesis against a number of emetogens (e.g. radiation and cytotoxic drugs) firstly in ferrets and later in dogs (Bountra et al., 1993; Watson et al., 1995). Unlike the many examples above of pre-clinical success followed by failure in Phase II clinical trials, the NK1 receptor antagonist MK-869 (Aprepitant), first trialed as an anti-depressant, passed Phase III clinical trials and is used in the clinic as a treatment for chemotherapy-induced vomiting (Hesketh et al., 2003; Poli-Bigelli et al., 2003).

1.2.4 Clinical successes and failures

As highlighted in the above sections the SP/NK1R pathway has been implicated in a vast array of conditions with NK1 receptor antagonist therapy showing promising initial results in pre-clinical trials only to reach phase II clinical trials where they perform no better than placebo or existing agents (Keller et al., 2006). NK1 receptor antagonists were an important focus for the large pharmaceutical companies at the turn of the 21st century, especially as a potential new branch of anti-depressant therapy, yet the only NK1 receptor antagonist therapy is currently the anti-emetic drug Aprepitant. The reason for the high failure rate between pre-clinical and clinical trials is most likely due to the species variation of the NK1 receptor. The rat and human NK1 receptor are ~95% homologous (Barr and Watson, 1993), yet the small differences have been shown to have a dramatic effect on antagonist binding properties (Fong et al., 1992).

1.3 Genetically modified mice

Genetically modified mice have had their genome manipulated by genetic engineering and are an invaluable tool in scientific research. They enable scientists to study the role of individual genes either by creating too much of the gene product, or by eliminating its presence and looking at molecular, cellular or behavioural changes, usually in

relation to human diseases. A good animal model should fulfill three criteria: a) mimic the clinical features of the disease in question (face validity), b) conform to theoretical rational e.g. have a similar pathophysiological differences to the relevant disease (construct validity) and c) respond to treatments in the same way a human with the disease would (predictive validity) (Willner, 1986).

The first 'transgenic mouse', a mouse with a simian virus incorporated in its genome, was created in the early 1970s (Jaenisch and Mintz, 1974), however the transgene could not be passed onto offspring. It was several years before Gordon and Ruddle, (1981) created a mouse with a mutation that could be passed to subsequent generations. Genetic modification of animal genomes has developed incredibly over the past three decades and is now a reliable and widespread laboratory technique. The work in this thesis looks at the phenotype of the NK1 receptor knockout mouse (NK1R-/-).

1.3.1 Types of genetically modified mice

There are several different types of genetically modified mice, of which the most common type used in scientific research are 'knockout' mice, in which an endogenous gene is either replaced or disrupted by insertion of an artificial piece of DNA. However if the loss of the gene results in a developmentally non-viable mouse, an alternative option is to create a knockin mouse. In this technique a transgene, containing a deliberate mutation, is inserted at a specific locus and results in expression of the mutated version of the gene product, but does not result in the death of the animal (Manis, 2007). Another method to overcome the problem of embryonic or post-natal lethality due to functional disruption of a gene is by creating a conditional knockout mouse (Friedel et al., 2011). There are two different approaches to create conditional knockout animals, either by using a tetracycline transactivator (tTA) system or the Cre-LoxP system with Cre recombinase (Morozov et al., 2003). In brief, tTA activates transcription when bound to a promoter containing the tetracycline operator, yet when in the presence of tetracycline or its analog doxycycline, the complex dissociates and transcription is halted (Gossen and Bujard, 1992). In the Cre-LoxP system firstly the target gene must be 'floxed', that is, two loxP must be integrated (within frame) at either end of a gene (usually for a small genes only, for larger genes the sites are placed around an essential exon). In the presence of Cre recombinase (which must also be

inserted into the genomic sequence) recombination occurs, which leads to the deletion of the sequence between the two loxP sites (Gu et al., 1994). The NK1R-/- mouse was created using targeted disruption in the first exon that renders the RNA transcript out of frame (for details see section 1.4.1).

1.3.2 The importance of background strain

There are a multitude of different background strains of mice used in scientific research, which is known to have a significant effect on behaviour. All mice used in laboratory tests derive from a handful of origins and are divided into two categories: inbred and outbred strains. The official definition of an inbred mouse strain is one that has undergone brother x sister mating for 20 or more consecutive generations (Lyon et al., 1996). After 20 generations, at least 98.6% of the loci in each mouse are homozygous, while many strains have been bred for more than 150 generations and are essentially homozygous at all loci (Beck et al., 2000). Since all the mice of a strain are genetically identical they have long been used for genetic studies and are the basis for creation of transgenic animals. Examples of commonly used inbred mouse strains are DBA, C57/BL6 and BALB/c. However, outbred strains (or stocks for the more accurate terminology) such as CD-1 and MF-1, should really only be used for mapping of quantitative trait loci (QTLs) of complex traits in line with the heterogeneous human genome. However, they are sometimes used in the maintenance of genetically modified mouse colonies due to their superior breeding performance (Chia et al., 2005). Examples of specific phenotypes for different strains include C57BL/6 mice that have a high preference for alcohol, while BALB/c mice are valuable in mutagenesis studies due to their sensitivity to a wide variety of mutagens. Embryonic stem (ES) cells from 129 mice are widely used in the creation of genetically modified mice because of their high success rate of germline transmission (Beck et al., 2000). It is therefore imperative that scientists carefully consider the phenotype of the background strain to use in experiments to prevent animal wastage, for example, C3H mice have a genetic defect that leads to early retinal degeneration so should not be used for any behavioural tests that require sight.

1.3.3 The flanking gene problem

In addition to the phenotype differences between background strains, there are other complications that arise from the creation of transgenic animals: epistatic interactions and the flanking gene problem. Epistasis is the influence of the genotype of the mouse at other, often unlinked loci, on the target-mutated gene. The flanking gene 'problem' arises when mutated ES cells (e.g. from a 129 background) are placed in the blastocyst from a different strain (e.g. C57BL/6). Due to the nature of genetic recombination, where the probability of recombination is inversely related to the distance between the loci of the genes, those genes that are situated close to the target gene will remain linked with the target gene. Therefore the alleles for these genes will remain homozygous for the 129-type with no influence from the C57BL/6 background in the homozygous null mutants (Gerlai, 1996). Backcrossing offspring onto a single background strain reduces the flanking gene problem, although even after 12 generations of backcrossing, 1% (16cM of the total 1600cM genome) of the mouse genome remains linked to the target gene (Crusio, 2004). It is therefore crucial that strict controls are used in all experiments that use transgenic animals to prevent false-positive results being attributed to the target gene, when it could be an indirect result from the creation and breeding strategy of the background strain and colony.

1.4 The NK1 receptor knockout mouse

The original NK1 receptor knockout mouse was created by De Felipe et al., (1998). The process is briefly outlined below but is reported in more detail in the Chapter 2 (section 2.1.1). The creation of the NK1 receptor knockout mouse enabled progress in the field of SP/NK1R research as the findings from previous NK1 receptor antagonist and cell ablation studies (see section 1.2) could be verified.

1.4.1 Creation of the NK1 receptor knockout mouse

A replacement vector was constructed of the cloned NK1 receptor gene with a cassette inserted into exon 1. The vector was then electroporated into embryonic stem (ES) cells, derived from a 129/Sv background. Negative selection left only those cells with the mutated version of the NK1 receptor gene that were then injected into C57BL/6 blastocysts to create a 129/Sv x C57BL/6 mouse line in which the gene encoding the

NK1 receptor was disrupted. Interbreeding of two heterozygous mice for the disrupted allele resulted in healthy homozygous NK1 receptor knockout mice.

1.4.2 The background strain of the NK1 receptor knockout mouse

In light of the importance of background strain of transgenic mice, the lineage of the NK1R-/- mouse must be considered. The original knockout mice were created from the ES cells of a 129 mouse, implanted into blastocysts of C57BL/6 mice. Therefore the flanking gene problem could arise and have an effect on the behaviour of the NK1R-/- mice. However the performance of mice from a 129 lineage was often inferior compared to that of C57BL/6 in a number of behavioural tests (Balogh et al., 1999). The strain was crossed onto an outbred MF1 strain for rapid dilution of the 129/Sv component, although in hindsight this has over-complicated the genetic background of the NK1R-/- mouse, however this has become the main colony kept at UCL. Another colony of NK1R-/- mice were developed through backcrossing for more than 10 generations onto a C57BL/6 background, thereby reducing the potential effect of the flanking gene problem (McCutcheon et al., 2008). A comparison of mice from the original C57BL/6 x 129/Sv (129B6) colony vs. those from the backcrossed C57BL/6 (B6) colony showed the NK1 receptor knockout mice on the 129B6 strain had elevated glucocorticoid receptor immunoreactivity, greater hippocampal neurogenesis and higher novelty-induced locomotion compared to the wildtype animals of the 129B6 strain, while no genotype differences were observed between the mice on the backcrossed B6 background (McCutcheon et al., 2008). Experimenters should therefore be cautious when drawing conclusions from the colonies of NK1R-/- mice on mixed backgrounds. Another group created a different colony of NK1R-/- mice on a pure 129/SvEv background (Santarelli et al., 2001).

1.4.3 Findings from the NK1 receptor knockout mouse

Many of the early experiments using the NK1 receptor knockout mice echoed those of the antagonist and cell ablation studies previously mentioned in Section 1.2 of this chapter.

1.4.3.1 Behavioural differences

1.4.3.1.1 Reduced nociception

The role of the SP/NK1R pathway in the modulation of pain signaling was well established prior to the creation of the NK1R-/- mouse, however both hyperalgesic and analgesic effects had been recorded when the system was disrupted. This was mirrored in experiments using the NK1R-/- mouse. NK1 receptor knockout mice displayed reduced behavioural responses to capsaicin-induced visceral pain and did not develop hyperalgesia (Laird et al., 2000), yet they also did not develop stress-induced analgesia while their response to acute pain was comparable to that of wildtype mice (De Felipe et al., 1998).

1.4.3.1.2 Depression

NK1 receptor antagonist treatment mimicked that of traditional anti-depressants by reducing stress-induced vocalisations (Kramer et al., 1998). The behaviour of NK1R-/- mice resembles that of wildtype mice given anti-depressants in a number of behavioural paradigms. In the forced swim test animals are forced to swim in a cylinder with no option of escape. After a period of time spent swimming and trying to escape the animal will 'give-up' and become immobile. The latency to become immobile is used as a reflection of 'behavioural despair', while antidepressant treatment lengthens the time spent swimming, making it a robust test for modeling depressive-like behaviour in mice (Porsolt et al., 1977). In line with the previous pharmacological studies into the SP/NK1R system having a role in the regulation of depressive behaviour (section 1.2.1.2), NK1R-/- mice had a longer latency to immobility than wildtype controls (Santarelli et al., 2002). In a similar vein of experiments, NK1R-/- mice spent more time struggling (an indication of an 'anti-depressive' phenotype) in the tail suspension test compared to wildtype animals (Rupniak et al., 2001).

1.4.3.1.3 Anxiety and stress

As for the reward and depression related experiments above, the behaviour of the NK1R-/- mouse mirrored the behaviour of wildtype mice treated with NK1 receptor antagonist in behavioural paradigms that test stress and anxiety. In the resident intruder test, used to measure stress related behaviours, the latency to attack was significantly longer in NK1R-/- mice than wildtype controls, thereby replicating the findings of the same test with NK1 receptor antagonist treatment (De Felipe et al., 1998; Rupniak et al.,

2001). Similarly, stress induced vocalisations after maternal separation were reduced in NK1R-/- mice compared (Rupniak et al., 2000; Santarelli et al., 2001) providing solid evidence for the SP/NK1R system playing an important role in the modulation of stress response. The findings from anxiety related behaviours in the elevated plus maze (EPM) are less clear. While (Santarelli et al., 2001) reported that NK1R-/- mice displayed increased time in the open arms of the elevated plus maze compared to wildtype controls, indicating a decreased level of anxiety related behaviours, (Murtra et al., 2000) found no difference in behaviour between wildtype and NK1 receptor knockout mice.

1.4.3.1.4 Reward and addiction

NK1 receptor knockout mice are insensitive to opiates, while responses to other drugs remain normal. In the conditioned place preference test NK1R-/- fail to form a preference for the chamber paired with morphine pre-treatment (Murtra et al., 2000). NK1R-/- mice also administered less morphine than wildtype controls in a self-administration lever press paradigm and did not sensitise to the locomotor stimulant effects of repeated morphine treatment (Ripley et al., 2002). The rewarding effects of alcohol are also impaired in NK1R-/- mice as consumption was significantly decreased in a two-bottle free-choice paradigm, and do not form a preference to the alcohol-paired chamber of the CPP paradigm when compared to wildtype controls (George et al., 2008; Thorsell et al., 2010). In all of the tests outlined above, the response of NK1R-/- to cocaine was indifferent to that of the wildtype controls, showing that the findings are not due to a global impairment in the reward pathway, but is specific to the opiate and alcohol reward systems.

1.4.3.1.5 Locomotor activity

Locomotor activity is one of the first behaviours to be tested in the battery of paradigms used to characterise a new transgenic animal, although it is often reported from a number of different tests. In the inaugural paper that characterised the NK1R-/- mouse, no difference in locomotor activity was recorded between the knockout mice and wildtype controls in the open-field paradigm (although this test is traditionally associated with testing anxiety-related behaviours) (De Felipe et al., 1998). Many studies have subsequently reported locomotor activity findings, with mixed findings. In the elevated-plus maze (also an anxiety-related behavioural test) no difference in locomotor activity (as indicated by total number of arm entries) was recorded between genotypes (Murtra et al., 2000; Santarelli et al., 2001). Activity chambers, where

activity is measured by breaks in infrared beams of light, provide a good measure of locomotor activity, since anxiety is reduced as a confounding factor. No difference in the locomotor activity of NK1R-/- mice compared to that of wildtype controls were reported in a number of studies (Rupniak et al., 2000; Ripley et al., 2002; Gadd et al., 2003), whereas (Herpfer et al., 2005) found that locomotor activity was significantly higher in NK1R-/- mice. This finding has been replicated in the light/dark exploration box (LDEB), although this is another paradigm used to measure anxiety and the recordings of hyperactivity of NK1R-/- mice were all taken from animals that had been administered an injection (Fisher et al., 2007; Yan et al., 2010). The activity of treatment naïve wildtype and NK1R-/- mice is investigated in Chapter 4 of this thesis in circular corridors. Recent telemetry data from the Hunt laboratory also suggests greater locomotor activity in NK1R-/-, although this was most significant in the dark phase of the daily cycle (unpublished data, 2012).

1.4.3.2 Neurochemical differences

It is important to try and relate observed behaviours such as those outlined above to the underlying neurological differences that result from the disruption of the SP/NK1R system. Given the link with the anti-depressive phenotype of the NK1R-/- mouse (section 1.4.3.1.2) and the known action of anti-depressant therapies in increasing central monoamine transmission, it followed to compare the monoaminergic systems of wildtype and NK1 receptor knockout mice using microdialysis.

1.4.3.2.1 Serotonin

Firing rate of serotonergic neurons in the dorsal raphé (DR) were increased in NK1R-/- mice compared to wildtype controls and was comparable to the activity of the 5-HT neurons of wildtype mice pre-treated with NK1 receptor antagonist (Santarelli et al., 2001), while paroxetine-induced 5-HT outflow in the frontal cortex was 4-6 fold higher in the NK1R-/- mice compared to wildtype controls, yet basal efflux of 5-HT did not differ between genotypes (Froger et al., 2001). The firing rate of dorsal raphé neurons is activated by locally released 5-HT, which activates presynaptic inhibitory 5-HT1A autoreceptors (Blier and de Montigny, 1985). Antagonism of the 5-HT1A receptor inhibited firing of DR neurons in wildtype mice, yet this effect was much reduced in NK1R-/- mice (Santarelli et al., 2001). This is probably due to reduced numbers of the 5-HT1A autoreceptor, as signified by reduced mRNA levels in the anterior raphé (Froger et al., 2001). These observations indicate that the presence of the NK1 receptor

has a direct effect on the expression of 5-HT1A receptors. Interestingly, immunohistochemical localisation studies showed that there was no co-localisation of NK1 receptors and tryptophan hydroxylase (a marker of serotonergic neurons) (Santarelli et al., 2001) in the dorsal raphé, suggesting that the modulation of the serotonergic system by the NK1 receptor is indirect.

1.4.3.2.2 Noradrenaline

Given that the noradrenergic neurons that project from the locus coeruleus also express NK1 receptors (Santarelli et al., 2001) and that one of the terminal fields are the raphé nuclei, they could provide the link between the NK1 receptor and modulation of the serotonergic system. Anaesthetised NK1R-/- mice had a greater basal efflux of noradrenaline (NA) in the cerebral cortex than wildtype counterparts (Herpfer et al., 2005; Fisher et al., 2007), this is most likely explained by increased levels of release rather than delayed or inefficient reuptake of the neurotransmitter since systemic desipramine treatment (a NA reuptake inhibitor) increased NA efflux to the same extent in both genotypes (Herpfer et al., 2005). Excessive noradrenaline release is ordinarily regulated by activation of presynaptic inhibitory α2-adrenoreceptors. However antagonism of these receptors with RX 821002 in NK1R-/- did not result in increased NA efflux as seen in wildtype mice (Fisher et al., 2007). Furthermore, binding of [^{35}S]GTPγS was significantly reduced in the locus coeruleus of NK1R-/- mice compared to wildtypes, signifying that fewer of the G-protein coupled $\alpha2_A$-adrenoreceptors were activated by agonist (adrenaline) (Fisher et al., 2007). These findings provide evidence for the regulation of NA efflux by the NK1 receptor, possibly due to desensitisation of α2-adrenoreceptors. However in a subsequent study using local infusion of potassium (K^+) to induce NA efflux, the initial dose resulted in equal levels of NA release in the prefrontal cortex in both genotypes, but when a second challenge of K^+ was administered, an increase of NA was noted in NK1R-/- mice but not in wildtypes (Yan et al., 2009). This effect was calcium dependent suggesting this phenomenon is related to exocytosis and the release of the transmitter rather than by regulation by α2-adrenoreceptors.

1.4.3.2.3 Dopamine

Compared to noradrenaline and serotonin, relatively little is known about any interaction between the NK1 receptor and the dopamine (DA) system. Microdialysis

studies have shown that basal efflux levels of DA are equivalent in the dorsal striatum of wildtype and NK1R-/- mice, but in the prefrontal cortex (PFC) DA efflux in NK1R-/- was half that of the wildtype control mice (Yan et al., 2010). When the mice were injected with d-amphetamine (a monoamine reuptake inhibitor and catecholamine (DA and NA) releasing agent), DA efflux in the dorsal striatum and PFC of wildtype mice increased, but this was not observed in the NK1R-/- mice (Yan et al., 2010). These findings highlight region specific interactions of the NK1 receptor and the dopaminergic system.

The work in our laboratory has found several features of the NK1R-/- mouse show a close relation to symptoms of the human condition attention deficit hyperactivity disorder (ADHD). The NK1R-/- mouse is hyperactive in the LDEB paradigm, which can be ameliorated with d-amphetamine (Yan et al., 2009), as well as having neurochemical changes that relate to the condition in humans (Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010). These findings led to the NK1R-/- knockout mouse being proposed as a novel rodent model of ADHD (Yan et al., 2009). This connection is further explored in the following section.

1.5 Attention Deficit Hyperactivity Disorder (ADHD)

1.5.1 Prevalence, symptoms and subtypes

Attention-deficit hyperactivity disorder (ADHD) is one of the most common neurobehavioural conditions in children and can result in impaired social and academic functioning in later life. Reports of the prevalence of ADHD in children range from as low as 1% to as high as 20%, although a large meta-analytical study found the worldwide prevalence of ADHD in children and adolescents to be 5.3% (Polanczyk et al., 2007). Approximately 20% of cases pervade into adulthood (Faraone et al., 2000), with inattentiveness as the most common prevailing symptom (Biederman et al., 2000). The disorder is more common in boys than girls with prevalence estimates at 10% and 4.2% respectively (Polanczyk et al., 2007). The core symptoms of hyperactivity, impulsivity and inattention usually present before the age of seven years. There are three subtypes of the disorder; an inattentive subtype, a hyperactive/impulsive subtype and a combined subtype where all three symptoms are present (American Psychiatric

Association, 1994). The diagnostic criteria for ADHD taken from DSM-IV are outlined in Table 1.2.

Table 1.2 Criteria for diagnosis of ADHD taken from DSM-IV. Symptoms must have persisted for a minimum of 6 months to a degree that is inconsistent with the developmental level of the patient. If 6 or more examples are present in both sections A and B then combined type ADHD is diagnosed, if 6 or more symptoms are met in section A, but not B, the hyperactive/impulsive subtype of the condition is diagnosed and vice versa for the predominantly inattentive subtype (American Psychiatric Association, 1994).

1.5.2 Aetiology of ADHD

The aetiology of ADHD is largely unknown, but hypotheses range from genetic to pathophysiological to environmental origins for the disorder. The clinical complexity of the condition probably reflects a multifarious basis, which incorporates pathophysiological differences caused by genetic differences, which may have been influenced by the environment during development! The Lancet review of ADHD by (Biederman and Faraone, 2005) provides a comprehensive look at the many proposed origins of the condition, while the main points are highlighted below.

The most widely accepted pathophysiological hypotheses for ADHD are disrupted function of the prefrontal cortex and its connection with the basal ganglia and limbic systems, based on the similarity of the symptoms of ADHD and the behaviour of patients with frontal lobe damage, and disruption of the monoaminergic transmission (Davids et al., 2003). In a meta-analysis of brain imaging studies (Seidman et al., 2005) reported decreased volumes of the dorsolateral prefrontal cortex, caudate, pallidum and cerebellum in children with ADHD, highlighting gross physical changes that may give rise to the symptoms of the condition. There is a multitude of evidence for disrupted monoaminergic transmission giving rise to ADHD-like symptoms in both human and animal studies, although it is the dopaminergic hypothesis of ADHD that is the most commonly quoted. The dopaminergic hypothesis arose from the clinical efficacy of stimulant drugs such as amphetamine in improving the behavioural deficits associated with the condition, although the disruption of a single neurotransmitter causing such a complex spectrum of behavioural deficits is now considered an overly simplistic explanation (Gonon, 2009).

Twin and adoption studies have estimated the heritability of ADHD between 60-91% (Thapar et al., 2005). Genetic linkage studies followed by allelic association studies have revealed many susceptibility genes for ADHD, mostly related to monoaminergic

transmission. These include the dopamine transporter (DAT1), dopamine receptors 2, 4 and 5 (DRD2, DRD4 and DRD5), the serotonin 1B receptor (5HT1B), dopamine β hydroxylase (DBH), α2A and 1A receptors (ADRA2A and ADRA1A), tryptophan hydroxylase 1 and 2 (TPH1 and TPH2) as well as several other genes such as synaptosomal-associated protein 25 isoform (SNARE protein SNAP25) (Sharp et al., 2009). The risk factor from each gene is small and therefore ADHD is likely to arise from a combination of factors associated with monoaminergic transmission in fronto-striatal brain regions that have undergone gene-gene and gene-environment interactions (Faraone, 2004; Thapar et al., 2005; Sharp et al., 2009).

Environmental factors have also been linked to the cause of ADHD, including obstetrical complications, older maternal age, and premature birth, although findings have been inconsistent and remain inconclusive (Zappitelli et al., 2001). There is also a correlation between parents that drink and smoke excessively having children with ADHD, therefore exposure to alcohol or tobacco smoke during early development may be a risk factor for the development of ADHD (Streissguth et al., 1994; Milberger et al., 1997).

1.5.3 Neurobiology of ADHD

The neurobiology of ADHD has been studied using a number of methods including electrophysiology, PET scans, lesions studies and pharmacological treatment in a number of behavioural tests and paradigms. Due to the nature of this thesis this section focuses on previous animal, rather than human studies, that have been conducted to assess the underlying neurobiology of the symptoms of ADHD.

1.5.3.1 Attention

A commonly used paradigm to assess attention in rodents is the 5-choice serial reaction time task (5-CSRTT), which is used in this thesis and is described in detail in chapter 4, while in non-human primate studies, visual attention studies involving measurements of gaze are widely used. Previous studies using the 5-CSRTT have helped to elucidate the neural basis of attention, although the definitive circuits are still conjecture. Structures of the basal ganglia play an important role in the modulation of attention, as a ‘disconnection’ of the circuitry via a dual excitotoxic lesion of the medial prefrontal

cortex and the dorsal striatum on the contralateral side significantly reduced levels of attention in the 5-CSRTT (Christakou et al., 2001). Early studies in to the role of neurotransmitters in the maintenance of attention demonstrated that cortical depletion of noradrenaline (NA) impaired attention (Carli et al., 1983). The noradrenaline basis of attention is corroborated by the findings from (Navarra et al., 2008) in which the selective NA reuptake inhibitor, atomoxetine, improved attention in the 5-CSRTT and elevated NA efflux when the attentional load of the task is increased (Dalley et al., 2001). However, the neural basis of attention is more complex than that. Both methylphenidate and atomoxetine that increase attention in the 5-CSRTT also increase extracellular levels of dopamine (DA) and acetylcholine (ACh) in the pre-frontal cortex (Bymaster et al., 2002; Robbins, 2002; Tzavara et al., 2006). Neurochemical inputs via the basal forebrain cholinergic neuronal projections have been proposed to boost 'signal-to-noise ratios', a key feature of attentional function (Everitt and Robbins, 1997; Noudoost et al., 2010). There is a large amount of evidence for the nucleus-basalis-neocortical cholinergic system having the lead role in the control of visual attention in the 5-CSRTT (Muir et al., 1994, 1995, 1996; McGaughy et al., 2002; Risbrough et al., 2002; Harati et al., 2008). A 70% reduction of cholinergic neurons in the nucleus basalis decreased both ACh efflux in the medial frontal cortex and reduced accuracy in the task, thus highlighting a direct relationship between impaired attentional function and ACh (McGaughy et al., 2002). Therefore it is impossible to disseminate the positive effects of methylphenidate and atomoxetine on attentional performance in the 5-CSRTT between the cholinergic and noradrenergic systems (Tzavara et al., 2006). There is ongoing research into which receptor subtype ACh acts through to mediate attention. Accuracy in the 5-CSRTT was reduced by the muscarinic ACh receptor antagonist scopolamine treatment, but was not effected by mecamylamine a nicotinic ACh receptor (nAChR) antagonist (Ruotsalainen et al., 2000), whereas mice carrying a deletion of the β2 subunit of nACh receptors also displayed impaired attentional performance in the test (Guillem et al., 2011). Dopamine is also included in the profile of neurotransmitters shown to regulate attention, via the sensory-cortical regions and PFC (Noudoost and Moore, 2011a). Work using macaque monkeys has pinpointed the frontal eye field (FEF) as the origin of spatially directed attention (Moore et al., 2003; Noudoost et al., 2010), thought to be modulated by the D1 class of DA receptors (Noudoost and Moore, 2011b). However, the 'visual attention' measured in these studies, where the monkey must focus on a single locus on a screen, is different to the 5-CSRTT set-up where the

animals must scan 5 apertures while waiting for the cue light and therefore are likely to involve different neural pathways.

1.5.3.2 Impulsivity

Discussion of human studies concerned with impulsivity and cognitive control of inhibition is covered in an excellent review by (Aron, 2007). In rodents there are several behavioral paradigms used to measure response inhibition including the go/no-go task, the stop-signal task, the 5-CSRTT and delay-discounting tests. Impulsivity and behavioral inhibition in rodents has been extensively covered in two comprehensive reviews (Dalley et al. 2011; Eagle & Baunez 2010), therefore this section serves to highlight a selection of findings. The brain regions most widely associated with impulsivity are the structures of the basal ganglia (Figure 3.3), especially the connectivity between the infra-limbic cortex and the striatum. Lesion studies of different structures have yielded results that highlight the discrete roles that these regions play in impulsive behaviour. For example, lesions of the orbitofrontal cortex and dorsomedial striatum impaired response inhibition in the stop-signal task and 5-CSRTT (Rogers et al., 2001; Chudasama et al., 2003; Eagle and Robbins, 2003a; Eagle et al., 2008), while other fronto-cortical regions such as the infralimbic and prelimbic cortex also play a role in modulation of impulsive responses (Chudasama and Muir, 2001; Chudasama et al., 2003; Eagle and Robbins, 2003a, 2003b). The role of monoamine transmission has also been widely investigated, with serotonin emerging as having an important role in the control of response inhibition and impulsivity. Global serotonin depletion increased premature responding in the 5-CSRTT (Harrison et al., 1997), while blockade of serotonin receptors has resulted in findings that are highly region and receptor specific as to whether improvements or deficits are recorded in impulsivity in the 5-CSRTT (Dalley and Roiser, 2012). The effect of drugs that target the monoamine systems including d-amphetamine (a monoamine reuptake inhibitor), methylphenidate (a monoamine reuptake inhibitor) and atomoxetine (a selective noradrenaline reuptake inhibitor) on behaviour in the 5-CSRTT is discussed in detail in the introduction of Chapter 5.

1.5.4 Co-morbid conditions

ADHD is not a unitary disorder, that is, its symptoms overlap with several other neuropsychiatric disorders. There are many reported co-morbid conditions present with

ADHD including personality disorder, tic disorders, sleep disturbances, depression, anxiety disorders, obsessive compulsive disorder, bipolar disorder, oppositional defiant disorder, conduct disorder and perseveration (Tannock and Schachar, 1992; Sharp et al., 2009; Taurines et al., 2010). However the most well studied co-morbidity is that of substance abuse and addiction in later life (Wilens and Morrison, 2011). One controversial theory is that this stems from the long-term exposure to psychostimulants during childhood treatment of ADHD, however two separate 10-year follow up studies both reported that stimulant treatment neither increases, nor decreases, the risk for substance abuse disorders in later life (Biederman et al., 2008; Mannuzza et al., 2008). It is interesting to note that the neurocircuitry of many of the above disorders, especially that of addiction, overlap with the proposed affected brain regions in ADHD (Koob and Volkow, 2010).

1.5.5 Treatment

The first line treatments for ADHD are psychostimulants, namely methylphenidate (Ritalin) or a mixture of amphetamine salts (Adderall). These drugs are monoamine reuptake inhibitors that work by prolonging the action of central dopamine and noradrenaline which helps to reduce hyperactivity, decrease impulsivity and increase attention, although reports suggest that first line treatment only works in approximately 70% of cases (Wilens, 2008). In healthy subjects these drugs increase activity levels, making their treatment of ADHD paradoxical.

1.5.6 Current animal models of ADHD

A good animal model of ADHD should mimic the symptoms of the condition (face validity), conform to the proposed underlying pathophysiology of the condition (construct validity) and display attenuation of symptoms by treatment that is effective in treating the human disorder and give insight into biological and behavioural aspects of the disorder that have not been observed in clinical situations (predictive validity) (Russell, 2011). There are several complications with analysing animal models of ADHD given the heterogeneity of the clinical symptoms and the different subtypes of the condition (see section 1.5.1). Also the aetiology of the condition is not definitively known, making it very difficult to satisfy the construct validity criterion. There are

many proposed animal models of ADHD, both in rats and mice. These include several genetic mutants (both naturally occurring and artificially produced), as well as animals that undergo brain lesioning or are exposed to neurotoxins, however none of them are fully comparable to clinical ADHD (Davids et al., 2003). Table 1.3 summarises each model in terms of how they satisfy the 'three validities'.

By far the most widely studied model of ADHD is the inbred spontaneously hypertensive rat (SHR) which is often compared to its derivative strain of normotensive Wistar-Kyoto (WKY) rats (Okamoto and Aoki, 1963). SHRs have strong face validity for ADHD as they are hyperactive, impulsive and display attentional deficits that mimic results from children with ADHD in a test of sustained attention (Sagvolden et al., 1993, 2005; Sagvolden, 2000). There is also evidence for ADHD-relevant changes in brain structure such as reduced volume of the prefrontal cortex and occipital cortex (Mignini et al., 2004) and changes in the sequence of the dopamine transporter (DAT) (Mill et al., 2005), thus satisfying the criteria for construct validity. However the main problem with the SHR model of ADHD is its predictive validity. Administration of psychostimulants, such as d-amphetamine and methylphenidate, does not ameliorate the ADHD-like symptoms (van den Bergh et al., 2006). The only other model in Table 1.3 that satisfies all three-core symptoms of ADHD, along with relevant predictive and construct validities is the thyroid hormone receptor β1 (TR-β1) transgenic mouse (Siesser et al., 2006) but the role of the thyroid in ADHD is ultimately unknown, thus limiting the clinical relevance of this model.

Given the strongly implicated role of dopamine in the aetiology of ADHD it is not surprising that many rodent models of ADHD concern disruption of the dopaminergic system (both genetically and pharmacologically induced) (van der Kooij and Glennon, 2007). Brain lesion and neurotoxin induced models include neonatal dopamine lesioning, neonatal hypoxia, prenatal BrdU treatment, and hippocampus irradiation and although they display some symptoms of ADHD, namely hyperactivity, the findings are rarely robust and do not fit the stringent criteria for good animal models of ADHD. The genetic models of ADHD that relate to the dopaminergic system (which include the SHR model outlined above) tend to be more reliable, but again do no cover the entire scope of the condition. For example, the activity of hyperactive wheel-running mice (derived from selective breeding of high voluntary wheel running) is reduced by

methylphenidate (Rhodes and Garland, 2003), but no other face-validity criteria are met thereby restricting its utility as a serious ADHD animal model. Along with the SHR model, the dopamine transporter knockout (DAT-/-) and dopamine transporter knockdown (DAT KD) mice are probably the most useful models. These mice are hyperactive and have impaired impulse response inhibition that can be ameliorated with amphetamine and methylphenidate treatment thereby satisfying all three aspects of a good ADHD animal model (Gainetdinov, 2010).

Table 1.3 Summary table of current animal models of ADHD divided into genetic and pharmacological models. Face validity is split into hyperactivity (hyp), inattention (inat) and impulsivity (imp). A question mark denotes where data are unknown. Table constructed from similar tables in (van der Kooij and Glennon, 2007; Sontag et al., 2010).

1.5.7 The NK1 receptor and ADHD

The findings that showed the NK1R-/- mouse to be hyperactive compared to wildtype controls (face validity) (Herpfer et al., 2005; Fisher et al., 2007), which was reduced with d-amphetamine treatment (predictive validity) (Yan et al., 2010), in conjunction with neurochemical abnormalities inline with those hypothesised to underlie the symptoms of ADHD (construct validity), lead to the NK1R-/- mouse being proposed as a novel model of ADHD. The link between the NK1 receptor and ADHD was furthered in a study of the TACR1 gene (the human equivalent of the NK1 receptor gene). In a study of 450 patients diagnosed with ADHD vs. 600 control subjects four single-nucleotide polymorphisms (SNPs) in the TACR1 gene showed association with ADHD (rs3771829, rs3771833, rs3771856, and rs1701137) (Yan et al., 2010). The NK1R-/- mouse not only therefore meets all the criteria to be a model of ADHD based on its hyperactive phenotype, but there is a strong link between the NK1 receptor gene and susceptibility for ADHD. The final piece of the jigsaw is to explore whether the NK1 receptor knockout mouse also displays behavioural deficits in impulsivity and attention.

1.6 Aims of this thesis

The work presented in this thesis serves to further explore the proposal that the NK1 receptor knockout mouse is a valid model of ADHD. Chapter 3 presents a comprehensive study of the distribution of the NK1 receptor and its preferred ligand substance P throughout the mouse brain. This provides a solid basis to highlight the

brain regions that are most likely to be affected by the functional disruption of the NK1 receptor and relating them to the role of these regions in the neurobiology of ADHD. The triad of diagnostic symptoms of the ADHD is investigated in terms of the phenotype of the NK1R-/- mouse. Activity levels of treatment naïve mice are measured using circular corridor apparatus (Chapter 4) while the 5-choice serial reaction time task (5-CSRTT) is used to measure impulsivity and attention in wildtype and NK1R-/- mice. The findings from the 5-CSRTT are further explored in Chapter 6 by testing wildtype mice in the 5-CSRTT after treatment with NK1 receptor antagonists to see if their behaviour reflects that of the knockout mouse. The final results chapter explores the perseverative phenotype of the NK1R-/- that was initially revealed in the 5-CSRTT. The concluding chapter draws together the findings with possible explanations of the underlying neurobiology that connects the NK1 receptor knockout mouse and ADHD-like symptoms.

2 Materials and Methods

2.1 Subjects

2.1.1 Creation of the NK1R-/- mouse

The original NK1R knockout (NK1R-/-) mouse was created via targeted disruption of the NK1R gene (De Felipe et al., 1998) and is summarised in Figure 2.1. The replacement vector comprised of a clone of the NK1 receptor gene with a cassette containing an internal ribosome entry site (IRES), the lacZ coding sequence and a neomycin resistance gene inserted into the unique *Stu*I site in exon 1 (Figure 2.1 inset). The vector was then electroporated into HM1 embryonic stem (ES) cells, derived from a 129/Sv background. This was followed by negative selection using G418- and GANC-containing media to remove cells without neomycin resistance and random integration of the vector (rather than homologous recombination) respectively. Cells containing the targeted mutation were identified and injected into C57BL/6 blastocysts to create a 129/Sv x C57BL/6 mouse line in which the gene encoding the NK1 receptor was disrupted. Interbreeding of two heterozygous mice for the disrupted allele resulted in healthy homozygous knockout mice.

2.1.2 Background strains

2.1.2.1 MF1 mice

The mice created by de Felipe et al (1998) were on a 129/Sv x C57BL/6 background. Since 129/Sv mice tend to perform badly in behavioural tests such as the Morris Water Maze (WMW) (Wolfer et al., 1997) the mouse line was crossed once onto an outbred MF1 background to rapidly dilute the 129/Sv component.

2.1.2.2 Backcrossed mice

The backcross colony was derived from the original 129/Sv x C57BL/6 mice by backcrossing on to a C57BL/6 (Harlan, Bicester, UK) background for more than 10 generations. C57BL/6 mice are known to perform well in cognitive tests and the homogenous background reduces the problem of flanking genes (see section 1.3.3).

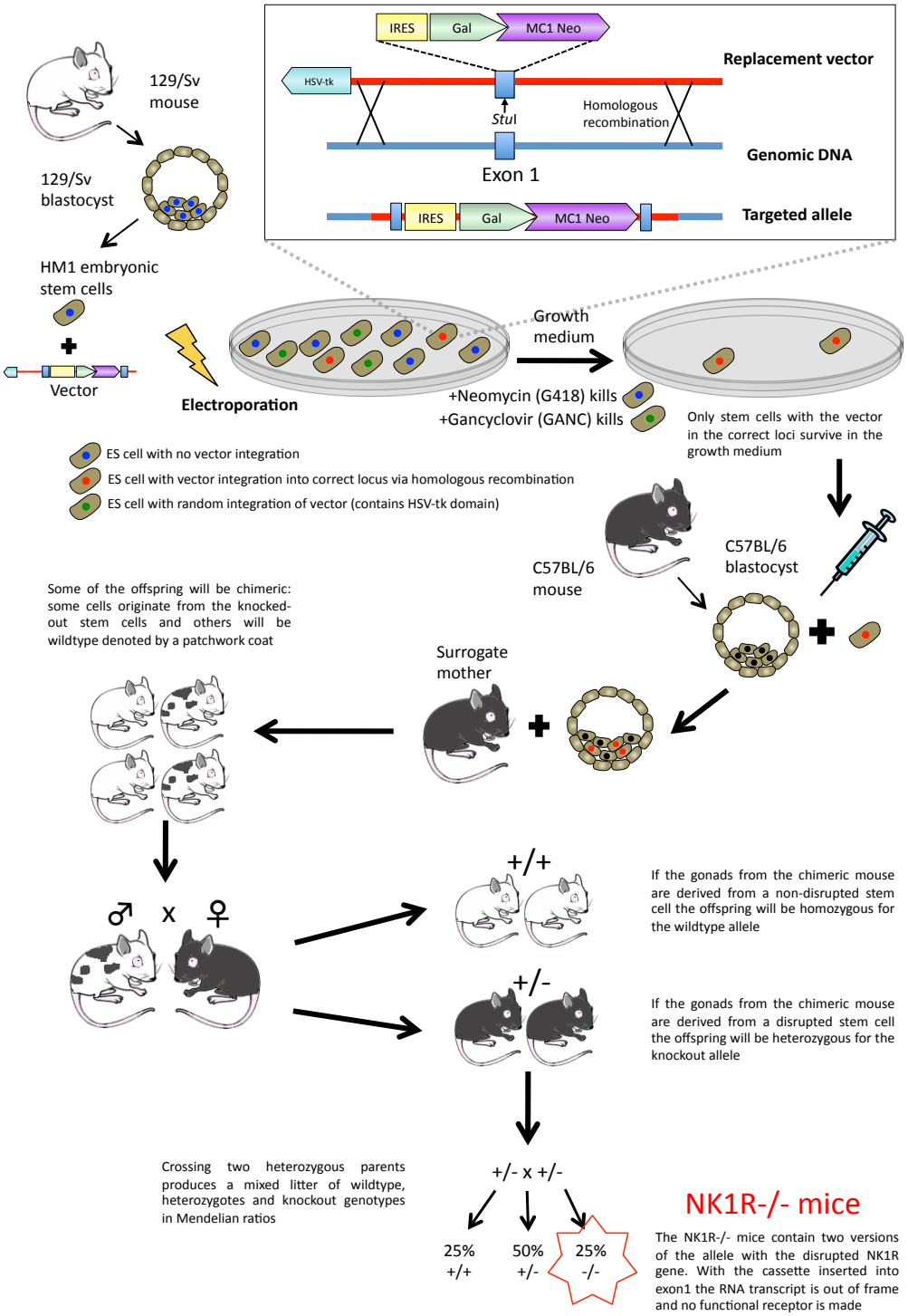


Figure 2.1 Schematic diagram of the creation of the NK1 knockout mouse. IRES, internal ribosome entry site; Gal, LacZ gene for galactosidase; MC1, promotor; Neo, neomycin resistance gene; HSV-tk, herpes simplex virus-thymidine kinase; *Stu*I, unique restriction enzyme site.

2.1.3 Housing

Animals were group-housed in the Biological Services Unit, UCL, London, with 2-5 animals per cage, kept on a 12-hour light:dark cycle (lights on at 8 a.m. with half light from 7-8 (a.m. and p.m.)) at 21 ± 2 °C and 45 ± 5% humidity. Cages were cleaned twice weekly. Food and water was available *ad libitum* (except where stated). Male mice (25-35g) were used for all experiments. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.1.4 Colony maintenance

2.1.4.1 Homozygous breeding

Monogamous breeding pairs were set up with homozygous adults from randomised, non-brother/sister pairings, to minimise the effects of inbreeding. Mating pairs were kept for approximately 8 months, or up to 6 litters, whichever came first. Pups were weaned at 3 weeks of age.

2.1.4.2 Heterozygous breeding

Maternal influences have an important role in the upbringing and therefore behaviour of the offspring. One important aspect of maternal care is the ultrasonic vocalisations made between mother and pups (Branchi et al., 2001). NK1R-/- pups have reduced ultrasonic calls compared to wildtype pups (Rupniak et al., 2000) so to eliminate the effects on mother-pup bonding and upbringing, a colony of heterozygous breeding pairs was established for the MF1 background mice. Offspring from these pairings were a mixture of wildtype, heterozygous and knockout mice and therefore required verification of their genotype before use in experiments. The backcrossed C57BL/6 colony was also maintained with heterozygous breeding.

2.1.5 Verification of genotype

2.1.5.1 Ear punching

Ear clippings were taken from pups aged at least P14 using a 2mm ear punch. The ear was swabbed with ice-cold alcohol prior to the punch. The tissue samples were placed in 0.5ml Eppendorf tubes and kept on ice until the next step of the protocol.

2.1.5.2 Tissue digestion with Hot Sodium Hydroxide and Tris (HotSHOT)

75µl of alkaline lysis reagent (25mM NaOH, 0.2mM disodium EDTA, dissolved in ultra-pure water, pH12.0) was added to each sample and then heated at 95°C for 30mins (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA). After the samples were cooled to 4°C, 75µl of neutralising reagent (40mM Tris-HCl dissolved in ultra-pure water, pH5.0) was added to produce a final buffer solution of 20mM Tris-HCl pH8.1 and 0.1M EDTA.

2.1.5.3 Polymerase chain reaction (PCR) for DNA amplification

6µl of sample was added to 19µl of master mix that contained three primers for the amplification of the NK1 receptor DNA: NK1-F, NK1-R and NeoF. The samples were then placed again in the thermocycler for DNA amplification. Full primer sequences, master mix recipe and PCR conditions are outlined in detail in Appendix A2.

2.1.5.4 Agarose gel electrophoresis

Samples were run on a 2% agarose gel (2g agarose dissolved in 100ml 0.5x Tris-borate-EDTA (TBE) buffer (National Diagnostics, Hull, UK)) with 8µl of ethidium bromide (EtBr), which fluoresces strongly under ultra-violet light when bound to DNA. 500ml of TBE buffer (with 2µl of EtBr) was poured into the gel tank. 4µl of loading buffer was added to each sample and a total of 15µl was loaded into the wells. The gel was run at 100-120mV using a FEC105 Voltage Power Pack for approximately 1 hour after which it was visualised and photographed on an ultra-violet transilluminator plate (UVP Ltd, Cambridge, UK). For homozygous wildtype and NK1R-/- mice a single band is seen corresponding to 350 and 260 bases respectively. For heterozygous animals 2 bands are present corresponding to 350 and 260 bases. Samples for genotyping were always run against positive control samples of known wildtype and NK1 receptor mice and a negative H_2O control (Figure 2.2).

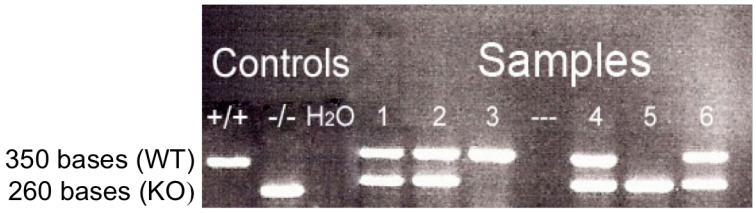


Figure 2.2 An example gel for verification of genotype of a litter of six pups. On the left are controls for +/+ (wildtype), -/- (NK1 receptor knockout) and a negative H_2O control. In this litter, there were 4 heterozygous mice (numbers 1,2,4 and 6), one wildtype and one knockout mouse giving a littermate pair (numbers 3&5 respectively). The litter of 6 was split into 2 cages that are separated on the gel by a blank lane.

2.1.5.5 Confirmation by immunohistochemistry

Another method for genotype verification is by immunohistochemical staining for the NK1 receptor (as outlined in section 2.2), although this should only be used as a secondary measure after genotyping by PCR. This method can only distinguish between wildtype and knockout mice, as heterozygous mice appear very similar to wildtype. Absence of staining for the NK1 receptor denotes tissues from a NK1 receptor knockout mouse (Figure 2.3B).

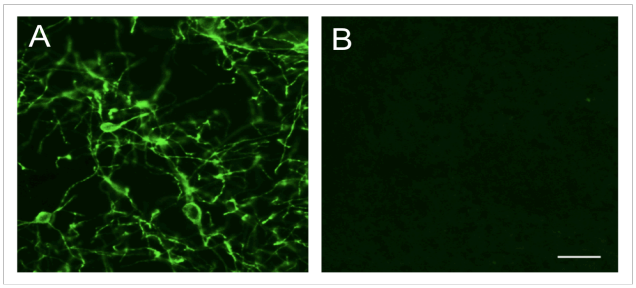


Figure 2.3 Representative immunofluorescent staining for the NK1 receptor in the dorsal striatum of a wildtype (A) and NK1 receptor knockout (B) mouse. Primary antibody was rabbit anti-NK1R (1:10000), with TSA amplification (section 2.2.3.2). Scale bar =40μm.

2.1.6 Culling

After experimentation, if tissue was required for immunohistochemical staining (see below) mice were perfused (section 2.2.1.1), otherwise mice were culled by CO_2 asphyxiation in a sealed chamber where the percentage of CO_2 was gradually increased. After cessation of breathing and visible heart beat, cervical dislocation was performed as a secondary measure.

2.2 Immunohistochemistry

The basis of immunohistochemistry (IHC) is to identify cellular constituents using a visible marker that labels specific antigen-antibody binding sites. The majority of immunohistochemistry in this thesis (Chapter 3) is staining for the NK1 receptor and it's preferred ligand Substance P, using avidin/biotin amplification (section 2.2.3.1) and chromogenic visualisation with nickel (section 2.2.4.1).

2.2.1 Tissue preparation

Good tissue preparation is fundamental in achieving successful immunohistochemistry. Fixation of tissue with paraformaldehyde (PFA) irreversibly cross-links the basic amino acid residues of proteins via formation of a methylene (CH_2) bridge. This maintains cellular structure and the *in vivo* locations of sub-cellular components as well as protecting against tissue degradation.

2.2.1.1 Perfusion

Mice were terminally anaesthetised by intraperitoneal (i.p.) injection of 0.3ml pentobarbital sodium (200 mg/ml; Euthatal, Harlow, UK) before being intracardially perfused with 20ml ice-cold heparinised 0.9% saline, followed by 20ml of 4% paraformaldehyde (PFA) in 0.1M PB.

2.2.1.2 Post-fix and cryopreservation

Brains were removed from perfused animals and post-fixed overnight in 4% PFA. Brains were then transferred to 30% sucrose in 0.1M PB containing 0.02% NaN_3 for cryoprotection and stored at 4°C for a minimum of 24 hours prior to sectioning.

2.2.1.3 Sectioning

Brains were mounted onto a pre-cooled sliding microtome (SM200R; Leica Microsystems, Milton Keynes, UK) and frozen with dry-ice. 40μm coronal sections were cut and collected in 5% sucrose solution (made in 0.1M PB) in serial sets of six.

2.2.1.4 Blocking

To reduce background staining and minimise non-specific binding of the primary antibodies the sections are first incubated in 'blocking solution'. This solution is made up in 0.1M phosphate buffer and comprising 0.03% Triton X-100 (for cellular permeabilisation) and 0.3% normal (non-immune) serum (preferably from the host species of the secondary antibody). If the stain is for chromogenic visualisation with DAB (section 2.2.4.1), 0.2% H_2O_2 must be added to the blocking solution to quench endogenous peroxidase activity of the tissue.

2.2.2 Direct staining

Specific antibodies to proteins are raised by injecting an animal such as a goat or rabbit, with a short peptide corresponding to part of the sequence of the protein under

investigation conjugated to a large carrier protein such as bovine serum albumin (BSA). Plasma is collected from the animal and the generated antibodies are purified. Primary antibodies are applied to tissue sections where they bind to the appropriate antigen. The tissue is then incubated in a secondary antibody that is conjugated to a visible marker. Visible markers can either be chromogenic or fluorescent (see section 2.2.4).

2.2.3 Amplification methods

Direct staining usually requires the antibodies to be used in high concentrations, leading to higher background staining, and an increase in expense. Amplification methods increase the sensitivity of the stain without loss of resolution or increase in background. Using amplification systems means that primary antibodies can be used at concentrations up to 1000-fold lower than for a direct stain.

2.2.3.1 Avidin/biotin system

Avidin is a glycoprotein found in egg white and has a high affinity for biotin, a small molecular weight vitamin. Each avidin molecule can bind up to four biotin molecules. Biotin is conjugated to a secondary antibody (raised against the primary antibodies host animal e.g. biotinylated anti-rabbit secondary antibody). A visible marker such as fluoroscein isothiocyanate (FITC) is conjugated to avidin, which binds to the biotinylated secondary antibody, thus allowing visualisation of the antigen of interest.

2.2.3.2 Tyramide Signal Amplification (TSA)

Tyramide signal amplification also uses the avidin/biotin system and further improves the signal-to-noise ratio. Horseradish peroxidase (HRP) is added to catalyse the addition of biotinylated tyramide solution to the antigens already bound to the biotinylated secondary antibody. The binding of the biotinylated tyramide is fast and covalent (the reaction intermediate dimerises with tyrosine residues on the endogenous protein). A marker is then used to visualise the complex (e.g. FITC-avidin).

2.2.4 Visualisation methods

2.2.4.1 Chromogenic staining (DAB staining)

3,3'-diaminobenzidine tetrahydrochloride (DAB) is a chromogenic electron donor in the presence of the enzyme HRP which catalyses the oxidation of DAB to form an

insoluble brown polymer. Using an HRP-avidin complex, the reaction product can be used to visualise complexes surrounding the primary antibody. Nickel ions can be added to the DAB reaction mixture to produce a more intense grey-black precipitate.

2.2.4.2 Fluorescent staining

Fluorescent dyes bound to avidin are often used to visualise labelled antigens. The dyes are excited at a specific wavelength of light that then emit light at a different wavelength, for example, FITC fluoresces green (515nm) when excited at a wavelength of 495nm. Because different dyes are excited by different wavelengths, they can be used to label separate antigens in the same tissue, a common method used to check for co-localisation of antigens.

2.2.5 Imaging

Sections were viewed using a Leica DMR microscope (Leica Imaging Systems Ltd., Cambridge, UK), equipped with a CCD camera (C-4742-95; Hamamatsu Photonics, Hamamatsu, Japan). Images were captured with OpenLab 4.0.4 software (Perkin Elmer, Waltham, MA, USA). Images for the NK1 receptor and substance P atlas were taken at x5 magnification and subsequently stitched together using Photoshop 7.0 (Adobe, San Jose, CA, USA).

2.3 Locomotor activity

Locomotor activity was measured over a 48-hour period using circular corridor apparatus.

2.3.1 Apparatus

A bank of eight circular corridors (Imetronic, Pessac, France) was used. Each corridor had a total diameter of 17cm, with a 5cm wide corridor and was 15cm high (Figure 2.4). Four infra-red beams were evenly spaced around the corridor 1cm above the floor. Beam breaks were detected and recorded via an electronic interface and logged using Imetronic software running on a connected PC. A consecutive break in two adjacent beams (one quarter-turn) was considered a unit of locomotion. The bank of corridors was enclosed and ventilated. Animals had access to food and water throughout the 48-hour experiment.

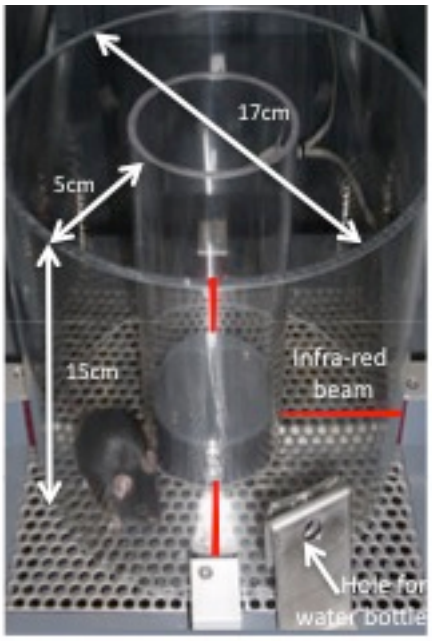


Figure 2.4 Set-up of circular corridor apparatus. During the experiment a water bottle is placed through the hole and food is placed in a hopper that hangs inside the corridor. Two consecutive beam breaks represent a unit of locomotor activity.

2.3.2 Lighting

All the corridors were individually lit at the same level (120 lux). The lights were programmed on a 12 hour light/dark cycle (lights off at 19.30 and on at 07.30) to mimic the timings of the animal house as closely as possible (see section 2.1.3).

2.4 5-choice serial reaction time task (5-CSRTT)

The 5-choice serial reaction time task requires the animal to scan five evenly spaced apertures until a light cue is presented, after which a nose-poke response is required in the correct spatial location (Bari et al., 2008). The 5-CSRTT can measure both attentiveness and impulsivity in a single test and therefore provides an excellent paradigm in which to test the hypothesis that the NK1 receptor knockout mouse is a model of ADHD.

2.4.1 Test Apparatus

The test apparatus consisted of four mouse operant chambers, each housed within a ventilated sound-attenuating box (Med Associates, St. Albans, VT, USA). The internal chamber had a curved wall on the left hand side, in which there were 5 equally spaced holes, each with an infrared detector inside them to detect nose-poke responses (
Figure 2.5A). These holes were lit with yellow stimulus lights, located inside each hole. On the opposite wall of the chamber was a larger hole into which the reinforcer was delivered (0.01ml of 30% condensed milk solution) (
Figure 2.5B), signaled by illumination of the hole. Head entries into the magazine, to collect the reward, were also recorded via the interception of an infrared beam. A house light, to illuminate the chamber, was mounted above the magazine. The presentation of stimuli and the recording of the responses were controlled by a Smart Ctrl Package 8IN/16OUT with an additional interface by MED-PC-IV for Windows (Med Associates, St. Albans, VT, USA).

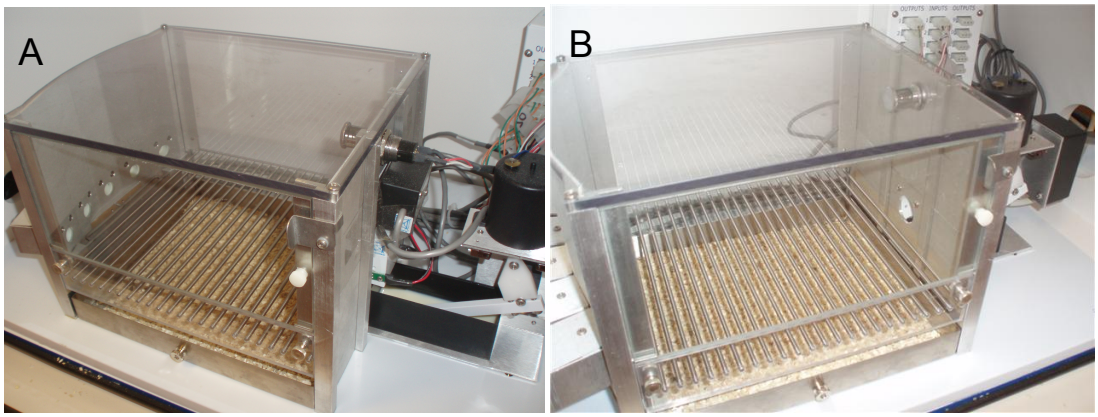


Figure 2.5 5-choice serial reaction time task apparatus, the five cue apertures are on the left-hand wall (A) with the magazine hole directly opposite (B).

2.4.2 5-CSRTT Habituation

During the first three sessions the animals were placed in the boxes for 30 minutes, and had access to the liquid food reward on a continuous schedule. To obtain the reward the animal had to nose poke into the food magazine, which raised the dipper and gave access to the milk solution for 10s. The house light, food magazine and stimulus lights remained on for the duration of the habituation sessions.

After the initial 3 days of apparatus habituation, the animals were trained on a non-spatial schedule. The 30-minute sessions began with the dipper arm raised and the magazine light on. To start the session the animal nose-poked into the magazine and ate the reinforcer. The stimulus lights in all five holes were then illuminated and the reward was only presented after a nose-poke into one of the five holes. To pass this procedural habituation training stage, the animal needed to earn more than 50 reinforcers on two consecutive sessions up to a maximum of 10 sessions.

2.4.3 5-CSRTT Training

After the initial habituation period, mice were trained through stages 1-6 of the 5-CSRTT, with each stage getting progressively harder and thus requiring more attention to be paid. To pass each stage the animal had to reach certain performance criteria (see Table 2.1).

Every training session began with the illumination of the food magazine light and the free delivery of the liquid reinforcer via the dipper arm. The first trial was initiated by a nose-poke into the food magazine by the animal. After a fixed interval (the inter-trial interval (ITI)), one of the holes was illuminated for a short amount of time, known as the stimulus duration (SD). The animal then had a set period of time (limited hold, LH) to respond with a nose-poke into the correct hole in order to receive the reward. After a correct response, the magazine light would come on and the milk reward was made available. Collection of the reinforcer initiated the next trial.

Stages	Parameters used			Passing criteria
	SD (s)	LH (s)	ITI (s)	
1	30	30	2	> 30 correct trials for 2 consecutive sessions
2	20	20	2	
3	10	10	5	> 50 correct trials for 2 consecutive sessions
4	5	5	5	> 50 correct trials, > 75% accuracy, < 25% omissions, for 2 consecutive sessions
5	2.5	5	5	
6	1.8	5	5	> 50 correct, > 75% accuracy < 25% omissions 7 sessions required with the final 3 reaching criteria

Table 2.1 Summary of training stages 1-6 parameters and criteria required to pass each stage

When the animal performed an incorrect response (nose-poking into one of the four non-illuminated holes), or an omission error (failing to respond to any of the five holes within the allowed time), or a premature response (response into the holes during the ITI, i.e. before the presentation of the stimulus), the house light was extinguished for 5s (Time Out, TO). Responses made into the holes during this period restarted the TO. After a TO period, the next trial was restarted by a nose-poke into the magazine. Perseverative responses, (additional responses into the holes after a correct response and before the collection of the reward), were recorded but had no programmed responses. Sessions lasted for 100 trials or 30 minutes, whichever came first.

2.4.4 5-CSRTT Tests

After the training phase, and a prolonged stable performance at stage 6 (baseline) for a minimum of 3 consecutive sessions, the animals were subject to two different tests that demanded increased attention. The long inter-trial interval (LITI), where the ITI is increased from 5 seconds to 7 seconds, and the variable inter-trial interval (VITI) with a random schedule of four ITI alternatives (2, 5, 10 or 15 seconds) (Table 2.2). The duration of the test sessions was increased to 45 minutes (or 100 trials). The stimulus duration for the VITI was the same as stage 6 (1.8s), but unfortunately due to a programming error the SD for the LITI was decreased to 1.0s, increasing the attentional load furthermore.

Tests	**Parameters used**		
	SD (s)	LH (s)	ITI (s)
Long ITI (LITI)	1*	5	7
Variable ITI (VITI)	1.8	5	2, 5, 10, 15

Table 2.2 Summary of parameters used in the tests of 5-CSRTT. * At the completion of the experiment it was realised the LITI stimulus was set at 1s rather than 1.8s

2.4.5 Behavioural scoring

The following variables of the 5-CSRTT training and tests were recorded and analysed:

- **Total number of trials required to pass the training phase**: the sum of all the trials completed at each training stage (for analysing the data obtained from Training Stage 1-6 only).
- **Total number of trials completed in each test session:** total correct response + total incorrect + total omission (for analysing the data obtained from the fixed long ITI and variable ITI tests, only).
- **% Accuracy:** correct responses / (correct + incorrect responses) x 100
- **% Omissions:** total omissions / (correct + incorrect responses + omission) x 100
- **% Premature responses:** premature responses / (correct + incorrect + omission + premature responses) x 100
- **Latency to correct response:** latency to nose-poke into the correct hole after the onset of stimulus (seconds)
- **Latency to collect reward:** latency to collect the reward after a correct response (seconds)
- **Perseveration:** total number of responses made into the holes after a correct response but before the collection of the reward.

2.5 Species-typical behaviours

The other behavioural tests used in this thesis in addition to the 5-choice serial reaction time task are marble-burying and burrowing. These behavioural paradigms are designed to measure species-typical behaviours such as digging, and unlike the majority of animal experiments, actually provide the animals with a form of environmental enrichment rather than a negative experience. Here the tests are used as a measure of perseverative behaviour i.e. the repetition of digging bouts in the marble-burying test which has been shown to be sensitive to animal species, strain, lesions and drug treatments (discussed further in Chapter 7). They are cheap, quick to run and require no specialist equipment.

2.5.1 Marble burying

2.5.1.1 Apparatus

The apparatus for the marble burying test comprised of a covered Perspex box (30 (l) x 19 (w) x 18 (h) cm), filled with sawdust (Litaspen Premium, Lillico Biotechnology, Surrey, UK) to a depth of 5cm. 20 black marbles (1.5 cm diameter) were placed on top of the sawdust in an evenly spaced pattern approx. 4cm apart (Figure 2.6A). The test was conducted with 2 test boxes next to each other.

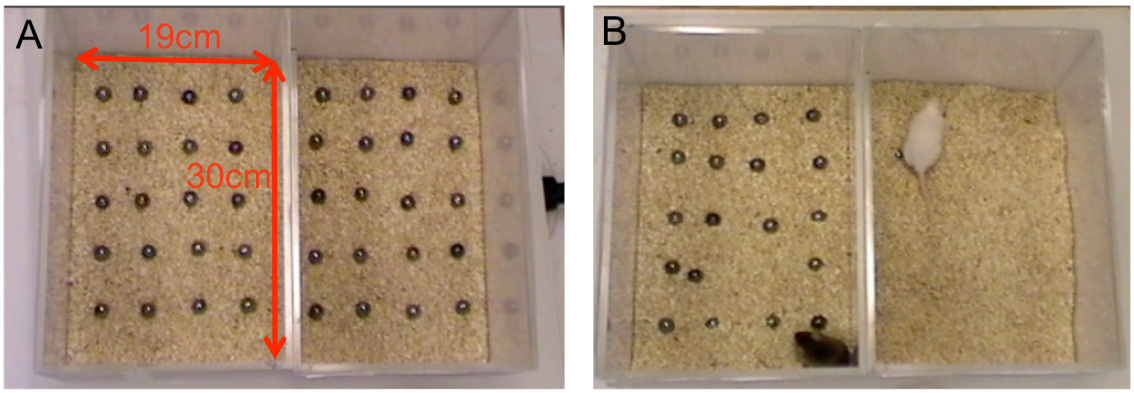


Figure 2.6 Experimental set-up for the marble-burying test (A). After 30 minutes the mice are removed and the number of marbles buried by more than two-thirds is recorded (B). In this example the NK1 receptor knockout mouse (left) buried 3 marbles and the wildtype (right) hand side buried all 20 marbles.

2.5.1.2 Procedure

The mice were habituated to the boxes without the marbles the day prior to the test for 15minutes. For the test the mice were placed in the boxes for 30minutes, after which the animals were returned to their home cages and the number of marbles buried by more than two-thirds was recorded. The tests were videoed using a Panasonic SDR-100 camcorder for behavioural scoring.

2.5.1.3 Behavioural scoring

The videoed tests were going to be used to score the latency to begin digging and the number of digging bouts, defined as a coordinated movement of the front paws followed by a kick of the hind paws, performed during the 30minute tests. However another behaviour, a displacement of sawdust by the front paws and muzzle, was prevalent when the videos were viewed. As this behaviour contributes to the burying of marbles the total number of 'nose-pushes' and the latency to the first nose-push were scored alongside digging bouts. The videos were scored in 5minute time bins to allow for further analysis.

2.5.2 Burrowing

2.5.2.1 Apparatus

Each burrow consisted of 20cm of black drainpipe (6.8cm diameter) with two 5cm machine screws bolted 1cm from the end to raise the opening 2.5cm from the floor. The opposite end of the burrow was sealed with a metal lid (Figure 2.7A&B). The burrowing substrate used in this experiment was gravel (8mm pea-shingle, B&Q, Hampshire, UK) (Figure 2.7C).

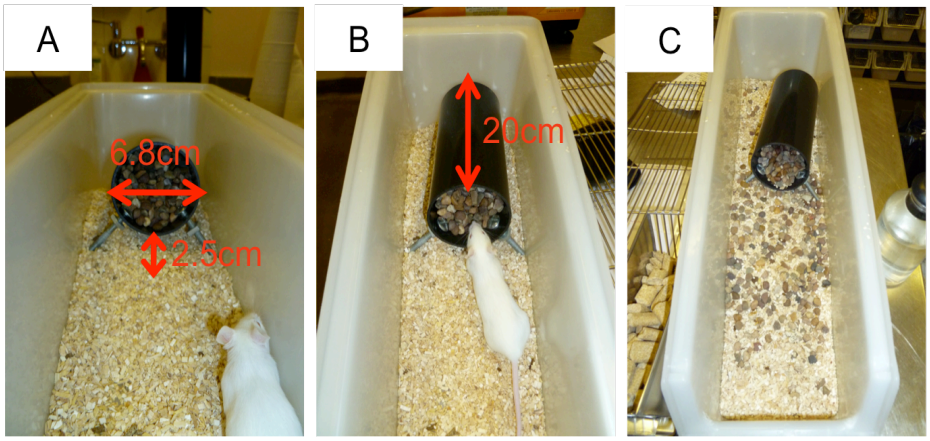


Figure 2.7 A full burrow in situ in the home cages with dimensions (A&B). During the test sessions, the pea-shingle is displaced from the burrow (C).

2.5.2.2 Procedure

Rodents burrow spontaneously, but their performance tends to improve with practice. To habituate the mice to the burrows and substrate, full burrows were placed in the home cages while the animals remained in group housing for two consecutive nights (burrows in at 5.00p.m and removed the next morning at 9.30a.m). The mice were then separated into individual cages for the burrowing tests, one overnight test and three 2-hour probe tests on consecutive days. Two different length tests are studied in case a ceiling effect is reached in the overnight test. Before each test the burrows were filled to approximately 1.5cm from the top and weighed before being placed in the cages. The probe tests were run from 3.00-5.00pm on three consecutive days. At the end of each test the burrows were removed and reweighed to calculate the gross weight of gravel

displaced from the burrow, which was subsequently calculated as a percentage of the initial weight.

2.6 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical tests were carried out using IBM Statistics version 19 for Mac (SPSS Inc, Chicago, Illinois, USA). $P<0.05$ was considered statistically significant. Specific statistical methods for each individual experiments are detailed in the results chapters.

3 Immunohistochemical localisation of the NK1 receptor and its preferred ligand substance P (SP) throughout the mouse brain

3.1 Introduction

The focus of this thesis is to investigate the potential role of SP and the NK1 receptor in the aetiology of ADHD. Therefore knowledge of the distribution of NK1 receptor and its ligand substance P throughout the brain is essential for the analysis and interpretation of behaviours implicated to the SP-NK1 receptor pathway. Previous studies of NK1 receptor distribution have been conducted in a range of mammalian species, but to date there is no published study of the distribution of the NK1 receptor, and its preferred ligand SP, throughout the mouse brain. This chapter serves to fill this void by using immunohistochemistry to describe the distribution of NK1 receptor and substance P throughout the mouse brain with comparison to previous NK1 receptor distribution studies in other species, while also considering those brain regions implicated in the aetiology of ADHD.

3.1.1 Previous NK1 receptor distribution studies

3.1.1.1 Radio-ligand binding

Early studies into the distribution of NK1 receptors in the central nervous system (CNS) examined the binding patterns of radio-labelled ligands of the NK1 receptor using autoradiography. In adult rat brain, binding of tritiated or iodinated substance P was noted in high concentration in the external layers of the olfactory bulbs, medial amygdala, dentate gyrus, superior colliculus, dorsal parabrachial nucleus and locus coeruleus. Moderate binding levels were seen in the nucleus accumbens, caudate putamen, globus pallidus, periaqueductal gray, lateral and medial septum and subiculum (Quirion et al., 1983; Mantyh et al., 1984; Rothman et al., 1984; Shults et al., 1984; Buck et al., 1986). One confounding aspect of these early findings is the lack of exclusivity in the binding properties of SP to the tachykinin family of receptors.

Although SP binds preferentially to NK1, it also exhibits limited binding to NK2 and NK3 receptors (Table 1.1 in the Introduction chapter). Thus, later autoradiographic distribution studies compared tachykinin-binding sites using NK1-, NK2- and NK3-specific ligands and more selective NK1 receptor ligands. In adult rat brain the findings for NK1 receptor distribution were largely identical to those noted previously due to the sheer predominance of the NK1 receptor in comparison to NK2 and NK3 receptors (Saffroy et al., 1988; Dam et al., 1990; Dietl and Palacios, 1991). Inter-species comparisons have highlighted the conservation of NK1 receptor distribution throughout the vertebrate CNS with an apparent increase in receptor density through evolution (Dam and Quirion, 1986; Dietl and Palacios, 1991; Rosen et al., 1993; Rigby et al., 2005).

3.1.1.2 In-situ hybridisation

In situ hybridisation uses labeled complementary DNA (cDNA) or RNA to localise specific DNA or messenger RNA (mRNA) sequences within a piece of tissue. In the rat brain hybridisation of oligonucleotides unique for the NK1 receptor yielded strong labeling in the diagonal band (both vertical and horizontal limbs, medial septal nucleus, nucleus basalis magnocellularis, suprachiasmatic nucleus, dorsal and ventral tegmental areas, hypothalamus, and structures of the basal ganglia (including the striatum) (Elde et al., 1990; Gerfen, 1991; Maeno et al., 1993; Mick et al., 1994). Findings from feline brain tissue reported laminated staining of the visual cortex and labeling of hypothalamic structures to varying degrees (weak labeling: posterior medial and lateral hypothalamus; moderate labeling: anterior medial hypothalamus and strong labeling of the basal amygdaloid complex) (Matute et al., 1993; Yao et al., 1999). Co-localisation of choline acetyltransferase (ChAT) and NK1R mRNA in the striatum was noted firstly in rat (Gerfen, 1991) and later confirmed in human striatal slices (Aubry et al., 1994). It must however be taken into consideration that mRNA levels may not necessarily correspond directly to the amount, nor final location, of the translated protein. This confounding factor is negated with immunohistochemical visualisation, since it is the in situ protein that is targeted.

3.1.1.3 Immunohistochemistry

Immunohistochemistry uses antibodies to bind to unique epitopes of proteins that are then visualised either by fluorescent or chromogenic methods. The high specificity and contrast of the staining enabled high-resolution images that clearly showed NK1 receptors on cell bodies and dendrites for the first time. (Nakaya et al., 1994) completed a comprehensive map of SP and NK1 receptor distribution in the rat brain which in large supported previous findings from other studies. Strong immunohistochemical staining was recorded in cortical amygdaloid nucleus, hilus of the dentate gyrus, locus coeruleus; moderately in the caudate-putamen, nucleus accumbens, olfactory tubercle, median, pontine, and magnus raphe nuclei; and sparsely in the cerebral cortex, basal nucleus of Meynert, claustrum, gigantocellular reticular nucleus, and lobules IX and X of the cerebellar vermis. Distribution of the NK1 receptor was comparable in guinea-pig brain to that of the rat (Yip and Chahl, 2001). Other immunohistochemical studies of the NK1 receptor and ligand have tended to focus on specific brain regions such the striatum, where NK1 receptors were found on 3% of neurons which were all large and aspiny (Shigemoto et al., 1993), these are now known to be cholinergic and somatostatinergic interneurons (Kaneko et al., 1993; Oda and Nakanishi, 2000). Interspecies comparisons have revealed differences, namely in the regions that control circadian rhythms. Staining of the intergeniculate leaflet was comparable in rat, hamster and mouse tissue, but double staining for SP and the NK1 receptor in the suprachiasmatic nucleus was only seen in hamster and rat, and not mouse (Mick et al., 1994; Piggins et al., 2001). This is further explored in Chapter 4 for this thesis. The NK1 receptor and SP are abundant in structures of the basal ganglia of non-human primates including the caudate, putamen, globus pallidus (internal and external segments) and ventral pallidum (Parent et al., 1995; Mounir and Parent, 2002; Lévesque et al., 2006). In human brain tissue, prefrontal and visual cortex, nucleus basalis, hippocampus and basal ganglia (including the striatum, globus pallidus, nucleus accumbens) all stain positively for the NK1 receptor (Burnet & Harrison 2000; Kowall et al. 1993; Mounir & Parent 2002; Parent et al. 1995; Tooney et al. 2000).

3.1.1.4 Positron emission tomography (PET)

Positron emission tomography is typically used in medical imaging and measures gamma rays released from a positron-emitting tracer. In the instance of distribution

studies for the NK1 receptor, radiolabeled antagonists are used such as ^{11}C-GR-205171 (Engman et al., 2012). The advantage of this technique is that it provides a non-invasive method of measuring receptor distribution. Early animal work with Syrian hamsters established that a modified, radiolabeled antagonist could penetrate the blood brain barrier (Livni et al., 1995) and the highest binding levels were found in the striatum and cortex of the guinea-pig brain (Solin et al., 2004). In humans, the strongest responses originate from the caudate and putamen, with moderate binding in the thalamus, globus pallidus, substantia nigra, and cortical regions that include the frontal, temporal, occipital and anterior cingulate cortices (Hargreaves, 2002; Hietala et al., 2005; Okumura et al., 2008; Engman et al., 2012). PET has also been used to establish the therapeutically relevant dose of aprepitant (the only NK1 receptor antagonist in clinical use) (Bergström et al., 2004).

3.1.2 Mismatch of SP and NK1 receptor

For an as-yet unknown reason, receptor density does not always correlate with that of its preferred ligand (Herkenham, 1987). This is the case for the NK1 receptor and substance P in several brain regions. This region most widely described with mismatched staining is the substantia nigra, which is rich in substance P, yet has very low levels of NK1 receptors. This has been noted with all of the techniques outlined above across a range of mammalian species (Mantyh et al., 1984; Rothman et al., 1984; Shults et al., 1984; Gerfen, 1991; Maeno et al., 1993; Nakaya et al., 1994). In rat brain tissue, other regions with high levels of SP relative to that of the receptor include the interpeduncular nucleus and the medial amygdaloid nucleus, whereas the dentate gyrus, inferior colliculus and dorsal tegmental area stain intensely for the presence of NK1 receptors, but weakly for SP (Nakaya et al., 1994). The most likely explanation is that due to its small size, SP diffuses across large distance relatively quickly so NK1 receptors in SP-poor regions may receive SP that is released form distant axon terminals (Herkenham, 1987).

3.1.3 Distribution of NK1R and SP in brain regions associated with the symptoms of ADHD

The focus of this thesis is to investigate the potential role of SP and the NK1 receptor in the aetiology of ADHD. The brain regions associated with control of attention and impulsivity mainly fall within the basal ganglia system and are therefore considered in greater detail in the discussion of this chapter. The specific cortico-striatal circuitry associated with the symptoms of ADHD are summarised in Figure 3.1.

Figure 3.1 Hypothesized cortico-striatal circuits responsible for different cognitive functions and the processes affected by lesioning specific cortical and subcortical structures in the rat. References: 1 Muir et al., (1996); 2 Rogers et al., (2001); 3 Chudasama et al., (2003); 4 Christakou et al., (2004); 5 Mobini et al., (2002); Winstanley et al., (2004) asterisk indicates depending on procedure used; 6 Cardinal et al., (2001); 7 Eagle et al., (2008b), Dagger denotes Bari and Robbins unpublished; 8 Eagle and Robbins (2003). Abbreviations: ACC Anterior cingulate cortex, d Dorsal, dm Dorso- medial, IL Infralimbic cortex, Nac Nucleus accumbens, OFC Orbitofrontal cortex, STR Striatum. Figure taken from (Bari and Robbins, 2011)

3.2 Materials and Methods

3.2.1 Subjects and tissue preparation

Male wildtype mice from the homozygous bred MF1 colony (129/Sv x C57BL/6 back-crossed with an outbred MF1 strain: see section 2.1.2), of approximately 10 weeks of age (n =3) were transcardially perfused, and their brains removed and sliced coronally using a freezing microtome into 40μm sections.

3.2.2 Immunohistochemistry

Immunohistochemistry was performed with primary antibodies raised against the NK1 receptor and its ligand, substance P, on every 6th section through the brain from the prefrontal cortex (approximately 2.5mm rostral to bregma) to the brainstem (approximately 7mm caudal to bregma). The DAB method of visualisation was used because of the greater contrast between stained, and non-stained brain regions. The sections were first incubated for one hour in blocking solution (0.1M PB containing 3% normal goat serum (NGS), 0.3% Triton X-100 plus additional hydrogen peroxide (2%)). This was followed by incubation over-night at room temperature (or 3 days at 4°C) in either rabbit anti-NK1 receptor (1:5000, a generous gift from P.W. Mantyh) or rat anti-Substance P (1:200, Abcam). Sections were subsequently incubated in biotinylated

secondary antibodies (1:250, goat-anti-rabbit and goat-anti-rat for NK1 receptor and SP respectively, Vector Laboratories, Burlingame, CA, USA) for 90 minutes and then in avidin-biotin solution (1:1000) from a Vectastain Elite ABC Kit (Vector Laboratories,) for a further 30 minutes. The signal was developed using a DAB Substrate Kit for Peroxidase, with added nickel for a blue/black stain (Vector Laboratories). The sections were mounted on gelatin-subbed slides, left to air-dry, then dehydrated through a series of alcohols and coverslipped using DPX mounting medium. A detailed protocol for this DAB stain is given in Appendix A3.

3.2.3 Imaging

The distribution of NK1 receptor immunoreactivity was comparative in the three brains used in this experiment. The best slices from the NK1- and SP-stained sets were selected for imaging and paired according to their position from bregma. Sections were viewed using a Leica DMR microscope (Leica Imaging Systems Ltd., Cambridge, UK), equipped with a CCD camera (C-4742-95; Hamamatsu Photonics, Hamamatsu, Japan). Images were captured with OpenLab 4.0.4 software (Perkin Elmer, Waltham, MA, USA). Four images per slice were taken for the NK1 receptor and substance P atlas at x5 magnification and later stitched together using Photoshop 7.0.

3.3 Results

Representative photomicrographs are shown in Figure 3.2, with SP stained images on the left- and NK1 receptor on the right-hand panel. An approximate value for the distance relative to bregma is given for each pair in the top left corner. Brain regions and bregma positions are taken from the mouse brain atlas (Franklin & Paxinos, 1997) and confirmed using The Allen Mouse Brain Atlas, an interactive mouse brain atlas

(found at http://mouse.brain-map.org/static/atlas). NK1 receptor staining was observed mostly in cell bodies and dendrites throughout the mouse brain, however regions of more diffuse staining were also seen such as in the central amygdaloid nucleus. Axonal SP staining is harder to discern in individual neurons even at higher magnification. Regions of relevance to the work in this thesis, along with regions of notable mismatched staining are summarised in Table 3.1 on page 78.

3.3.1 Cerebral Cortex

In general, staining for the NK1 receptor or SP is weak in the 6 defined layers that make up the neocortex, with a slight graduation from the surface (lamina I) through to weaker staining in the deeper laminae (Figure 3.2 F'). Staining of the NK1 receptor is stronger in the somatosensory and piriform cortices (Figure 3.2E' & F' respectively) but is not discernable in the tissue stained for SP (Figure 3.2E'-F').

3.3.2 Basal Ganglia

NK1 receptors and substance P are found abundantly in the structures that comprise the basal ganglia, although staining of receptor and ligand appears to be inversely correlated. NK1 receptor staining is strong in the caudate putamen (Figure 3.2E'-L'), moderate in the nucleus accumbens (Figure 3.2D') and vertical nucleus of the diagonal band of the pallidum (Figure 3.2F'), and weak in the globus pallidus (Figure 3.2I'-L'), ventral pallidum (Figure 3.2G'-I') and the substantia nigra (Figure 3.2S'-U'). Conversely, the staining intensity of SP is extremely intense in the substantia nigra (Figure 3.2S-V) and ventral pallidum (Figure 3.2G-I), strong in the shell of the nucleus accumbens, moderate in the core of the nucleus accumbens (Figure 3.2E) and weak in the caudate putamen, globus pallidus (Figure 3.2E-L) and vertical nucleus of the diagonal band of the pallidum (Figure 3.2F). Staining is negligible for both the NK1 receptor and SP in the subthalamic nucleus (Figure 3.2O&O'). The importance of the staining of the basal ganglia structures is discussed in terms of the relation to the behaviours measured in the subsequent chapters of this thesis in the discussion section.

3.3.3 Limbic system

In the hippocampus, SP staining is only visible in the molecular layer of the dentate gyrus whereas NK1 receptor staining is present in the granule layer of the dentate gyrus, pyramidal layer and lacunosum stratum of CA1-CA3 fields (Figure 3.2L'-T'). NK1 receptors are expressed abundantly in the central and cortical amygdaloid nuclei (Figure 3.2J'-M'), but not the medial amygdaloid nucleus, where SP staining is more intense (Figure 3.2K'). The septum also has regional staining for the NK1 receptor and SP. SP staining in intense in the lateral, but not medial septum, and is especially dense in the ventral region (Figure 3.2G-H), whereas the NK1 receptor is only found in the internal section of the lateral septum, and in the medial septum (Figure 3.2H')

3.3.4 Thalamus and hypothalamus

NK1 receptor staining is minimal in the thalamus, except in the habenula where staining is greater in the medial rather than lateral section (Figure 3.2M'-N') and the intergeniculate leaflet of the ventral thalamus (Figure 3.2P'-Q'). Substance P staining is also found in the intergeniculate leaflet (Figure 3.2P-Q) and in the paraventricular and intermediodorsal nuclei of the dorsal thalamus (Figure 3.2M). Overall, the hypothalamus stains strongly for SP and moderately for NK1 receptors, however the SP stain is so intense that it is difficult to discern the individual nuclei within the hypothalamus (Figure 3.2I-P) and is particularly intense and dispersed in the periventricular fiber system (Figure 3.2O). Both receptor and ligand stain strongly in pre-optic structures (Figure 3.2H/H'-I/I'). NK1 receptors are stained strongly in the dorsomedial but not the ventromedial hypothalamic nuclei (Figure 3.2N'-O').

3.3.5 Midbrain

3.3.5.1 Motor related structures

The periaqueductal gray (PAG), and the motor-related component of the superior colliculus located above it, are moderately stained for NK1 receptors and strongly stained for SP (Figure 3.2R/R'-X/X'). Similarly the ventral tegmental area is also stained more intensely for SP than the NK1 receptor (Figure 3.2T&T'). The substantia nigra pars reticular, previously mentioned as part of the basal ganglia, is intensely stained for SP yet devoid of staining for the NK1 receptor (Figure 3.2S/S'-V/V').

3.3.5.2 Sensory related structures

The sensory-related components of the superior colliculus (optical, superficial gray and zonal layers) are strongly stained for NK1 receptors and very weakly for SP (Figure 3.2S'-X'). The inferior colliculus is comparably stained for both NK1 receptors and SP in the dorsal and external nuclei, but not in the central nucleus (Figure 3.2Y/Y'-Z/Z').

3.3.5.3 Behavioural-state related structures

The main structures of the midbrain that are associated with behavioural-state also have mismatched staining of receptor and ligand. Strong staining of NK1 receptors is seen in the interpeduncular nucleus, yet only moderate staining for SP (Figure 3.2T&T'). Conversely, the substantia nigra par compacta and dorsal raphe nucleus are stained more intensely for SP, than NK1 receptors (Figure 3.2S/S'-U/U' and W&W' respectively).

3.3.6 Hind Brain and cerebellum

3.3.6.1 Pons

The pons and medulla in the hindbrain can also be divided into sensory-, motor- and behavioural state-related structures. Most notably the parabrachial nucleus, a component of the sensory-related pons is stained for both NK1 receptors and SP, especially in the lateral division (Figure 3.2Z&Z'). Also part of the sensory-related pons is the superior olivary complex that is stained strongly for NK1 receptors and moderately for SP (Figure 3.2Z&Z'). Amongst the motor-related structures of the pons region, the dorsal tegmental area is clearly defined with moderate staining of the NK1 receptor, but weak staining for SP (Figure 3.2Z&Z'). The behavioural-related structures of the pons that are stained for SP or NK1 receptor include the locus coeruleus which is strongly stained for both receptor and ligand (Figure 3.2Z&Z').

3.3.6.2 Medulla

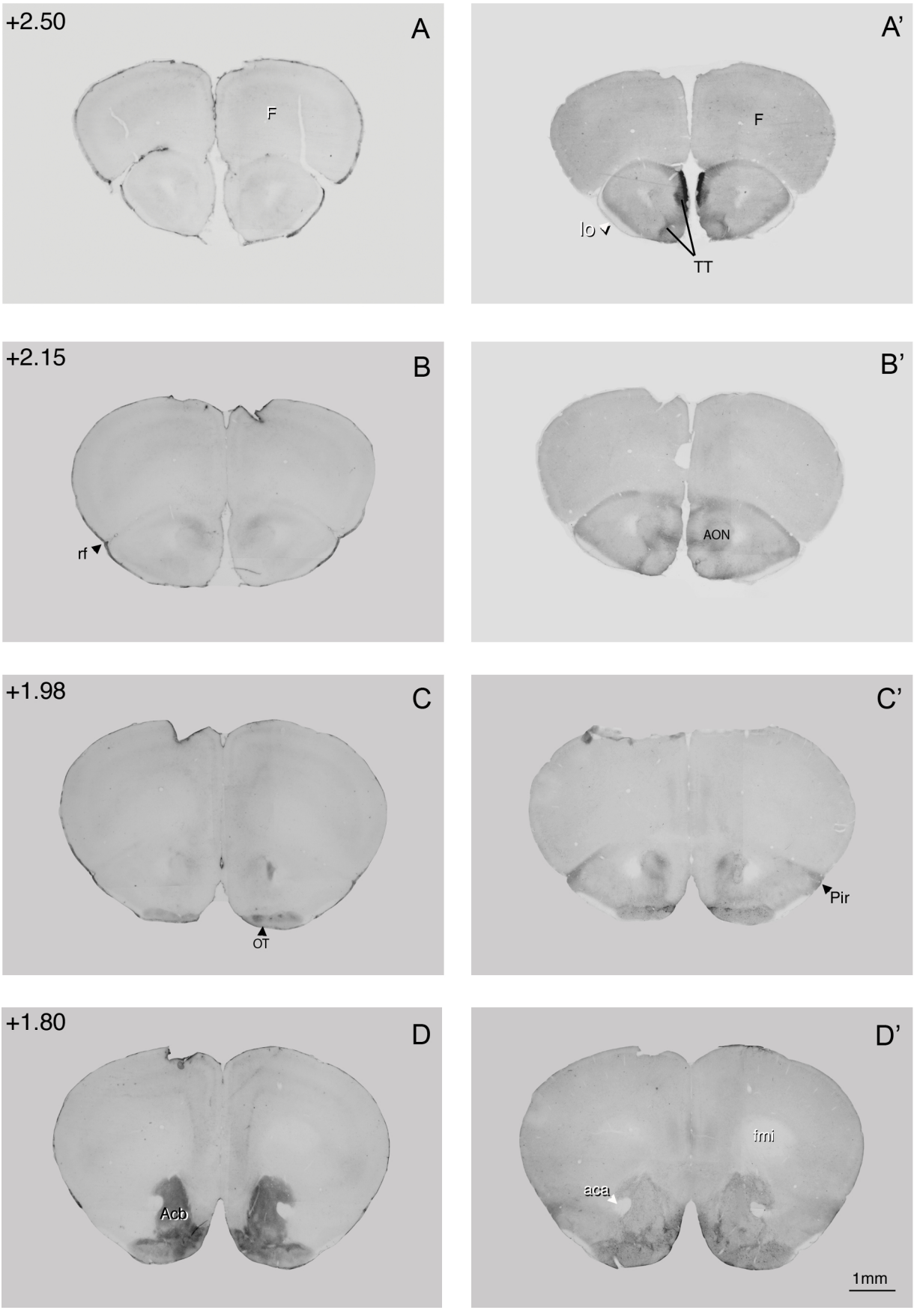
Structures of the medulla with significant staining include area VII (or 7) the facial motor nucleus, which is moderately stained for both SP and NK1 receptors, whereas the parapyramidal nucleus is strongly stained for SP, but only weakly for the NK1 receptor (Figure 3.2AA&AA'). The nucleus ambiguus, another structure of the motor-related components of the medulla, is moderately stained for the NK1 receptor and SP (Figure 3.2BB&BB').

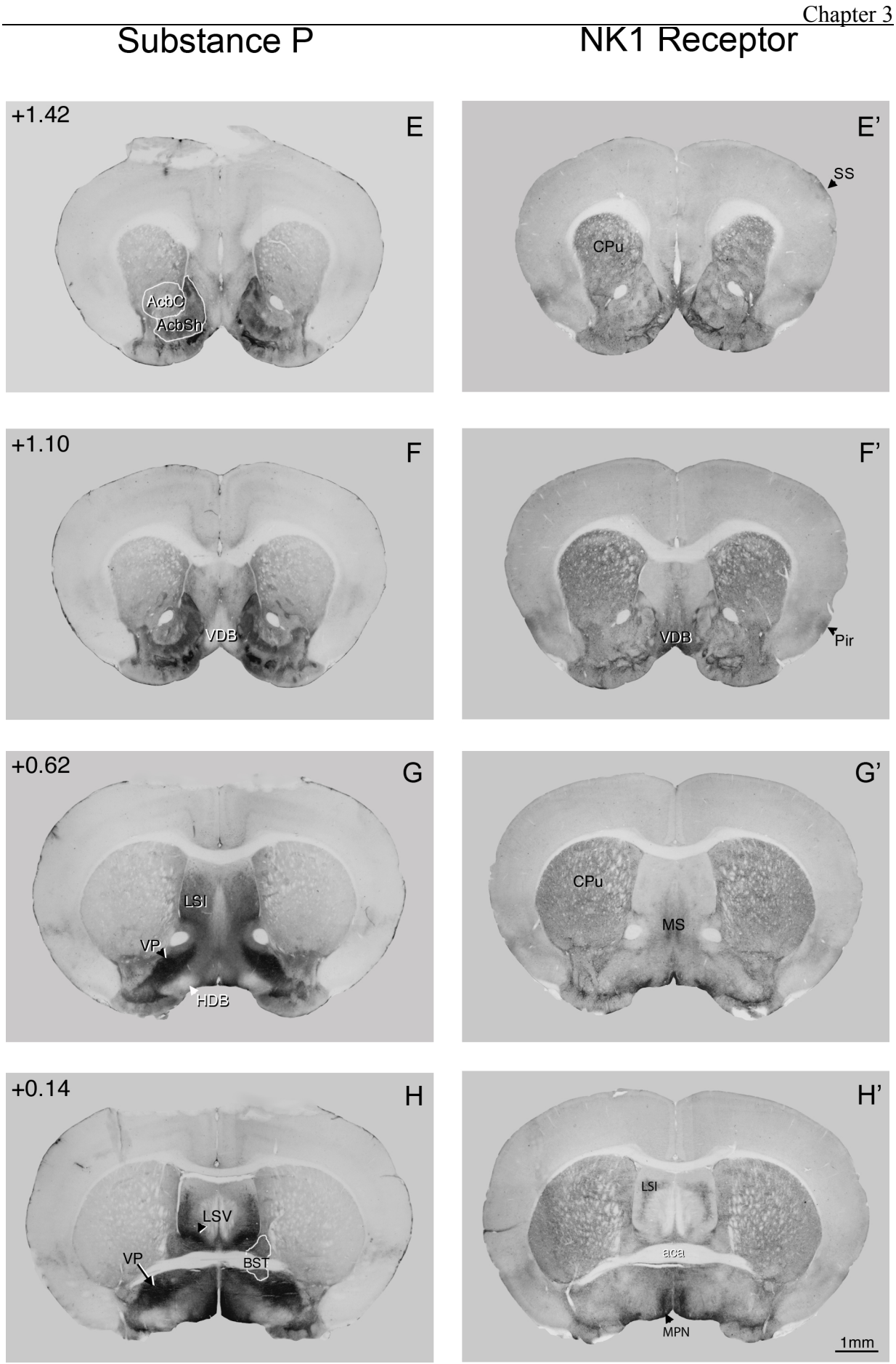
3.3.6.3 Cerebellum

Lamination of the cerebellum is visible in the tissue stained for the NK1 receptor, whereas no SP staining is visible. NK1 receptors are stained uniformly in the granular layer more than the molecular layer of each lobule (Figure 3.2AA&AA') The cerebellar nuclei (fastigial-, interposed- and dentate-nuclei) are also positively stained for the NK1 receptor, but only weakly (Figure 3.2AA&AA').

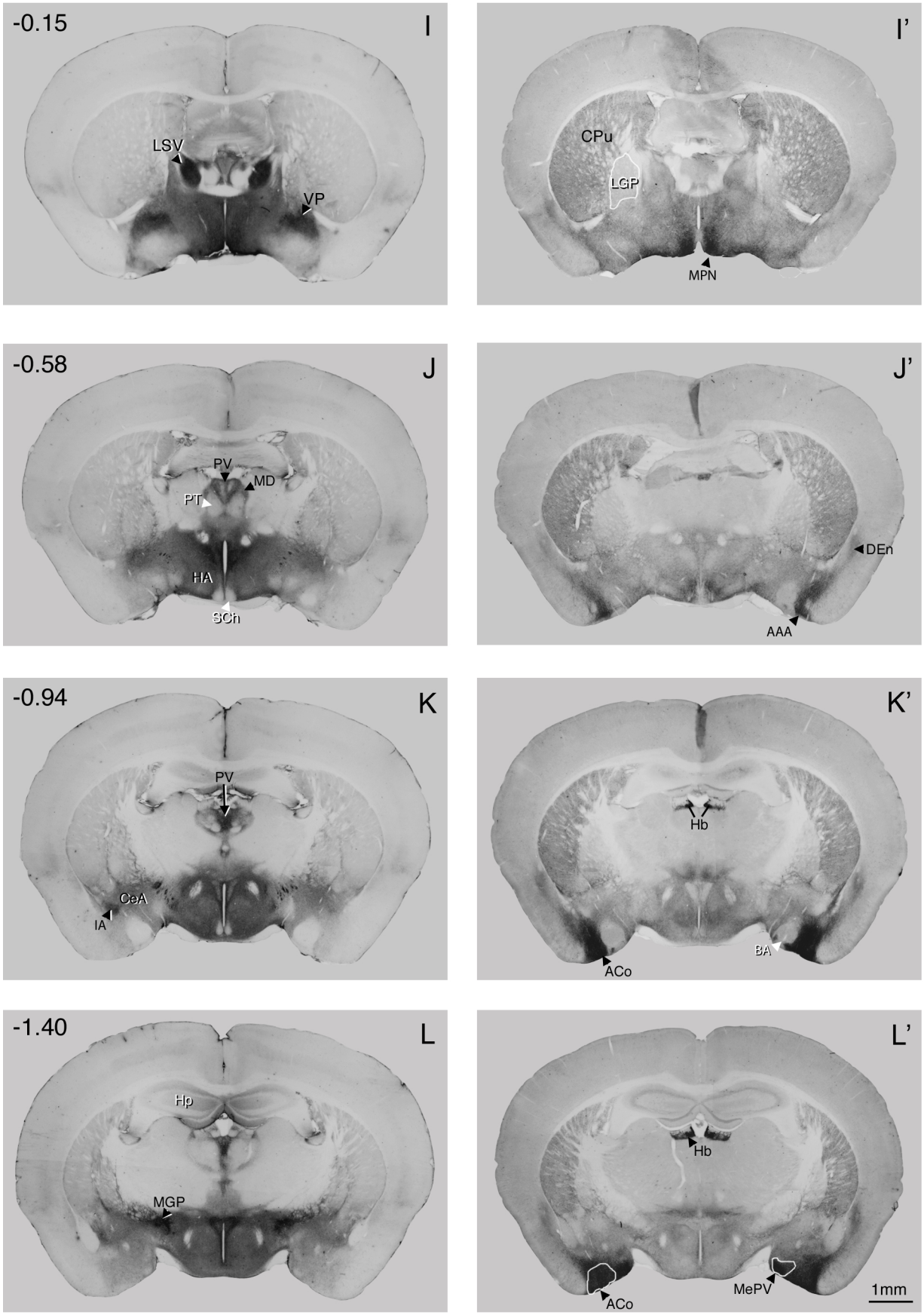
Figure 3.2 Immunohistological localisation of Substance P and the NK1 receptor throughout the mouse brain. Approximate values from bregma are shown in the top left corner. Labeling of brain regions and abbreviations taken from The Mouse Brain Atlas (Franklin and Paxinos, 1997). A list of abbreviations used can be found on page 78. Rat anti-SP antibody 1: 200; rabbit anti-NK1 receptor antibody 1:5000. Scale bar = 1mm.

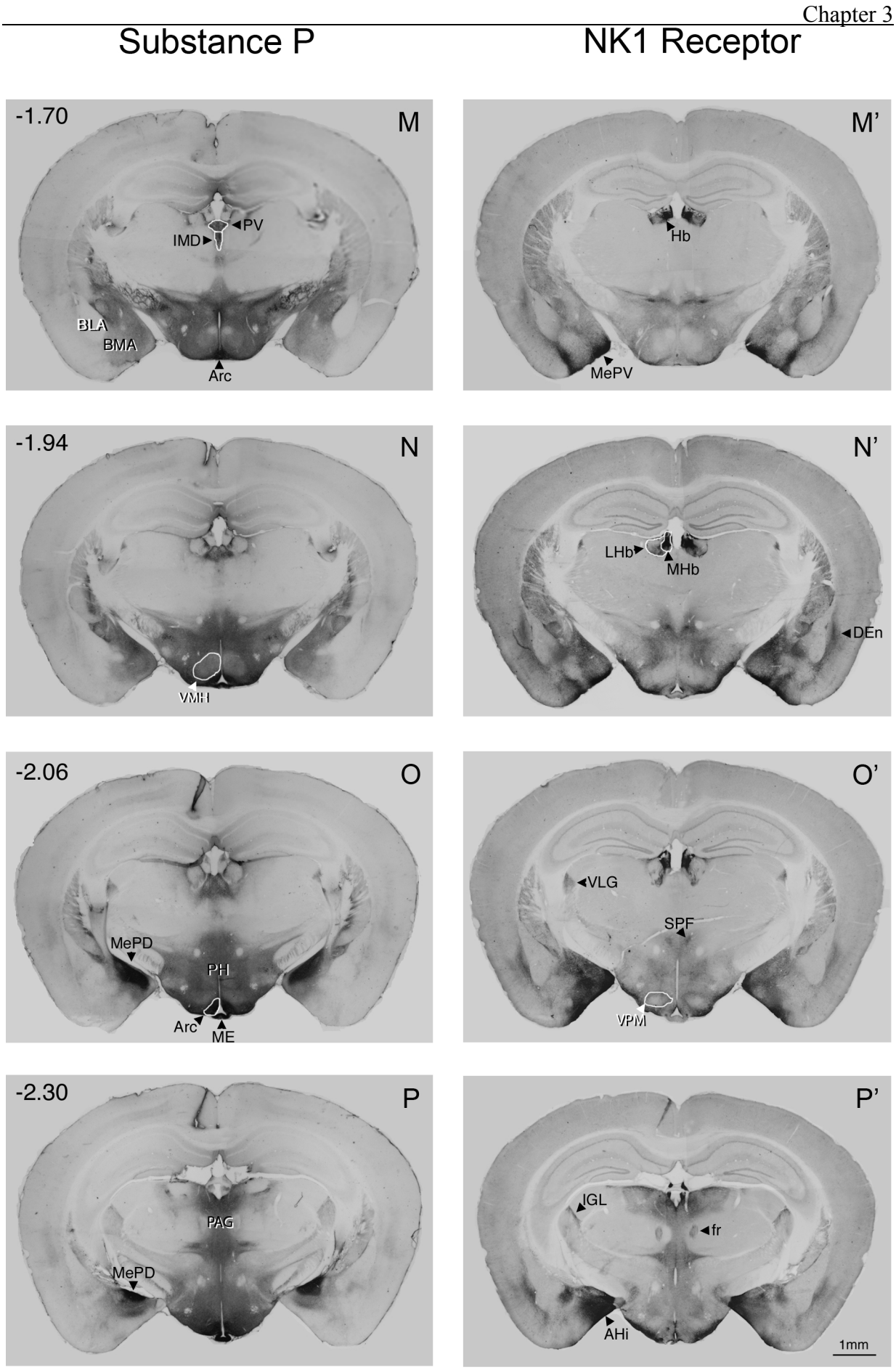
Substance P NK1 Receptor



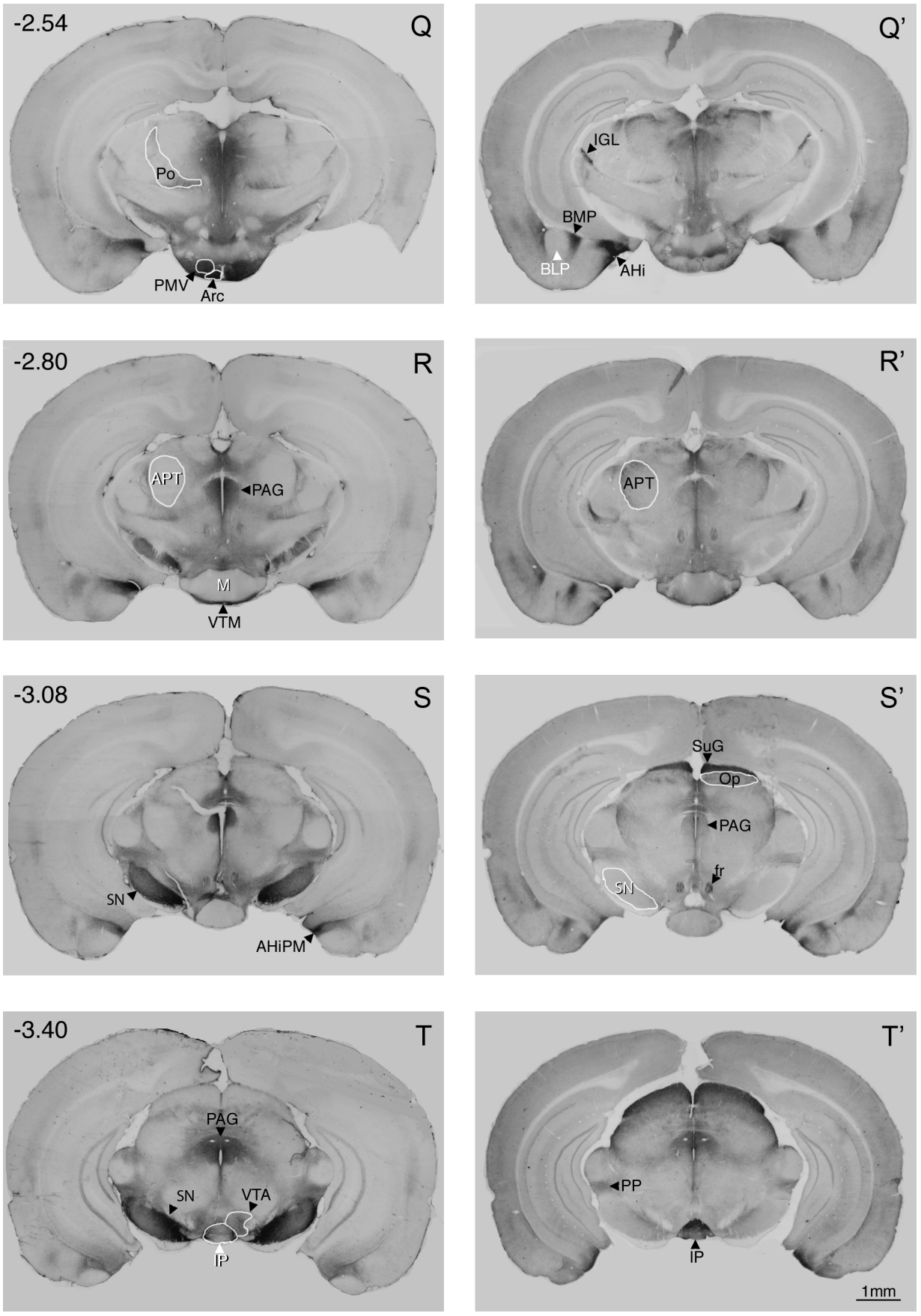

Substance P
NK1 Receptor
+1.42
E
AcbC
AcbSh
E'
SS
CPu
+1.10
F
VDB
F'
VDB
Pir
+0.62
G
LSI
VP
HDB
G'
CPu
MS
+0.14
H
LSV
VP
BST
H'
LSI
acai
MPN
1mm

Substance P NK1 Receptor



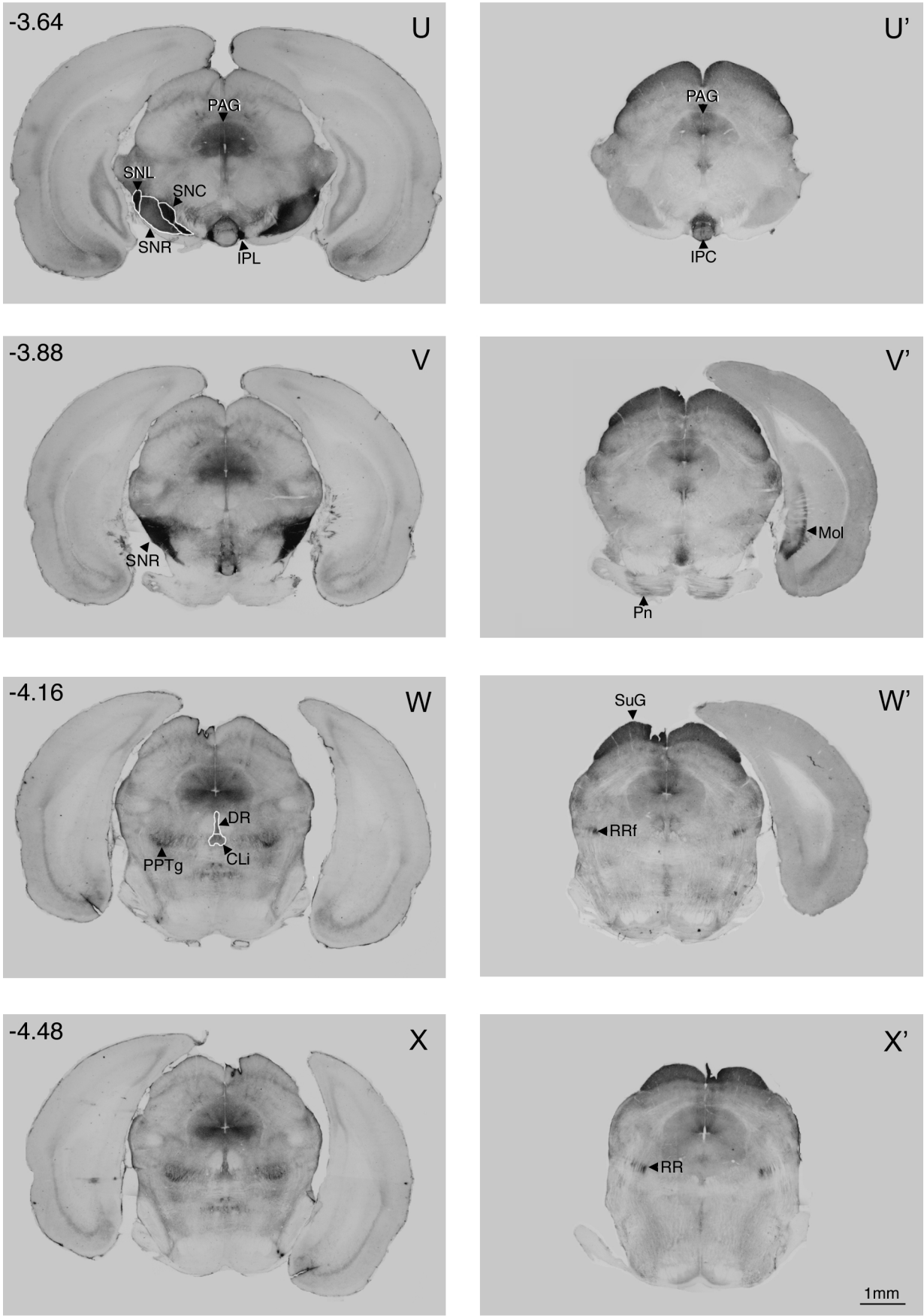

Substance P
NK1 Receptor
-1.70
M
PV
IMD
BLA
BMA
Arc
M'
Hb
MePV
-1.94
N
VMH
N'
LHb
MHb
DEn
-2.06
O
MePD
PH
Arc
ME
O'
VLG
SPF
VPM
-2.30
P
PAG
MePD
P'
IGL
fr
AHi
1mm

Substance P NK1 Receptor

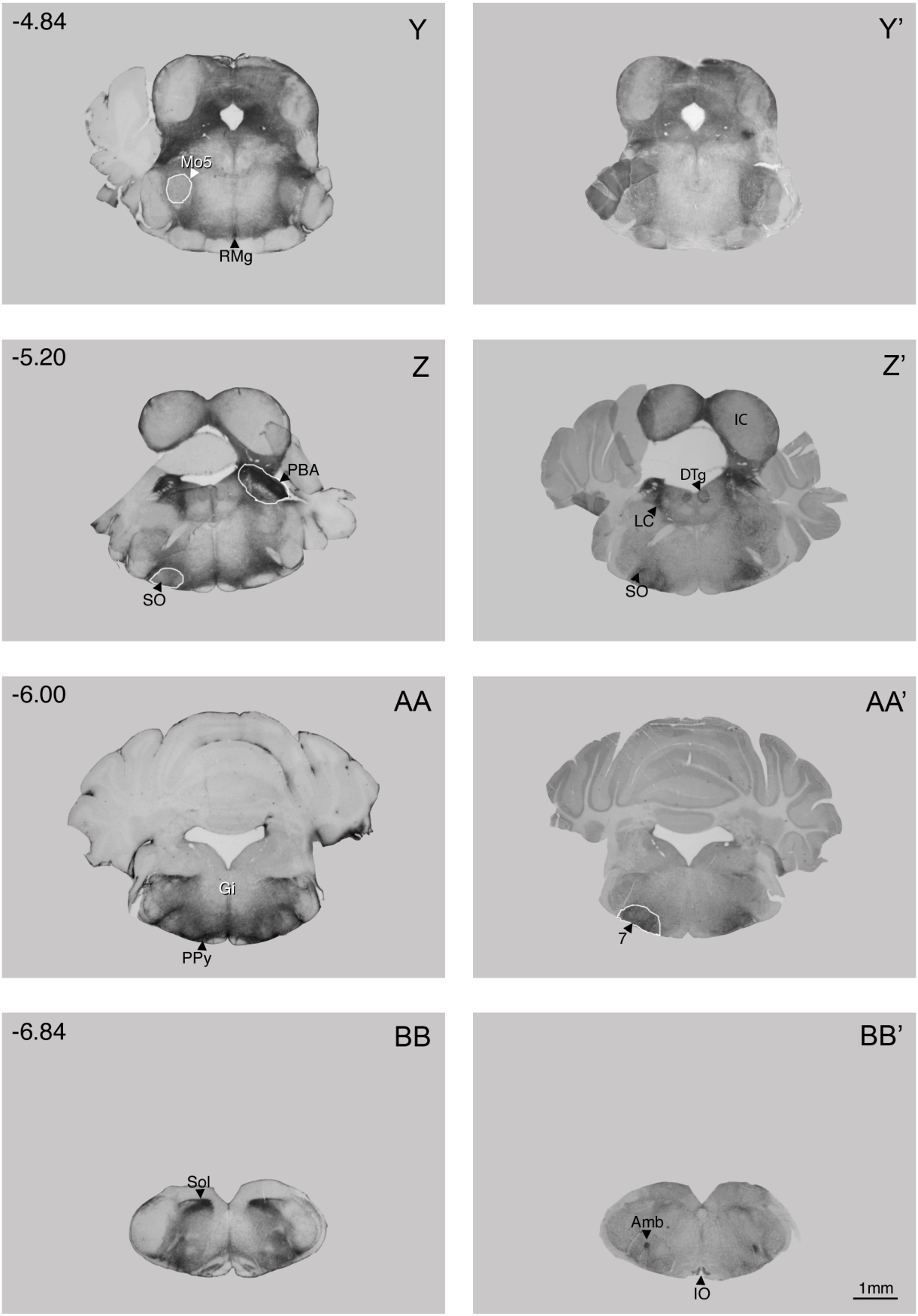


Substance P

NK1 Receptor



Substance P NK1 Receptor



7	facial motor nucleus
AAA	anterior amygdaloid area
aca	anterior commissure, anterior part
Acb	nucleus accumbens
AcbC	nucleus accumbens, core
AcbSh	nucleus accumbens, shell
ACo	anterior cortical amygdaloid nucleus
AHi	amygdalohippocampal area
AHiPM	amygdalohippocampal area, post-medial alveus
AON	anterior olfactory nucleus
APT	anterior pretectal nucleus
Arc	arcuate hypothalamic nucleus
BLA	basolateral amygdaloid nucleus, anterior part
BMA	basomedial amygdaloid nucleus, anterior part
BLP	basolateral amygdaloid nucleus, posterior part
BMP	basomedial amygdaloid nucleus, posterior part
BST	bed nucleus of the stria terminalis
CeA	central amygdaloid nucleus
CLi	caudal linear nucleus raphe
CPu	caudate putamen
DEn	dorsal endopiriform nucleus
DR	dorsal raphe nucleus
DTg	dorsal tegmental nucleus
F	frontal cortex
fmi	forceps minor corpus callosum
fr	fasciculus retroflexus
Gi	gigantocellular reticular nucleus
HA	hypothalamic area
Hb	habenula
HDB	diagonal band nucleus, horizontal limb
Hp	hippocampus
IA	intercalated amygdala nucleus
IO	inferior olivary complex
IC	inferior colliculus
IGL	intergeniculate leaflet
IMD	intermediodorsal thalamic nucleus
IP	interpeduncular nucleus
IPL	interpeduncular nucleus, lateral
IPC	interpeduncular nucleus, core
LC	locus coeruleus
LGP	lateral globus pallidus
LHb	lateral habenular
LSI	lateral septal nucleus, intermediate part
LSV	lateral septal nucleus, ventral part
lo	lateral olfactory tract
M	mamillary nucleus
MD	mediodorsal thalamic nucleus
ME	median eminence
MePD	medial amygdaloid nucleus, posterodorsal part
MePV	medial amygdaloid nucleus, posteroventral part
MGP	medial globus pallidus
MHb	medial habenular
Mo5	motor nucleus of the trigeminal
Mol	molecular layer of the dentate gyrus
MPN	medial pre-optic nucleus
MS	medial septal nucleus
Op	optic layer of the superior colliculus
OT	olfactory tubercle
PAG	periaquductal gray area
PBA	parabrachial area
Pir	piriform cortex
PH	posterior hypothalamic area
PMV	pre-mammillary nucleus
Pn	pontine gray
Po	posterior thalamus
PP	peripeduncular nucleus
PPTg	pedunculopontine tegmental nucleus
PPy	parapyramidal nucleus
PT	parataenial thalamic nucleus
PV	paraventricular thalamic nucleus
rf	rhinal fissure
RMg	raphe nucleus magnus
RR	retrorubral nucleus
RRf	retrorubral field
SCh	suprachiasmatic nucleus
SN	substantia nigra
SNC	substantia nigra, pars compacta
SNL	substantia nigra, lateral
SNR	substantia nigra, pars reticulata
SO	superior olivary complex
Sol	solitary tract
SS	somatosensory cortex
SuG	superficial gray, superior colliculus
TT	components of taenia tecta
VDB	diagonal band nucleus, vertical limb
VLG	ventrolateral geniculate leaflet
VMH	ventromedial hypothalamus
VP	ventral pallidum
VPM	ventral premamillary nucleus
VTA	ventral tegmental area
VTM	ventro tubero mammillary nucleus

Brain region	NK1	SP	Brain Region	NK1	SP
Forebrain			Basolateral nucleus	-	-
Olfactory system			Basomedial nucleus	-	+
Olfactory bulbs	NE	NE	Medial amygdaloid nu.		
Olfactory tubercle	++	++	Anterior part	-	-
Cerebral cortex			Posteroventral part	+++	+
Frontal cortex	+	+	Posterodorsal part	++	+++
Motor cortex	+	+	Subthalamus		
Sensory cortex	++	+	Zona Incerta		
Visual cortex	+	+	Ventrolateral part	++	+
Piriform cortex	++	+	Ventromedial part	-	+
Entorhinal cortex	+	+	Dorsolateral part	+	+
Tenia tecta	+++	+	Dorsomedial part	-	+
Hippocampal circuit			Hypothalamus		
CA1-CA3	+	-	Ventromedial nucleus	-	++
Dentate gyrus	+	+	Arcuate nucleus	+	+++
Cerebral nuclei			Posterior hypothalamic nu.	++	++
Caudate putamen	++	++	Periventricular nucleus	++	+++
Nucleus accumbens			Suprachiasmatic nucleus	-	-
Core	++	++	Lateral hypothalamic area	++	+++
Shell	+++	++	Pre-mammillary nucleus	-	++
Globus pallidus	+	+	Mammillary nucleus	++	-
Ventral pallidum	+	+++	Supramammillary nucleus	+	++
Septal regions			Ventro-tubero mammillary	++	++
Medial septal nucleus	++	++	**Midbrain**		
Lateral septal nucleus			Anterior pretectal nucleus	+	-
Dorsal part	+	+	Marginal part	++	-
Intermediate part	+	++	Pretectal olivary nucleus	-	+
Ventral Part	-	+++	Posterior pretectal nucleus	+	+
Basal forebrain regions			Substantia nigra		
Nucleus of the diagonal band			pars compacta	+	+++
Vertical limb	+++	-	pars reticulata	+	+++
Horizontal limb	+++	-	Ventral tegmental area	-	++
Medial preoptic area	++	+	Peripeduncular nucleus	++	+
Medial preoptic nucleus	+++	+++	Interpedunclar nucleus	+++	+
Epithalamus			Superior Colliculus	+++	+
Medial habenular nucleus	+++	+	Inferior Colliculus		
Lateral habenular nucleus	++	+	Central nucleus	++	-
Thalamus			External/dorsal nuclei	+++	++
Paraventricular nucleus	-	++	Periaqueductal gray	++	+++
Parataenial nucleus	-	-	**Hind brain**		
Mediodorsal nucleus	-	++	Pontine nuclei	-	-
Centromedial nucleus	-	++	Parabrachial area	++	+++
Centrolateral nucleus	-	+	Superior olivary complex	++	+
Ventral lateral geniculate nu.	++	++	Inferior olivary complex	++	-
Dorsal lateral geniculate nu.	-	-	Dorsal tegmental nucleus	++	+
Intergeniculate leaflet	++	+	Ventral tegmental nucleus	++	+
Amygdala			Locus coeruleus	++	+++
Cortex-amygdala transition	+++	+	Motor nucleus of the trigeminal	++	-
Amygdalohippocampal area	+++	+	Ambiguus Nucleus	+++	-
Central nucleus	+	++	Facial motor nucleus	++	++
Intercalated amygdaloid nu.	-	+++	Parapyrimidal nucleus	+	+++

Table 3.1 Summary table of noteworthy brain regions and the relative intensity of immunohistochemical staining (+++ strong, ++moderate, +weak staining). Regions where there is significant mismatch between receptor and ligand are highlighted in orange (more NK1R than SP) and green (more SP than NK1R).

3.4 Discussion

There have been many studies into the distribution of NK1 receptors and its preferred ligand Substance P, in several species of animal. As well as detailed distribution patterns of NK1 and SP, these studies have shown that a high level of NK1 receptor expression does not necessarily correspond to a high level of SP (and vice versa). However, to date, there has been no direct comparison of NK1 and SP expression patterns throughout the mouse brain. The brain regions that stain most intensely for the NK1 receptor include the caudate putamen, amygdaloid nuclei, habenula interpeduncular nucleus, and the superior olivary complex, whereas SP staining is most intense in the accumbens, ventral pallidum, septum, periaqueductal gray, parabrachial nucleus and substantia nigra. The structures of relevance to the behaviours measured in the subsequent chapters of this thesis, including attention and impulsivity are considered in section 3.4.2.

3.4.1 Linking the distribution of the NK1 receptor and SP to clinical conditions

Findings were largely in concordance with previous studies of NK1 receptor distribution in the rat brain (Nakaya et al., 1994). In the mouse cortex, NK1 receptor staining appears relatively weak, yet in rat and human distribution studies the NK1 receptor reportedly stained intensely (Nakaya et al., 1994; Okumura et al., 2008; Engman et al., 2012). It is therefore the density of the positively stained neurons that should also be taken into account, since a few intensely stained neurons will not clearly show at the magnification used in this chapter. There is significant staining of either SP or the NK1 receptor in the majority of structures that comprise the basal ganglia, which is in concordance with the findings from the rat, non-human primate and human studies (Nakaya et al., 1994; Mounir and Parent, 2002). The conservation of staining patterns of the SP/NK1 receptor system through evolution intimates the importance of the role that this receptor and ligand has in the functionality of the basal ganglia. The staining patterns of the NK1 receptor and SP in the individual structures of the basal ganglia, and how this could give rise to the symptoms of ADHD are discussed in more detail below (section 3.4.2.1).

The structures of the limbic system have a wide range of functions from learning and memory in the hippocampus to the control of emotion, motivation and reward in the amygdala. The system is also linked to the decision-making components of the prefrontal cortex (orbitofrontal cortex) (Haber and Brucker, 2009). The distribution of ligand and receptor are essentially identical between rodent species, although the density of staining is slightly reduced in mouse compared to rat in some regions such as the amygdaloid nuclei. Substance P and the NK1 receptor play an important role of the functionality of the limbic system, as demonstrated by pharmacological manipulations and in the phenotype of the NK1R-/- mouse. For example both wildtype mice dosed with NK1 receptor antagonists and NK1R-/- mice both show decreased anxiety and stress in the resident intruder test (De Felipe et al., 1998; Rupniak et al., 2001). In the elevated plus maze (EPM) NK1 receptor antagonists increased the amount of time spent in the open arms of the maze (an anxiolytic effect) which was replicated in the behaviour of NK1 receptor knockout mouse compared to wildtype animals, however ablation of NK1 receptor containing neurons in the amygdala resulted in the opposite effect as lesioned mice displayed an anxiogenic profile on the EPM (Santarelli et al., 2001; Gadd et al., 2003). The nucleus accumbens receives input neurons from the basolateral amygdala and plays an important role in reward and addiction. There is moderate staining for the NK1 receptor in the core and shell, while staining for SP is strong, especially in the shell of the nucleus accumbens. In the conditioned place preference (CPP) paradigm, NK1R-/- mice do not develop a preference for the chamber associated with morphine (Murtra et al., 2000), furthermore ablation of NK1 receptor containing neurons of the amygdala, but not nucleus accumbens, also induced this preference deficit (Gadd et al., 2003). The nucleus accumbens sends information to the ventral pallidum (VP), which is one of the main brain regions that shows a large discrepancy between the staining intensity of receptor and ligand. NK1 receptor staining is non-existent in the VP whereas SP staining is almost saturated. The VP is part of the limbic system also associated with reward and addiction. These findings highlight the importance of the limbic system in many of the conditions that are associated with disruption of NK1R/SP brain circuitry.

3.4.2 NK1R/SP circuitry and ADHD

This section takes a closer look at the two most widely accepted hypotheses for the aetiology of ADHD as outlined in the introduction chapter of this thesis (see section 1.5.2), firstly dysfunction of the basal ganglia circuitry and secondly disruption of monoamine transmission.

3.4.2.1 Basal ganglia

The basal ganglia comprise the globus pallidus, substantia nigra pars compacta and pars reticulata, the subthalamic nucleus and the striatum. Together the basal ganglia are involved in the selection of actions by evaluating competing 'action plans' and selecting the best option, which are influenced by previous experiences (Surmeier et al., 2011). They also regulate the initiation and control of movements, link the thalamus to the motor cortex and are also involved in cognitive and emotional behaviours (Graybiel, 2008). These effects are produced through an intricate network of excitatory and inhibitory connections that are outlined below and summarised schematically in Figure 3.3.

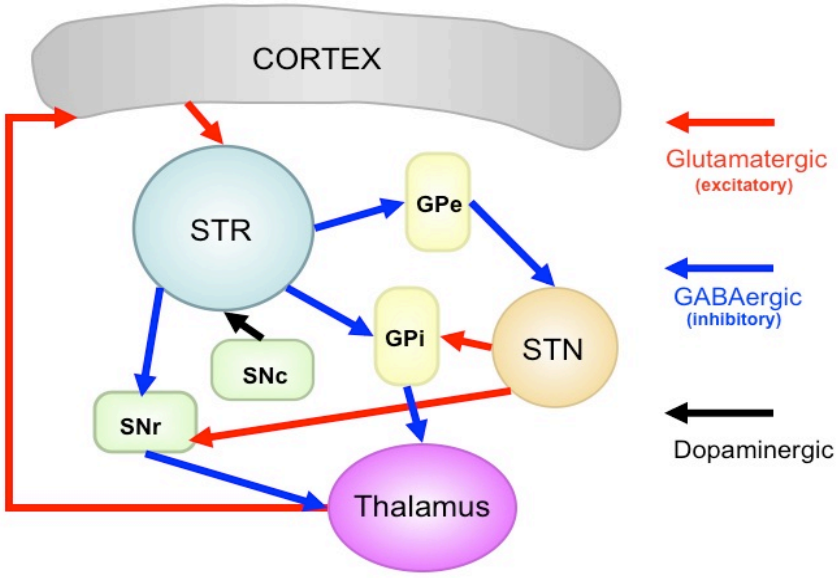


Figure 3.3 Schematic diagram of the main basal ganglia circuitry. The striatum (STR) receives excitatory input from the cortex. The direct pathway originates from D1-expressing GABAergic medium spiny neurons (MSNs) of the striatum that project to the substantia nigra pars reticulata (SNr) and the internal segment of the globus pallidus (GPi). Inhibition of the output nuclei allows the glutamatergic excitatory feedback from the thalamus to the cortex, thus inducing movement. The D2-expressing MSNs of the indirect pathway project to the external segment of the globus pallidus (GPe) that connect to the thalamus via the subthalamic nucleus (STN). Inhibition of the GPe disinhibits the STN that then sends excitatory messages to the SNr and GPi that inhibit the thalamus and thus result in reduced movement. Other targets of the SNr and GPi include the superior colliculus and pedunculopontine nucleus (not shown).

The basal ganglia receives the majority of input signals from the cerebral cortex (including sensory, motor and associational areas) (Bolam et al., 2000), but some signals originate from the subthalamic nucleus, particularly the intralaminar thalamic nuclei (Smith et al., 2004). In the cortex, significant NK1 receptor and SP staining is barely visible when observed at the low magnification used in this chapter, however NK1 receptor positive neurons are present in the rat cortex, and given the conservation of staining patterns between the rat and mouse we can tentatively assume that they are also present in the mouse cortex too. In terms of the 5-choice serial reaction time task (used in latter chapters of this thesis), the micro-circuitry of sustained attention originates in the prelimbic and anterior cingulate cortical regions and projects to the dorsal-striatum, while impulsive behaviour is controlled by the circuitry of the infralimbic cortex and the nucleus accumbens (Bari and Robbins, 2011). From the results seen in this chapter, with SP and NK1R staining appearing very weak in the cortex but abundant in the striatum and nucleus accumbens, it most likely that any behavioural deficits of the NK1R-/- mouse associated with attention and impulsivity originate from these heavily stained regions rather than the initial cortical input.

All projections from the cortex are glutamatergic and are received by the striatum. The striatum is the largest nucleus of the basal ganglia and is divided into the dorsal striatum (caudate putamen) and the ventral striatum (nucleus accumbens). 90% of striatal neurons are GABAergic medium spiny neurons (MSNs), with the other 10% comprised of four different types of interneuron: cholinergic, parvalbumin-expressing GABAergic, calretinin-expressing GABAergic and neuropeptide Y (NPY)/ somatostatin/nitric-oxide synthase (NOS)-expressing GABAergic interneurons (Gerfen & Surmeier 2011; Kawaguchi et al. 1995). NK1 receptors are expressed on cholinergic interneurons and NPY-expressing GABAergic interneurons (Kawaguchi et al., 1995). GABAergic interneurons inhibit the activity of MSNs, while the tonically active cholinergic interneurons serve to increase the excitability of MSNs (Pisani et al., 2007). Given that NK1 receptors are found on both types of interneuron, they could play a role in ‘fine tuning’ the activity of the basal ganglia circuitry at the level of the striatum.

Information passes through the striatum to the output nuclei of the basal ganglia through two different methods: the direct and indirect pathways. The medium spiny neurons in the striatum are divided into two distinct populations dependent on their differential

expression of dopamine receptors and their axonal projections. The MSNs of the direct pathway release dynorphin and Substance P in conjunction with GABA, mainly express D1 receptors (which are associated with excitatory G-proteins) and project straight to the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr) which are the GABAergic output nuclei of the basal ganglia. In contrast, the MSNs of the indirect pathway release enkephalin in conjunction with GABA and mainly express D2-receptors (which are associated with inhibitory G-proteins) and project only to the external capsule of the globus pallidus (GPe). The inhibition of the GPe disinhibits the subthalamic nucleus, which then provides excitatory glutamatergic signals to the GPi and SNr. The output nuclei (GPi and SNr) provide inhibitory GABAergic projections to the thalamus, superior colliculus and pedunculopontine nucleus (Gerfen & Surmeier 2011).

The striatal inhibition of the SNr-GPi complex coupled with SNr-GPi inhibition of the thalamus therefore results in a net reduction of inhibition of the thalamus via the striatum. The thalamus projects excitatory glutamatergic neurons to the cortex itself. The direct pathway, therefore, results in the excitation of the motor cortex by the thalamus. In contrast, the end-result of activation of the indirect pathway is inhibition of the thalamus and, therefore, decreased stimulation of the motor cortex. Therefore disruption of basal ganglia circuitry directly links to the symptoms of ADHD, namely hyperactivity and impulsivity.

3.4.2.2 Origins of monoamines

Although presented in separate sections disruption of monoamine transmission and dysfunction of the basal ganglia are not mutually exclusive. For example, the striatum receives dopaminergic input from the core of the substantia nigra, which in turn activates the direct and indirect pathways of the basal ganglia circuitry, thus disruption of the monoaminergic transmission can feed into other circuits and have further indirect effects. In this section the origins of the neurotransmitter systems are considered in relation to SP and the NK1 receptor while more detailed discussion of the monoaminergic systems and ADHD are covered in the subsequent results chapters.

The dopaminergic hypothesis of ADHD arose from the efficacy of psychostimulants in alleviating the symptoms of ADHD. The three main origins of dopamine in the brain are the substantia nigra pars compacta, the ventral tegmental area and the hypothalamus. All of these regions stain at least moderately for SP and the NK1 receptor, which could potentially have a regulatory effect on the release of dopamine. The main origin of noradrenaline is the locus coeruleus (LC) with axons targeting a number of different regions including the cortex, amygdala, hippocampus and septum (Sara, 2009). Interestingly the only major structure not to receive innervation from the LC is the basal ganglia. The LC stains strongly for both SP and the NK1 receptor, again revealing potential for SP/NK1R control of neurotransmitter release. Serotonin originates from the raphe nuclei located in the brain stem. The dorsal raphe nucleus is stained more strongly for SP then for the NK1 receptor and projects out to the amygdala and periaqueductal gray regions, both of which are associated with the control of stress and anxiety both of which are behaviours linked to the SP/NK1 receptor system.

3.4.3 Mismatched distribution of ligand and receptor

In line with previous observations in other species, the distribution of NK1 receptors and substance P are not always correlated in the mouse brain. Regions where there appears to be an abundance of SP, relative to that of the NK1 receptor in the mouse brain include the nucleus accumbens, ventral pallidum, medial amygdaloid nucleus, and the substantia nigra. The latter is a well-characterised area of ‘mismatch’ and has been reported in a number of mammalian species (Mantyh et al., 1984; Rothman et al., 1984; Shults et al., 1984; Gerfen, 1991; Maeno et al., 1993; Nakaya et al., 1994). Although we are still unsure of the full explanation for a mismatched density between receptor and ligand, it is by no means a unique phenomenon for the NK1R/SP system, as reviewed by (Herkenham, 1987). The most likely explanation is that NK1 receptors in regions with little SP receive substance P released from distant axon terminals. Conversely the regions with strong NK1 receptor staining and weak SP staining include the cortical amygdaloid nucleus, dorsal tegmental nucleus, and the superior and inferior colliculi. SP is a regarded as a neuromodulator, therefore unlike neurotransmitters, it can be released non-synaptically and due to its small size it can diffuse considerably long distances (Herkenham, 1987; Landgraf and Neumann, 2004).

3.4.4 Methodological considerations and conclusions

This chapter has looked at the distribution of the NK1 receptor, and its preferred ligand substance P throughout the mouse brain using immunohistochemistry. In this study the brain was only considered caudally from +2.50mm relative to bregma, therefore the olfactory bulbs, in which NK1 receptors are prevalent in the rat, are not considered here. It is therefore impossible to rule out other interspecies differences in this region. This technique, especially with DAB chromogenic visualisation, provides a high level of contrast between background levels, and regions where the receptor and/or ligand are present. The reliability of the results is based on the specificity of the primary antibodies, in this case rabbit anti-NK1 receptor and rat anti-SP, for their respective epitopes. This was tested in a control experiment where the protocol was repeated, omitting the primary antibodies. No staining was seen in the control tissue (data not shown), showing that the staining for the NK1 receptor and substance P is not an artifact of any other stage of the protocol and is a product of the primary antibodies binding to their target. To further investigate the distribution of NK1 receptors and SP in the mouse brain, a subsequent study could include the olfactory bulbs, as well as looking at the regions of relevance to conditions associated with the NK1 receptor, such as the basal ganglia and amygdala at higher magnifications to categorise the density and types of stained neurons. Yet from the images obtained at the magnification here, we can say that the NK1R/SP system could influence behaviours such as impulsivity and attention based on their presence in relevant brain regions for these behaviours such as the striatum and nucleus accumbens. A fluorescent double-stain for NK1 receptors and SP could also be imaged using high magnification confocal microscopy to further study the proximity of SP-positive axons and NK1 receptors, both in matched and mismatched regions of staining.

4 Measuring the locomotor activity of NK1R-/- mice

4.1 Introduction

Previous work in our lab demonstrated that NK1 receptor knockout mice had greater levels of activity in the light/dark exploration box (measured by total number of line crosses) than the wildtype controls (Herpfer et al., 2005; Fisher et al., 2007). A later study also demonstrated higher activity levels of the NK1R-/- mice in the LDEB, expressed as lines crossed per unit of time (Yan et al., 2010). However all of these locomotor measurements were taken after injections had been administered.

As noted in the previous chapter, the NK1 receptor is expressed in the thalamic intergeniculate leaflet, which is involved in circadian pace-making (Piggins et al., 2001) and NK1 receptor antagonist treatment can alter circadian rhythms (Challet et al., 1998). This chapter looks at whether the activity levels and/or circadian rhythm patterns of treatment naïve NK1R-/- mice differ from that of wildtype mice over a 48-hour period.

4.1.1 NK1 receptor and circadian rhythms

Circadian rhythms are controlled by the hypothalamic suprachiasmatic nucleus, which receives photic information directly via the retinohypothalamic tract and indirectly from retinally innervated cells in the thalamic intergeniculate leaflet. SP is a possible modulator within the circadian rhythm system. In rats, early neuroanatomical investigations revealed SP-immunoreactivity in fibres and terminals of the SCN and highlighted a population of SP-positive retinal-ganglion cells that projected directly to the SCN (Ljungdahl et al., 1978; Takatsuji et al., 1991). Antagonist studies also implicated a role for the SP/NK1R system in circadian rhythm modulation in hamsters. NK1 receptor antagonist GR-205,171 inhibited light-induced phase advances in hamster circadian wheel running activity rhythms. However, systemic injection of the antagonist did not induce non-photic changes in circadian rhythm shift indicating that tachykinin NK1 receptor antagonists are only effective when a light stimulus is applied to the pacemaker system (Gannon and Millan, 2005). NK1 receptor antagonist treatment has

also been shown to induce phase-advances in wheel running in hamsters kept in constant light conditions (Challet et al., 1998) providing more evidence that the SP/NK1R system modulates photic responses of the SCN.

However, caution must be applied when making interspecies comparisons into the role of the NK1R/SP system in circadian rhythm modulation, since NK1 receptor and SP staining patterns across rodent species in the SCN and intergeniculate leaflet are slightly different. In rats, SP and NK1 receptor staining is intense in the ventral, retinally innervated portion of the SCN, while in hamsters and mice the staining for NK1 and SP in this region is sparse. Staining patterns were found to be comparable across hamsters, rats and mice in the intergeniculate leaflet for both SP and the NK1 receptor (Piggins et al., 2001). These data indicate that SP could play more of a role in direct circadian rhythm modulation in the rat than in the hamster or mouse, but the indirect modulation via the intergeniculate leaflet may be conserved.

4.1.2 NK1 receptor and locomotor activity

There is contrasting evidence as to the role that the NK1 receptor plays in regulating locomotor activity. Intracerebroventricular injection of NK1 receptor agonists has been shown to enhance locomotor hyperactivity in rats, mice and guinea pigs (Naranjo and Del Rio, 1984; Elliott and Iversen, 1986; Piot et al., 1995), while an intraperitoneal injection of NK1 antagonist also elicited an increase in locomotor activity in mice (Yan et al., 2010). Many previous comparisons of activity levels of NK1R-/- mice and wildtype controls have reported no differences in locomotor activity (Rupniak et al., 2000; Ripley et al., 2002; Gadd et al., 2003).

In this experiment, wildtype and NK1R-/- mice were placed in circular corridors to monitor their activity levels over a period of 48-hours. This experimental set-up enables a simultaneous study of circadian rhythm patterns as well as locomotor activity levels. Given that the NK1R-/- mouse is a proposed model of ADHD and children with the condition often have sleep-problems (Cortese et al., 2009), here we test whether its phenotype matches the hyperactive profile of the condition before investigating attentional and impulsive behaviour that make up the triad of core symptoms.

4.2 Materials and methods

4.2.1 Subjects

Six wildtype and NK1R-/- mice were used from the homozygous bred MF1 colony (mixed background of 129/Sv x C57BL/6 x MF1) (see section 2.1.2.1). All mice were 8-10 weeks old at the time of experiment.

4.2.2 Apparatus

The experimental setup is described in section 2.3 of the materials and methods chapter. In brief, the 5cm wide circular corridors contained four evenly spaced infrared photo beams 1cm above the floor. Locomotor activity was recorded by a computer interface. Quarter turns, as measured by the consecutive breaking of two adjacent beams, was taken as a unit of locomotor activity. The animals had free access to food and water throughout the experimental period.

4.2.3 Procedure

Mice were placed in the circular corridors at 13.30hrs to measure their locomotor activity for a period of 48-hours. The lighting of the corridors was programmed on a 12-hour light/dark cycle to mimic that of the animal house lights as closely as possible (lights off at 19.30 and on at 07.30). This is illustrated in
Figure 4.1. Data were collected in 5-minute time bins that were then combined into 1- and 6-hour time bins for statistical analysis.

4.2.4 Statistics

Total activity over the 48-hours was analysed using an independent t-test with genotype as the between subject factor. To test for a difference in activity levels across the 48-hr time period a repeated measures univariate analysis was used with genotype as the between subject factor and time-bin as the within-subject factor. Statistical significance was set at $P<0.05$.

4.3 Results

As expected, the activity levels of the animals was in line with the changes in lighting with large increases in activity after the lights went out at 19.30 on both days. Activity during the light phases is significantly lower for both genotypes. A full statistical analysis is discussed further in section 4.3.3.

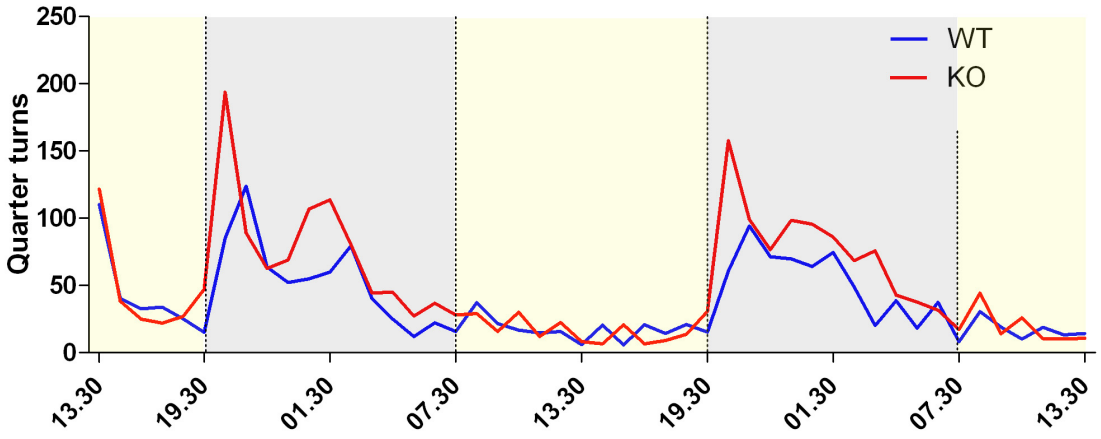


Figure 4.1Illustrative figure of the activity of six wildtype (blue line) and six NK1R-/- (red line) mice over a 48-hour period. Periods of light and dark are designated by yellow and grey backgrounds respectively.

4.3.1 Total activity

There was no statistical difference in the total number of quarter turns travelled by each genotype in the circular corridors over the 48-hour time period, although NK1R-/- mice tended to have greater activity levels than the wildtype animals (Figure 4.2).

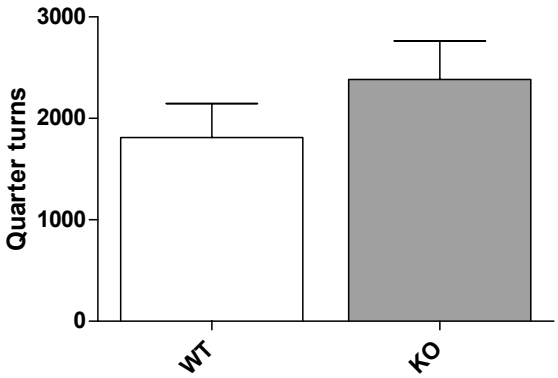


Figure 4.2 Total locomotor activity of wildtype and NK1 receptor knockout mice over a period of 48-hours. N=6.

4.3.2 6-hour time bin analysis

There was an overall interaction between time bin and genotype ($F_{(7,70)}$=2.29, P<0.05) in the repeated measures univariate test. Independent t-tests were conducted for each 6-hour time bin to look for genotype differences, but none emerged. The activity during the period of 19.30-01.30 on both occasions was higher for both wildtype and knockout mice than all other time bins (paired t-tests).

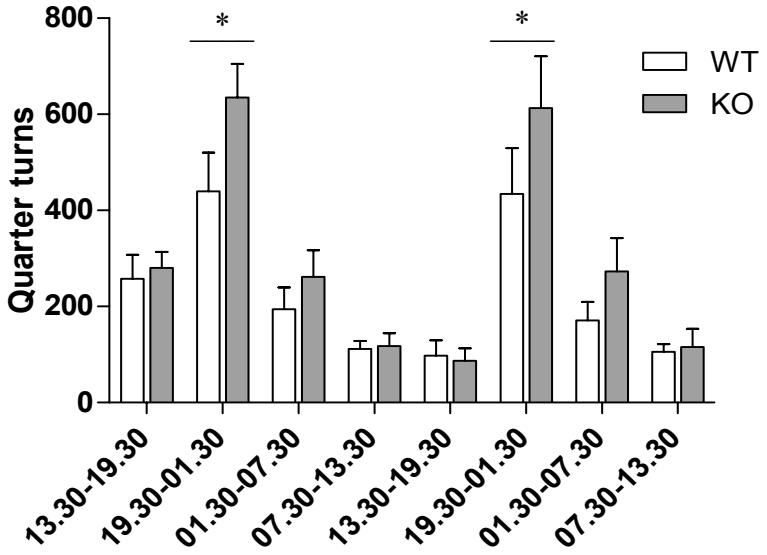


Figure 4.3 Locomotor activity data for WT and NK1R KO mice across 48-hrs divided into 6-hr time bins. Activity of both genotypes was highest during the period just after lights out at 19.30-01.30. N=6

4.3.3 1-hour time bin analysis

The split of the activity into hour time bins is the clearest display of the varying activity levels over the 48-hr period (Figure 4.4). There was a significant interaction between time bin and genotype ($F_{(6.2,62)}$=2.54. P<0.05), with further analyses revealing a difference in activity levels between genotypes in the hour directly after the dark phase began. The activity of NK1 receptor knockout mice was approximately twice that of the wildtype mice during this hour after lights out on both occasions within the experiment (hour7; $t_{(10)}$=3.6, P<0.05 and hour31; $t_{(10)}$=2.78, P<0.05).

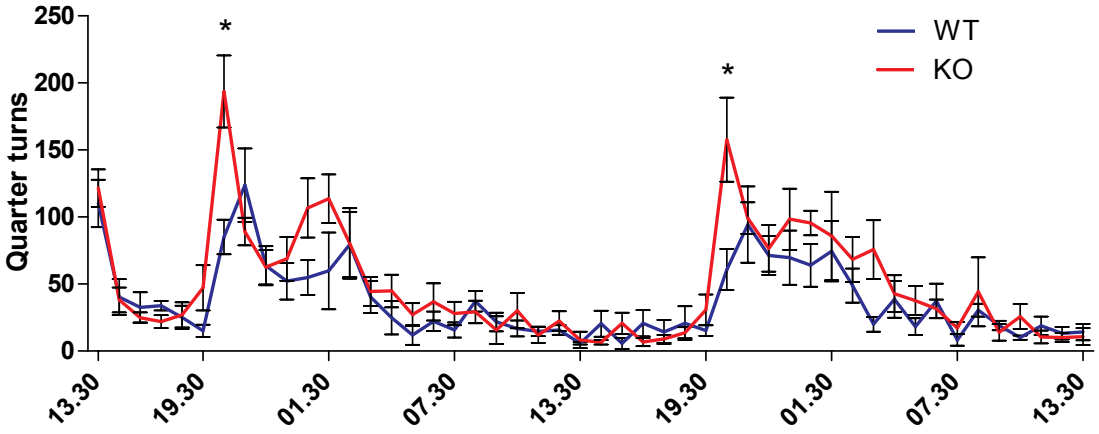


Figure 4.4 Locomotor activity data for WT and NK1 receptor KO mice across 48-hrs divided into 1-hr time bins. Activity of NK1 receptor knockout mice was significantly higher in the hour after lights out compared to wildtype mice. N=6

4.4 Discussion

4.4.1 The NK1 receptor and circadian rhythms

From the illustrative figure (Figure 4.1) we can see that placing the animals in the circular corridor apparatus for 48-hours did not disrupt the circadian changes in locomotor activity. Both NK1R-/- and wildtype mice showed a significant increase in locomotor activity in the early period of the dark-phase, which is ordinarily associated with natural 'foraging' time (Benstaali et al., 2001). Although the 48-hour time period enables a visualisation of locomotor activity in terms of circadian activity, this experiment was never meant to be an in depth circadian rhythm study. With this experimental set-up and small sample size, only very crude differences in circadian phases would ever show up. Substance P and NK1 receptors are found in the intergeniculate leaflet of the mouse, which is part of the indirect pathway from the retina to the suprachiasmatic nucleus, the control center of circadian rhythms. The NK1R/SP system has previously been considered as having a role in the modulation of circadian rhythms (Challet et al., 1998; Gannon and Millan, 2005), although the locomotor activity profiles of wildtype and NK1R-/- mice do overlap there is a difference in time bin at which the peak activity is reached. The peak of activity in NK1R-/- is in the hour immediately after lights-out, while the peak for wildtype mice is in the second hour of the dark phase. This would suggest that the NK1R-/- mice are more sensitive to the change of light conditions, which could arise from the lack of

SP/NK1R modulation of the rhythm through the IGL. More specific experiments related to the control of circadian rhythms such as keeping the mice in constant light or dark conditions could reveal further genotype differences in circadian modulation via the SP/NK1R system.

4.4.2 The NK1 receptor and locomotor activity

Across the 48-hour period there was no difference in the total locomotor activity of the NK1R-/- mice and wildtype controls. However given the extended period of study time for any differences to emerge there would have to have been a robust difference across the majority of the time bins. When the data were analysed in 1-hour time bins the locomotor activity of the NK1R-/- mice was significantly higher than that of the control animals in the hour immediately following the onset of the dark phase, but no differences in activity levels were seen during the light phase ('day') when experimental studies are ordinarily conducted. Therefore, although the NK1R-/- mice have previously been shown to be more active than wildtypes in the light/dark exploration box paradigm (Yan et al., 2010), although this was measured after the administration of injection, the data presented here from treatment naïve animals show that the knockout mice are not always more active than the control animals and caution should be applied when labeling them as 'hyperactive'.

4.4.3 Methodological considerations

The circular corridor experimental set-up enables us to consider activity levels over an extended period of time. There was no difference in activity levels of NK1R-/- and wildtype mice across the total 48-hr period, nor when this was broken down into 6-hour time bins. This is important because other short-term activity related experiments in the light/dark exploration box (LDEB) have reported a hyperactive phenotype for the NK1R-/- mouse (albeit after injections had been given). However the LDEB is usually used to measure anxiety related behaviours and is therefore designed to have an aversive effect on the mice, which is likely to influence activity levels. Also neither the circular corridor, nor the LDEB experiment, takes into consideration the speed at which the animals were moving. This is an important consideration since we cannot determine whether the 'hyperactivity' of the NK1R-/- is due to an increase in the speed of

movement or just an increase in movement per se. This could be measured with computerised tracking software such as EthoVision (TrackSys Ltd., UK).

Similarly the circular corridors are far removed from the sawdust lined home-cage environment where intrinsic activity levels are best reflected. Telemetry experiments, where a probe is inserted subcutaneously to monitor activity levels by interacting with a telemetry mat placed beneath the home cage circumvent the above raised issues, but surgery is required, thus providing a possible confounding factor. Therefore the best method to measure locomotor activities would be to use a computerised home-cage infrared boxes and a large number of mice over an extended period of time. The next chapter looks at the phenotype of the NK1R-/- mouse in terms of the other core symptoms of ADHD, namely impulsivity and attention using the 5-choice serial reaction time task.

5 Testing the NK1R-/- mouse in the 5-choice serial reaction time task (5-CSRTT)

5.1 Introduction

Previous work has shown that NK1R-/- mice display locomotor hyperactivity that is attenuated by treatment with d-amphetamine or methylphenidate (Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2009). These findings, supported by neurochemical abnormalities in the NK1R-/- led to the proposal that the NK1 receptor knockout mouse is a valid animal model of attention-deficit hyperactivity disorder (ADHD) (Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2009, 2010). The aim of this chapter was to further explore this hypothesis by using the 5-choice serial reaction time task (5-CSRTT) to measure attention and impulsivity (which alongside hyperactivity make up the three core diagnostic criterion of ADHD) in NK1R-/- mice versus wildtype controls.

5.1.1 Development of the 5-choice serial reaction time task

The 5-choice serial reaction time task for rodents was developed in the early 1980's (Carli et al., 1983) as an analog of Leonard's 5-choice serial reaction time task used to assess 'vigilance' in humans (Wilkinson 1963), and also contains elements of a continuous performance test (CPT) of sustained attention. CPT is an 'umbrella-term' for a range of attention/vigilance tasks (Riccio et al., 2002) including Rosvold and colleagues' early CPT studies where a child's behaviour was classified as 'on-task' (i.e. paying attention) or 'off-task' (loss of visual attention for more than 2 seconds from the task) (Rosvold et al., 1956) and the widely used X-CPT (responding to an 'X' on a computer screen, while withholding a response to other letters of the alphabet) (Conners 1985). Children with ADHD achieve lower scores in the CPT as measured by increased impulsive and incorrect responding (Epstein et al., 2006).

The rodent version of the 5-choice serial reaction time task requires the animal to scan five evenly spaced apertures until a light cue is presented, after which a nose-poke

response is required in the correct spatial location (Bari et al., 2008). Low response accuracy, and/or a high number of missed (omitted) responses would indicate a deficit in attention, whereas a high number of nose-poke responses prior to the onset of the stimulus light is indicative of an impulsive phenotype.

5.1.2 Measuring impulsivity and attention in rodents using the 5-CSRTT

In the three decades that the 5-CSRTT has been used in rodents, a wide variety of physiological and pharmacological studies of attention and impulsivity have been conducted. The main aim of these studies has been to elucidate the nature of the deficits shown by children with attention deficit/hyperactivity disorder (ADHD) and the effects of drugs used in the clinic to treat the disorder (Robbins, 2002). Studies using the 5-CSRTT tend to fall into 4 categories 1) systemic pharmacological manipulations, 2) physiological manipulations (ablations or lesions) of specific brain regions, 3) phenotyping of knockout mice and 4) comparisons between background strain (usually mice). These divisions (exceptcomparisons of background strain) tend to look at individual neurotransmitter systems e.g. the noradrenergic or dopaminergic systems, and the role these systems potentially play in attention and impulsivity. Although many studies are outlined below, this topic has been comprehensively reviewed in (Robbins, 2002).

5.1.2.1 Manipulations of the noradrenergic system

ADHD in the clinic is treated with a targeted increase in noradrenaline (NA) using both selective noradrenaline reuptake inhibitors (e.g. atomoxetine) and non-selective monoamine reuptake inhibitors that also work on the dopaminergic and to a lesser extent the serotonergic neurotransmitter systems (e.g. methylphenidate (Ritalin®) and d-amphetamine (Adderall®)). It is therefore not surprising that a plethora of studies have been conducted in the 5-choice serial reaction time task using the above drugs. However results appear to depend on the particular parameters used for the tests and small differences in protocol can drastically effect the experimental outcome as highlighted below. The data are summarised in Table 5.1. All experiments presented here were conducted in rats.

Methylphenidate (MPH) (a monoamine reuptake inhibitor)

Although methylphenidate is used to treat ADHD it has been used to artificially *induce* impulsivity, which was preventable with a β-, but not an α1-andrenoreceptor antagonist (Milstein et al., 2010). The increase in premature responding after methylphenidate treatment was somewhat corroborated in the work of Navarra and colleagues, but only when the inter-trial interval was at its longest (10s) (Navarra et al., 2008). Interestingly, when MPH was orally administered, no increase in premature responding was observed (Robinson, 2011). Furthermore, MPH has also been shown to *decrease* 'anticipatory responses' (a similar measurement to premature responding, but one that was not 'punished') (Bizarro et al., 2004). In terms of attention, MPH again displays variable results between studies. No changes were reported in %accuracy after 2mg/kg i.p. in rats, nor after oral MPH (1.0-10mg/kg p.o.) (Milstein et al., 2010; Paterson et al., 2011; Robinson, 2011), yet improvements in attention (increased %accuracy) have been reported after treatment within the same dose range (Bizarro et al., 2004; Navarra et al., 2008).

d-Amphetamine (d-AMP) (monoamine reuptake inhibitor and catecholamine releaser)

Intra-peritoneal injection of d-amphetamine in rats has been shown to both improve and worsen impulsive responding in the 5-choice serial reaction time task (Grottick and Higgins, 2002; Bizarro et al., 2004; Paterson et al., 2011). The effect of d-amphetamine on attention appears to be more robust, with studies reporting improvement in percentage accuracy after treatment with low doses of d-AMP (0.1-1.0mg/kg i.p.) (Grottick and Higgins, 2002; Bizarro et al., 2004). Higher doses were also effective at increasing accuracy, but only when the sensitivity of the test was increased (more nose-poke responses (either 3 or 10) were required to release the reward) (Koffarnus and Katz, 2011).

Reboxetine (selective noradrenaline reuptake inhibitor)

In rats, low doses of reboxetine (0.1-0.3mg/kg i.p.) have been shown to decrease premature responding, however the number of 'missed' responses ('omissions', an index of attention) also increased (Robinson, 2011). A higher dose (10mg/kg i.p.) improved premature responding and attention, but also increased omission and latencies, perhaps suggesting a sedatory side effect of the high dose (Liu et al., 2009).

Atomoxetine (selective noradrenaline reuptake inhibitor)
Results from the 5-CSRTT after atomoxetine treatment are similar to those of reboxetine inasmuch that premature responses (impulsivity) and accuracy (attention) were both improved, but omissions and latencies worsened (Navarra et al., 2008; Robinson, 2011). However, other studies have reported a decrease in premature responding after atomoxetine treatment, without detrimentally affecting other measures (Robinson et al., 2008; Paterson et al., 2011).

Table 5.1 Description of previous findings on impulsivity and attention in the 5-CSRTT after pharmacological manipulation of the noradrenergic system. ↑ indicates either an increase in impulsivity or an improvement in attention, ↓ indicates either a decrease in impulsivity or attention, i.p., intraperitoneal, p.o., per os, [a] only when the ITI was 10s, [b] not premature responses but 'anticipatory responses' that were not punished, [c] over extended sessions of 250 trials, [d] impulsivity decreased, but the total number of reinforcers also decreased, [e] only animals that displayed sub-optimal performance were tested, [f] accuracy improved, but errors of omission increased.

Although pharmacological studies provide a vital insight into the role of neurotransmitter systems in human diseases, treatment tends to be systemic, and therefore cannot reveal which brain regions within the noradrenergic system are responsible for the effects observed. Studies that use a targeted depletion of a specific neuronal population can help narrow down the brain regions that are important for specific behaviours. Surprisingly, drastic depletion of cortical noradrenaline of up to 95% either with intracortical infusion of dopamine-β-hydroxylase conjugated to saporin (DβH-saporin) or 6-hydroxydopamine (6-OHDA) lesion of the dorsal noradrenergic bundle did not impair any aspect of performance in the 5-choice serial reaction time task (Carli et al., 1983; Cole and Robbins, 1987, 1992; Milstein et al., 2010). Therefore other brain regions within the noradrenergic system such as the locus coeruleus may have a greater role in determining the behaviours in the 5-CSRTT.

5.1.2.2 Manipulations of the dopaminergic system

One strong hypothesis for the cause of ADHD is the dopaminergic theory as discussed in section 1.5.2. It is therefore important to consider the effect of manipulating the dopaminergic system in relation to performance in the 5-CSRTT.

Systemic blockade of D4, but not D1 or D2, receptors reduced methylphenidate-induced impulsivity, but did not effect accuracy (% correct or % omissions) (Milstein et al.,

2010). Nafadotride, a D2/3 antagonist when delivered systemically has no effect on impulsivity in the 5-CSRTT, yet when administered via microinfusion into the nucleus accumbens core and shell (NAcbC and NAcbS) of rats selected for their high impulsive nature, the antagonist both decreased and increased impulsivity respectively. Furthermore a DA D2/3 partial agonist (aripiprazole) administered in the same regions had no effect on impulsivity, yet systemic injection decreased impulsive behaviour (Besson et al., 2010). This one study highlights the complex and opposing nature of receptors within a single brain region, and the importance of the route of administration as to the action of a drug. Antagonism of D1 but not D2 receptors in the core and shell regions of the nucleus accumbens decreases premature responding in the task (Pattij et al., 2007a). The dopaminergic system also has a role in the control of behaviours other than impulsivity in the 5-CSRTT. D1 and D2 receptors within the core of the nucleus accumbens also appear to modulate different aspects of behaviour in the 5-CSRTT. Manipulating the activity of D1 receptors in the NAcbC had selective effects on attentional accuracy, while stimulation of D2 receptors increased perseverative responding (Pezze et al., 2007). In addition to the importance of the dopaminergic system the NAcb in the control of behaviours associated with the 5-CSRTT, manipulations of the DA system in the medial prefrontal cortex also modulate attentional performance (Granon et al., 2000).

In the synaptic cleft, dopamine is metabolised by the enzyme catecholamine-o-methyl transferase (COMT), which is inhibited by the drug tolcapone. Based on the selective effect of tolcapone on cortical dopamine versus noradrenaline (Tunbridge et al., 2004) it was given to rats that demonstrated sub-optimal baseline behaviour in the 5-CSRTT to try to improve their performance. Tolcapone had no positive effect on accuracy or premature responding, and in fact worsened performance by increasing the percentage of omitted responses (Paterson et al., 2011).

Depletion of dopamine using 6-OHDA has been carried out in both the ventral (including the nucleus accumbens) and dorsal (caudate-putamen) striatum. Despite the lesions being comprehensive, no changes in baseline response accuracy were observed for either sub-region (Cole and Robbins, 1989; Baunez and Robbins, 1999). Depletion of dopamine in the ventral striatum did however elicit a small decrease in premature responding (Cole and Robbins, 1989).

5.1.2.3 Manipulations of the serotonergic system

Serotonin is a member of the monoamine family of neurotransmitters, although non-selective monoamine re-uptake inhibitors are thought to only have small effects on the serotonergic system. The behavioural findings from the 5-CSRTT following manipulation of the serotonergic system paint a diverse and complex picture, possibly due to the large number of 5-hydroxytryptophan (5-HT or serotonin) receptor subtypes and the non-selectivity of the agents used to investigate the system.

In one early study there were deficits in accuracy following administration of the non-selective 5-HT2A/C agonists LSD (0.1mg/kg, i.p.) and quinzapine (2.5mg/kg) that were attenuated by the 5-HT2A/C-receptor antagonist ritanserin (Carli and Samanin, 1992). However further studies using an alternative 5-HT2A/C agonist, DOI, did not replicate the initial findings and in fact found no change in accuracy, yet premature responding was elevated (Koskinen et al., 2000). Serotonergic agents often bind to more than one sub-type of receptor e.g. LSD acts on both 5-HT2A/C and 5-HT1A receptors. Carli and Samanin (2000) continued their initial work by further investigating the 5-HT1A receptor and found treatment with the agonist OH-DPAT severely impaired behaviour in the 5-CSRTT (Carli and Samanin, 2000). The previously mentioned methylphenidate-induced impulsivity observed by Milstein et al. (2010), was not reduced by co-treatment with 5-HT1A, nor 5-HT1B antagonists. Attention was also not affected by this antagonist treatment (Milstein et al., 2010).

The complex findings that come from the pharmacological blockade of the serotonergic system are echoed in that from central 5-HT depletion studies. Two different neurotoxins have been used for central 5-HT depletion, para-chlorophenylalanine (PCPA) and 5,7 dihydroxytryptamine (DHT) that have resulted in conflicting results. PCPA treatment, leading to a 99% reduction in frontal cortical serotonin (but also reduction of cortical dopamine and noradrenaline) reduced accuracy, without affecting premature responding, yet intracerebroventricular administration of DHT (after pre-treatment with a noradrenergic and dopaminergic uptake inhibitors) robustly increased impulsivity but not accuracy in rats in the 5-CSRTT (Jäkälä et al., 1992; Harrison et al., 1997). Therefore serotonergic control of impulsivity in the 5-CSRTT is not driven from the cortex.

5.1.2.4 Manipulations of the cholinergic system

The cholinergic system has not been as comprehensively studied as the monoamine systems above, but given the influential role of cholinergic interneurons in the striatum, a region highly associated with those behaviours measured in the 5-CSRTT, it is an imbalance that should be addressed.

The cholinergic studies that have been completed mainly report deficits in response accuracy after treatment (both systemic treatment and lesion studies) but conflicting results have arisen. Systemic treatment with scopolamine, a muscarinic antagonist, has produced inconsistent findings in the 5-CSRTT. One group reported impairments in response accuracy with a low dose of the drug (0.1mg/kg s.c.), yet with the same injection route and dose others only found a deficit in attention when the stimulus light was presented at the same time as a white-noise distracter (Jones and Higgins, 1995; Mirza and Stolerman, 2000). The variable findings from systemic scopolamine treatment have also been reported in mice, where differences in background strain and attentional load produced inconsistent results after scopolamine treatment (Humby et al., 1999). Following on from the findings that muscarinic acetylcholine receptor antagonism causes attentional deficit, it is surprising that mAChR agonists, nor the cholinesterase inhibitor physostigmine have not shown any beneficial effects on baseline performance in the 5-CSRTT (Muir et al., 1995; Robbins, 2002), however one group have shown improved accuracy and decreased omissions with nicotine treatment (an cholinesterase inhibitor) (Bizarro et al., 2004). Cholinesterase inhibitors however do improve the 5-CSRTT performance of rats with lesions of the nucleus basalis magnocellularis (nbm), a major cholinergic centre (Muir et al., 1995). The effects of nicotinic acetylcholine receptors (nAChR) are also inconsistent. The non-competitive nAChR antagonist, mecamylamine, has been shown to have a detrimental effect on accuracy (Jones et al., 1995; Grottick and Higgins, 2002), yet other groups reported no change in response accuracy (Mirza and Stolerman, 2000). A recent study has even implicated the cholinergic system in impulsivity, more specifically the α4β2 nicotinic acetylcholine receptor subtype, although the brain region responsible for this is yet to be elucidated. Candidate areas with high levels of endogenous acetylcholine and α4β2 receptors include the ventral tegmental area, the dopaminergic terminals in the nucleus accumbens and the infralimbic cortex (Tsutsui-Kimura et al., 2010).

A lack of a suitably specific neurotoxin hindered early work on lesions of the cholinergic system, since different excitotoxins varied greatly in potency and selectivity for cholinergic neurons (Everitt and Robbins, 1997). AMPA was discovered to be a good excitotoxin and lesion of the basal forebrain, but not the vertical limb of the diagonal band, resulted in poor performance in the 5-CSRTT, thus showing that the effects of cholinergic depletion are region specific (Muir et al., 1994, 1996).

5.1.2.5 Using mice in the 5-CSRTT

All of the above experimental work in the 5-CSRTT has been conducted using rats. However with a few minor changes to the 5-CSRTT parameters, mice can learn and perform the task equally well (Humby et al., 1999; Sanchez-Roige et al., 2012). Under low attentional demand, mice can develop a stable baseline performance with high levels of accuracy, short response times and low levels of premature responding (Humby et al., 1999; Marston et al., 2001; de Bruin et al., 2006; Hoyle et al., 2006; Davies et al., 2007).

Unlike in rats, where there is a relatively small selection of background strains used in biomedical research, there is a phenomenal number of different mouse lineages. It is commonly known, but not often seriously considered, that background strain can have a significant influence on the behavioral performance in a single experimental paradigm. There have been several studies that have looked at the baseline performance of different rat and mouse strains in the 5-CSRTT. Wistar and Long Evans rats perform to a similar level in the 5-CSRTT, but in comparison Brown Norwegian/Lewis crosses displayed decreased responding, speed and impulsivity (Didriksen and Christensen, 1993). Sprague-Dawley rats required significantly fewer training sessions to learn the task than Long Evans rats (Auclair et al., 2009). Mouse background strain has a significant effect on behaviour in the 5-CSRTT as outlined in Table 5.2.

Table 5.2 Table outlining a selection of mouse background strain comparisons conducted in the 5-CSRTT

The fact that mice can perform equally as well as rats in the 5-CSRTT allows for the testing of transgenic and genetically modified mice in a behavioural paradigm that can assess attention, response inhibition, compulsion, response times and motivation.

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

Previous findings within the lab have shown that NK1R-/- mice display locomotor hyperactivity in the light/dark exploration box (LDEB) (Herpfer et al., 2005; Fisher et al., 2007). This finding, observed in two separate studies, provided the first evidence towards the NK1R-/- mouse being proposed as a rodent model of ADHD (face validity). Furthermore this hyperactivity was attenuated by treatment with d-amphetamine and methylphenidate providing predictive validity for the NK1R-/- mouse being a model of ADHD (Yan et al., 2010). The proposal was further consolidated when it was shown that NK1R-/- mice display similar neurochemical profiles to patients with ADHD, including reduced DA efflux in the frontal cortex (Yan et al., 2009, 2010).

5.1.4 Experimental aims

The ability of the 5-choice serial reaction time task to measure both attention and impulsivity in the same experimental set-up makes it the ideal choice for testing the NK1R-/- mouse as a rodent model of ADHD. Given the previous findings that the hyperactivity of the NK1R-/- mouse is attenuated with psychostimulants that are used to treat ADHD in the clinic we would hypothesise that the NK1R-/- mouse would display increased impulsivity and attentional deficits in the 5-CSRTT compared to wild-type counterparts. All of the behaviours recorded in the 5-CSRTT will be looked at in their entirety to form a complete profile to investigate further the ADHD-like behaviour of the NK1R-/- mouse.

5.2 Materials and Methods

5.2.1 Subjects

The animals used in the 5-CSRTT were from the original, homozygous bred, MF1 colonies as outlined in section 2.1.2. 12 wildtype (WT) and 12 knockout (KO) animals (males, aged approximately 8 weeks old at the start of each experiment) were tested in this experiment. One WT mouse from cohort 2 died in the early stages of training. The mice were kept on a restricted diet to maintain body weights at 90% of the animals free-

feeding weight to ensure they remained motivated for the task. Animals were weighed every day prior to the start of the experiment.

5.2.2 5-CSRTT Procedures

The training / testing session occurred once a day, Monday to Friday. All mice were brought to the lab at 09.00, weighed, and placed in the test room for acclimatisation. Each mouse was placed in the 5-CSRTT chambers at the same time every day. Six wildtype and six knockout mice were trained / tested between 10.00 and 12.00, and six of each genotype between 13.00 and 15.00. The mice were habituated, trained and tested in the 5-choice serial reaction time task as outlined in full in the Materials and Methods chapter (section 2.4) and was based on the protocol from (Oliver et al., 2009). Figure 5.1 is a schematic diagram of the possible outcomes from the 5-choice serial reaction time task.

Figure 5.1 Schematic diagram of the sequence and potential outcomes from the 5-choice serial reaction time task. A trial is initiated with a head entry into the magazine, which is followed by a brief light stimulus in one of five apertures on the opposite wall to the magazine after an inter-trial interval (ITI) of 5 seconds (at baseline). The inter-trial interval can be extended in a long inter-trial interval (LITI) test or presented in an unpredictable variable inter-trial interval (VITI) test. If the animal responds before the stimulus is illuminated a premature response is recorded. After the presentation of the stimulus the mice have 5 seconds to respond within the 'limited hold' time (LH). If a nose poke response is made into the hole where the stimulus was presented a correct response is recorded and a reward released. If the animal nose pokes into the wrong hole, or fails to respond within the LH time period a time out (TO) period of complete darkness is initiated. A premature response also incurs a time out period. (Figure modified from (Bari et al., 2008)).

5.2.3 Testing regime

After each animal had satisfactorily reached the criteria to pass training and performed at a stable baseline, they were tested with a single inter-trial interval probe test of the long and variable ITI tests. Half the cohort was first tested with a long ITI (LITI NI-1), the other with the variable ITI (VITI NI-1). One week later, after performing satisfactorily at baseline, the corresponding test was conducted. There was then an unscheduled 5-week break in testing due to various reasons. The mice were on ad lib food for 3 weeks, followed by a week to re-establish the 90% body weight and a final week to re-establish satisfactory baseline performances. They were then tested in the LITI and VITI tests again with no injection treatment (LITI and VITI NI-2) to make sure the results were comparable to the first experience of the tests. They then went on

to be tested in a fully randomised drug regime with 2 different NK1 receptor antagonists (RP 67580 (RP) or L-733,060 (L-733)) at 2 different doses with both the LITI and VITI tests. The animals either received another no-injection trial (NI-3), vehicle, RP 5mg/kg, RP 10mg/kg, L-733 5mg/kg and L-733 10mg/kg. The results from training and the initial LITI and VITI tests are presented here, whilst data from the antagonist study are presented in the next chapter.

5.2.4 5-CSRTT Statistics

The behaviours scored in the 5-CSRTT, were previously outlined in section 2.4.5 but are here as a reminder.

- **Total number of trials required to pass the training phase**: the sum of all the trials completed at each training stage (for analysing the data obtained from Training Stage 1-6 only).
- **Total number of trials completed in each test session:** total correct response + total incorrect + total omission (for analysing the data obtained from the fixed long ITI and variable ITI tests, only).
- **% Accuracy:** correct responses / (correct + incorrect responses) x 100
- **% Omissions:** total omissions / (correct + incorrect responses + omission) x 100
- **% Premature responses:** premature responses / (correct + incorrect + omission + premature responses) x 100
- **Latency to correct response:** latency to nose-poke into the correct hole after the onset of stimulus (seconds)
- **Latency to collect reward:** latency to collect the reward after a correct response (seconds)
- **Perseveration:** total number of responses made into the holes after a correct response but before the collection of the reward.

For the training data, a repeated measures 2-way ANOVA with 'stage' as the within-subject factor and genotype as the between subject factor was used. Statistics were carried out on raw data where possible, but if the variance of the groups differed (i.e. Levene's test for the equality of error variance was significant), the data was transformed (Square-root, Lg10 or Lg10(score+1) (the latter being used if any of the raw data values were zero)) to normalise the variance. If this did not correct the problem, non-parametric statistical tests were used. These exceptional cases are highlighted in the results section. Significant differences for the within subject factor 'stage', were not pursued. Statistical significance was set at $P<0.05$. For the column

graphs, significant genotype differences are denoted by a black bar between the columns that represent a statistical difference of at least $P<0.05$.

5.3 Results

5.3.1 Training

Of the behaviours measured across training stages 1-6 overall genotype differences were found in the total number of sessions required to pass training, latency to collect reward ($F_{(1,21)}=8.76$, $P<0.01$) and perseveration ($F_{(1,21)}=6.0$, $P<0.05$). Each behaviour is discussed below with the relevant post-hoc analyses for latency to collect the reward and perseveration.

Total number of sessions required to pass training. NK1 receptor knockout mice required more training sessions to reach satisfactory baseline criterion than the wildtype control animals ($t_{(21)}=2.63$, $P<0.05$) (Figure 5.2A).

Accuracy is calculated as the (number of correct responses / (number of correct responses + number of incorrect responses)) yet only takes into account the accuracy of when a response is made. Although conventionally considered as an index of attention, this measure does not take omissions or premature responses into consideration. No overall genotype difference was found meaning both genotypes were equally accurate over the six stages of training (Figure 5.2B).

Omissions is calculated as the (number of omissions / (number of omissions + number of correct responses + number of incorrect responses)). Although NK1R-/- mice failed to respond to the stimulus light more often than wildtype controls, especially at stages 2, 3 and 4, this did not yield a significant genotype difference (Figure 5.2C).

Premature responses is calculated as the (number of premature responses / (number of premature responses + number of omissions + number of correct responses + number of incorrect responses)). Premature responding corresponds to impulsivity. Across the six training stages there was no significant difference in the impulsive behaviour of wildtype and NK1 receptor knockout mice (Figure 5.2D).

Latency to make a correct response is an index of both attention and motivation. No overall genotype difference was observed for this measure showing that both genotypes respond to the cue light at the same time (Figure 5.2E).

Latency to collect reward is typically used as a measure of motivation but as suggested below it is strongly linked with perseveration too. NK1R-/- mice were consistently slower to collect the reward after a correct response had been made. This occurred at all stages of training (1-6), but was statistically different at stages 1, 5 and 6 (Figure 5.2F). This could be due to a motivational deficit, however the latency to make a correct response did not differ from that of the wildtype mice, making this an unlikely reason. It is most likely to be due to the increased perseverative responding (see below) after a correct response had been recorded, which would delay the collection of the reward.

Perseveration is recorded as repeated, yet unnecessary, nose poking in the stimulus hole after a correct response has been made. NK1R-/- mice consistently recorded a higher number of repeated nose pokes into the stimulus hole after the initial correct response compared to wildtype controls, although this was only statistically significant at stages 1 and 5 of training (Figure 5.2G).

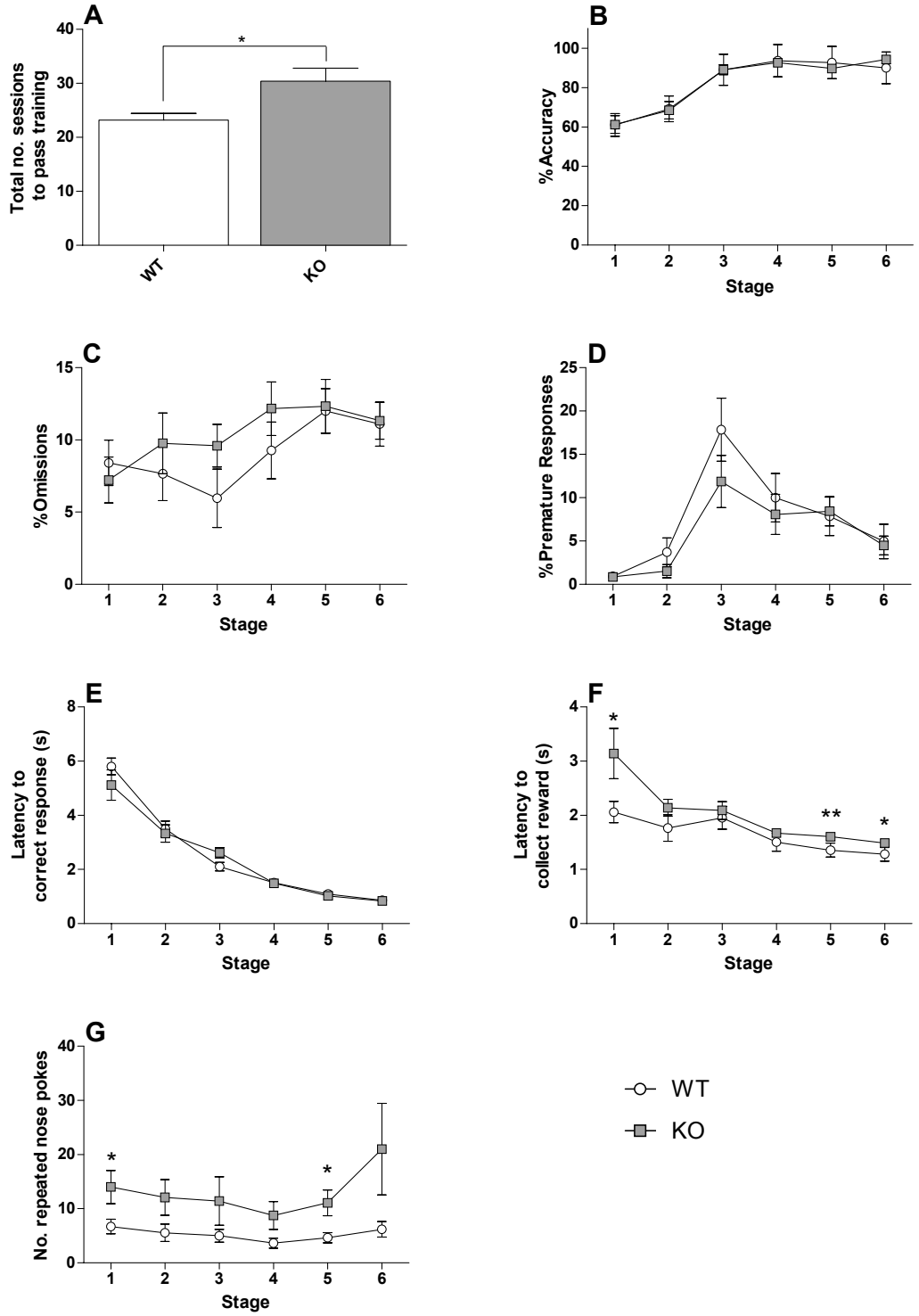


Figure 5.2 A comparison of the behaviour of WT and KO mice in the 5-CSRTT during training stages 1 to 6. (A) number of sessions required to pass training, (B) percentage accuracy, (C) percentage omissions, (D) percentage premature responses, (E and F) latencies to make a correct response and to collect the reward respectively and (G) perseverative nose pokes made after a correct response. WT - wildtype (white circles) and KO - knockout (grey squares). * P<0.05, ** P<0.01 N=11-12

5.3.2 Long and variable inter-trial interval tests (LITI and VITI NI-1)

Once the animals had reached a stable baseline performance with the parameters of the task as of that for stage 6 they were subjected to the test phases of the 5-choice serial reaction time task. The long inter-trial interval (LITI) test increases the attentional load of the task as the wait time between trial initiation and the stimulus light being illuminated is increased from 5s to 7s, while in the VITI test the interval is either 2, 5, 10 or 15 seconds presented in a randomised order. The data from the two tests were first analysed with a 2-way ANOVA with genotype as the between subject factor and 'test' (LITI and VITI) as the within subject factor to check whether any behaviours differed between the tests.

Total number of trials is an indication of motivation and sustained attention. Across the two 5-CSRTT tests, there was a significant effect of genotype ($F_{(1,21)}$=5.43, P<0.05) and test ($F_{(1,21)}$=6.98, P<0.05). Further post-hoc analysis revealed that the NK1R-/- mice completed significantly fewer trials in the VITI test than in the LITI, which also resulted in a significant genotype difference in the VITI test ($t_{(12.1)}$=2.69, P<0.05) (Figure 5.3A).

Accuracy The difficulty of the test did not effect the high accuracy of the responses made, and there was no genotype difference in the accuracy of the responses made in either test (Figure 5.3B). Again this does not take into account omissions or premature responses.

Omissions Both wildtype and NK1R-/- mice made more errors of omission in the unpredictable VITI test than in the LITI test ($F_{(1,21)}$=29.8, P<0.001). There was also a significant genotype difference in both tests ($F_{(1,21)}$=15.8, P<0.001) with NK1R-/- mice failing to respond to the stimulus lights more than the wildtype controls (LITI: $t_{(21)}$=2.17, P<0.05; VITI: $t_{(21)}$=3.17, P<0.01) (Figure 5.3C). This fits with the hypothesis of the NK1R-/- mouse model of ADHD, as a high percentage of omissions indicate a lack of attention to the task, thus replicating one of the core symptoms of ADHD.

Premature responses The initial 2-way ANOVA revealed significant overall differences in 'genotype' and 'test' ($F_{(1,21)}$=7.5, P<0.05 and $F_{(1,21)}$=13.8, P<0.001 respectively). Post-hoc analyses showed that there was no difference in the behaviour of

wildtype and NK1R-/- mice in the LITI test, but a significant increase in premature responses made by the NK1R-/- mice in the VITI test compared to the LITI test (KO *c.f.* LITI $t_{(11)}$=3.7, P<0.01) elicited a genotype difference ($t_{(21)}$=3.3, P<0.01) (Figure 5.3D). Therefore in their first experience of the unpredictable VITI test the NK1R-/- mice display an impulsive phenotype.

Latency to correct response Both wildtype and NK1R-/- mice were slower to respond to the cue light in the VITI test compared to the LITI test ($F_{(1,21)}$=39.4, P<0.001, WT: $t_{(10)}$=7.1, P<0.001 and KO: $t_{(11)}$=4.35, P<0.001). Both genotypes responded in a similar manner in the LITI test, but in the VITI test NK1R-/- mice were significantly slower to make a correct response than the wildtype controls ($t_{(13.7)}$=2.5, P<0.05) (Figure 5.3E).

Latency to collect reward As in seen in all stages of training, NK1R-/- mice were slower to collect the reward after a correct response in both the LITI and VITI tests ($F_{(1,21)}$=13.2, P<0.01; post-hoc LITI $t_{(21)}$=3.0, P<0.01; VITI $t_{(21)}$=3.9, P<0.001), but the difficulty of the task did not alter the response times in either genotype (Figure 5.3F).

Perseveration As seen in training, NK1R-/- mice made more repeated nose pokes into the stimulus hole after a correct response had already been made in the LITI and VITI tests (effect of 'genotype' $F_{(1,21)}$=4.2, P<0.05; post-hoc LITI $t_{(14.0)}$=1.9, P<0.05; VITI $t_{(11.4)}$=2.0, P<0.05), but this was not changed by the different difficulties of the tests (Figure 5.3G).

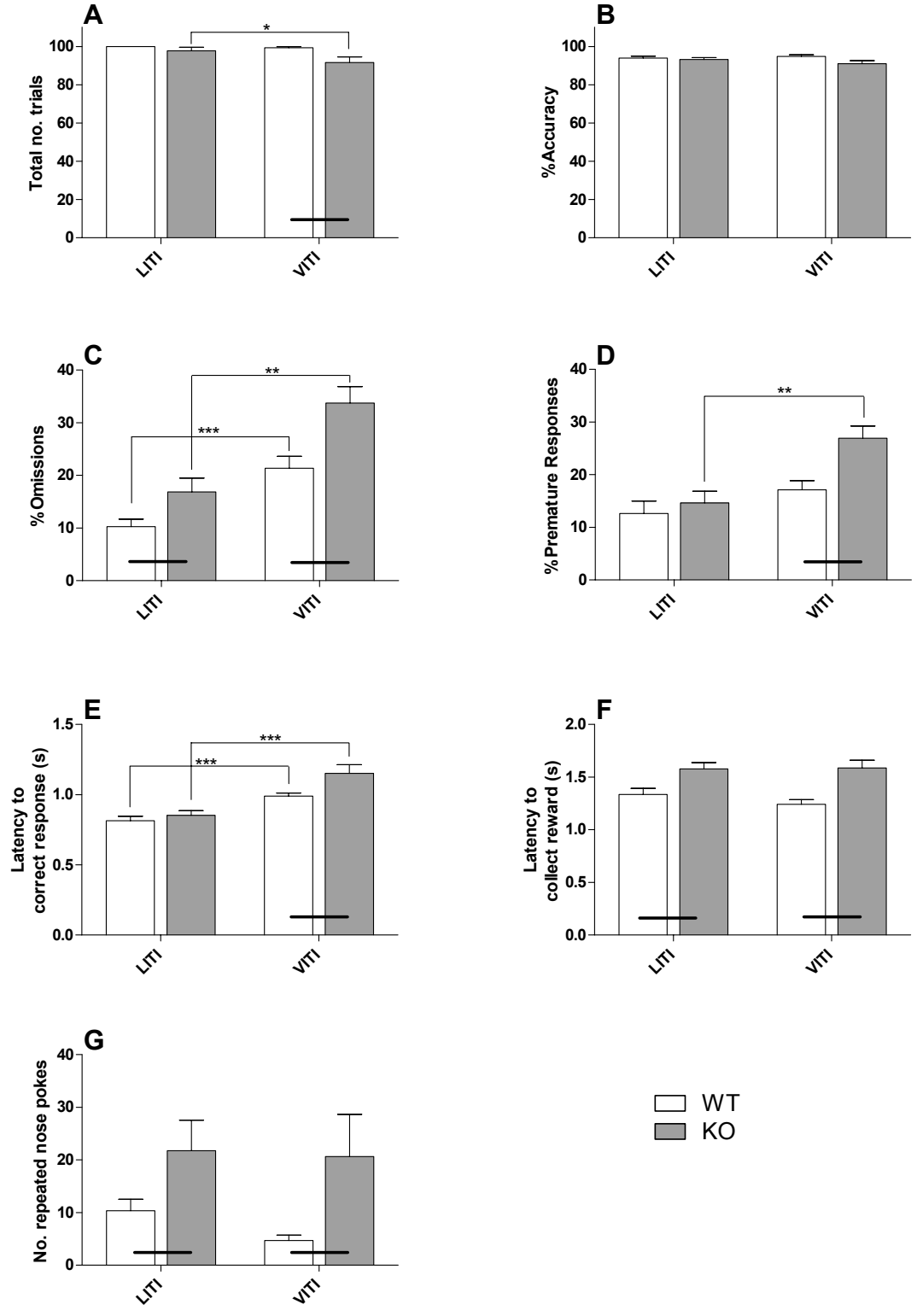


Figure 5.3 A comparison of the behaviour of WT and KO mice in the 5-CSRTT during the animals' first experience of the long and variable inter-trial interval tests (LITI and VITI NI-1). (A) Total number of trials completed in the session, (B) percentage accuracy, (C) percentage omissions, (D) percentage of premature responses, (E) latency to a correct response, (F) latency to collect reward and (G) number of perseverative nose-poke responses. WT - wildtype and KO - knockout. Genotype differences are represented by solid black bars between the columns. * P<0.05, ** P<0.01, ***P<0.001 N=11-12

Table 5.3 summarises the findings from training and the LITI and VITI tests. The increasing difficulty of the tests draws out more behavioural differences between the NK1R-/- mice and their wildtype controls. The only genotype differences during training were increased perseverative responding by the NK1R-/- mice, which was accompanied by a longer latency to collect the reward probably as a direct consequence of the perseverative behaviour. These differences prevailed across the two test phases too. The accuracy of responses did not waver across training and the two tests and remained extremely high throughout. Increasing the inter-trial interval from 5 to 7s (LITI test) meant that the NK1R-/- mice performed more errors of omission than their wildtype controls, indicative of an inattentive phenotype that also remained in the VITI test. NK1R-/- mice display an impulsive phenotype, but only in the VITI test when the cue lights were presented in an unpredictable order.

Behaviour	**Training**	**LITI test**	**VITI test**
% Accuracy	No genotype difference	No genotype difference	No genotype difference
% Omissions	No genotype difference	KO ↑	KO ↑
% Premature responses	No genotype difference	No genotype difference	KO ↑
Latency to correct response	No genotype difference	No genotype difference	KO slower to respond to the cue light
Latency to collect reward	KO slower	KO slower	KO slower
Perseveration	KO ↑	KO ↑	KO ↑

Table 5.3 Summary of findings from training LITI and VITI tests

5.3.3 Splitting the VITI into separate ITI times

The fact that the LITI test and VITI tests produced different results, i.e. the impulsive phenotype of the NK1 receptor knockout mice was only drawn out in the VITI test, we can say that the difficulty of the task has a bearing on the behavioural responses. Prolonging the ITI time in the LITI test from 5 to 7s did not draw-out any underlying differences in impulsivity between the genotypes, but when the ITI was varied and presented in an unpredictable, randomised schedule the NK1R-/- committed significantly more premature responses than the wildtype controls. There are two

possible reasons for this; either the NK1R-/- mice cannot adjust to the irregular presentation of the stimulus, or that the knockout mice cannot withhold their response during the longer ITI times of 10 and 15 seconds. Here the VITI data are further analysed into the different ITI trials of 2, 5, 10, and 15seconds. This should provide more of an insight into the level of control the mice have over their behaviour. The data were analysed with a 2-way repeated measures ANOVA with 'genotype' as the between subject factor and 'ITI' as the within-subject factor.

Total number of trials Both genotypes completed fewer trials at the longer ITI intervals (main interaction of 'genotype' and 'ITI': $F_{(1.7,35.3)}$=5.97, P<0.01) (Figure 5.4A). The computer system presents the cue lights on a randomised schedule, with an equal ratio of ITI times. The decrease in total number of trials arises because these data do not include the number of premature responses, which were significantly higher during the longer ITI times (see below).

Accuracy The accuracy of responses was not dependent on the ITI length, but a previously unknown overall 'genotype' effect arose ($F_{(1,21)}$=5.1, P<0.05) with NK1R-/- mice being slightly less accurate across each split. Post-hoc analysis revealed that this was only significant for the 15s ITI split ($t_{15.2)}$=2.2, P<0.05) (Figure 5.4B).

Omissions Significant overall effects of 'genotype' and 'ITI' were found in the initial ANOVA ($F_{(1,21)}$=15.7, P<0.001 and $F_{(3,63)}$=15.7, P<0.001 respectively). Post-hoc analyses revealed that NK1R-/- mice omitted more than wildtypes at 5, 10 and 15 second ITI splits, but not at 2s. There was no statistical interaction between genotype and ITI so, in general, as the ITI time increased so did the errors of omission committed by both genotypes. The percentages of omissions committed at 10s and 15s ITI splits were significantly higher than that of 2s and 5s (Figure 5.4C).

Premature responses As the length of the ITI increased, so did the number of premature responses made by both genotypes ($F_{(3,63)}$=155.8, P<0.001). At the 2s ITI split, no premature responses were made by either genotype. Given that there was a significant interaction between genotype and ITI split, post-hoc analyses were conducted to compare each data set. Each comparison was statistically significant from each other except the premature responses made by NK1R-/- mice at 10 and 15s. There

was also an overall effect of genotype ($F_{(1,21)}$=10.9, P<0.01), with NK1 receptor knockout mice performing more premature responses at 5, 10 and 15s ITI splits, however this only reached significance at the 10s ITI ($t_{(21)}$=3.93, P<0.001) and only just failed to reach significance at the 15s ITI (P=0.054) (Figure 5.4D).

Latency to correct response Non-parametric statistical analysis had to used on the data for the latency to correct response because Levene's test of variance was violated and could not be corrected with either square-root of log10 transformation of the data. Interestingly, even though a significant genotype difference was found in the analysis of the pooled VITI data (Figure 5.3E), when analysed separately with non-parametric Mann-Whitney tests no genotype differences emerged. However the Wilcoxon signed rank tests for comparison of the ITI splits showed that the latency to respond after the 2s ITI was greater than that after a 5 or 15 second ITI, possibly reflecting the position of the mice within the chambers (*c.f.* 2s: 5s Z=4.17, P<0.001 and 15s Z=2.56, P<0.05) (Figure 5.4E).

Latency to collect reward The length of ITI did not have any effect on the latency to collect the reward, but NK1R-/- mice were robustly slower at every ITI split (Figure 5.4F).

Perseveration A significant interaction between 'genotype' and 'ITI' emerged for the perseveration data set ($F_{(1.3,28.1)}$=3.8, P<0.05). Perseverative responses were consistent across all ITI splits for the wildtype mice but not for the NK1R-/- mice. There was a significant decrease in number of perseverative responses made after the longer ITI times (10 and 15s) than for the shortest ITI time of 2s (10s $t_{(11)}$=2.9, P<0.05 and 15s $t_{(11)}$=2.7, P<0.05). The NK1 receptor knockout mice committed a greater number of perseverative responses than the wildtype controls at every ITI split, but this only reached statistical significance at the 2s interval ($t_{(11.7)}$=2.3, P<0.05) and just failed to reach significance at the 5s split (P<0.053) (Figure 5.4G).

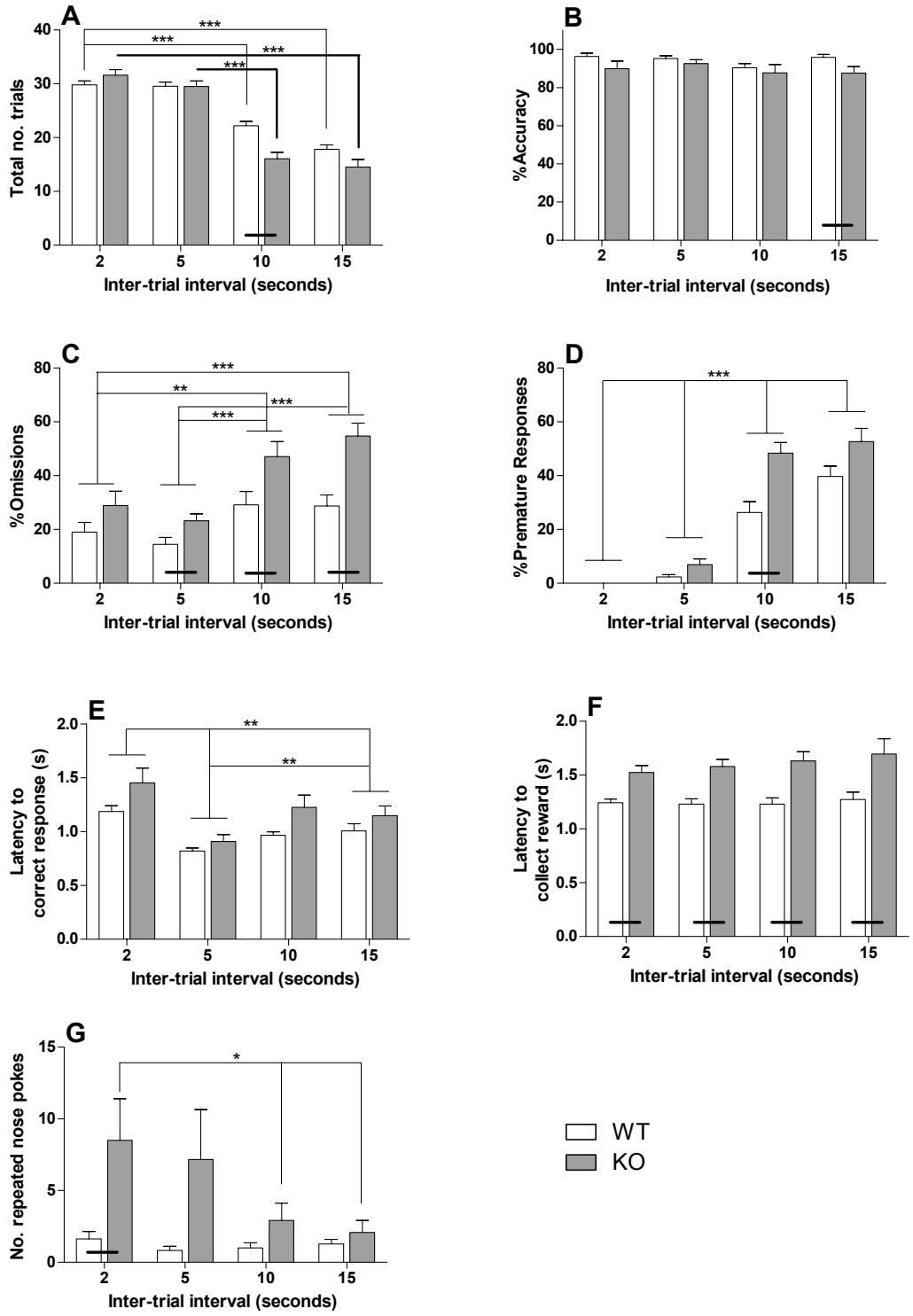


Figure 5.4 A comparison of the behaviour of WT and KO mice in the 5-CSRTT during the animals' first experience of the variable inter-trial interval test (VITI NI-1) split into the individual inter-trial interval trials of 2, 5, 10 and 15 seconds. (A) Total number of trials (B) percentage accuracy, (C) percentage omissions, (D) percentage of premature responses, (E) latency to a correct response, (F) latency to collect reward and (G) number of perseverative nose-poke responses. WT - wildtype and KO - knockout. Genotype differences are represented by solid black bars between the columns. * P<0.05, ** P<0.01, ***P<0.001 N=11-12

Both accuracy and the latency to collect the reward were not affected by the different lengths of ITI wait times. Unsurprisingly the % omissions and % premature responses increased as the length of the ITI was increased. The latency to respond to the cue light differed with ITI, with both genotypes taking longer to respond after a 2s ITI than 15s, possibly because of their position within the chamber. There was also a significant difference in response time after a 5s and 15s ITI. The perseverative responses made by the NK1 receptor knockout mice were significantly fewer after the longer ITI splits than after 2 or 5s ITI wait times.

5.4 Discussion

As predicted from the hypothesis that the NK1R-/- mouse is a model of attention deficit hyperactivity disorder (ADHD) the knockout mice displayed attentional deficits and higher impulsive responses in the 5-choice serial reaction time task (5-CSRTT), but this was dependent on the difficulty of the test parameters. What also emerged was a robust perseverative phenotype in the NK1R-/- compared to the wildtype counterparts at every phase of the experiment.

5.4.1 Task acquisition

23 out of 24 mice (one died) that started the experiment successfully reached the criteria for a stable baseline at the end of training. The lack of functioning NK1 receptor did not impede the ultimate acquisition of the task as However the NK1R-/- mice did require a greater number of training sessions to reach the test phase of the experiment. There was no difference in the behaviour of the NK1R-/- mice compared to the wildtype counterparts in accuracy, premature responding, and latency to respond to the cue light. Errors of omission were generally higher in the NK1R-/- mice, but this failed to reach statistical significance. Although no other experiments in the 5-CSRTT have been conducted on the exact background strain as the mice tested here (C57BL/6 x 129/Sv, crossed once onto an MF1 strain), a study of C57BL/6 x 129/Sv mice showed that they are capable of learning the procedure (Humby et al., 1999) suggesting that any behavioural deficits of the NK1R-/- mice presented here are unlikely to be related to the background strain of the animals used. The results in the previous chapter demonstrated that there was no difference in the overall activity levels at the times at which the

5-CSRTT was conducted therefore this should not impact the behaviour in the task. When the VITI data are split into individual ITI times, there is a decrease in the number of trials completed at the longer ITI times. Despite the computer programme presenting the cues in a randomised but balanced order, the decrease arises because more premature responses are made during the longer ITI times, after which the animal is punished with a time-out period of darkness. This resets the computer to present the next ITI time and the response is not recorded in the total number of completed trials. This is perhaps a flaw in the experimental programming since it creates a bias for the total number of trials completed at the shorter ITI times. It would be more beneficial if a premature response simply reset the ITI and did not cycle to the next trial, however this would perhaps promote a greater learning aspect to the task and could therefore mask an underlying impulsive phenotype.

5.4.2 NK1R-/- mice display deficits in attention

No one single behaviour in the 5-CSRTT measures 'attention' per se, but by considering the accuracy of the responses made, alongside the percentage of omitted responses and the latency to respond to the cue light we can gain insight into the attentional profile of the animals. There were no differences in the behaviour of wildtype and NK1R-/- mice in the measures associated with attention during the training stages of the experiment. This demonstrates that the level of attentional demand required during the training stages of the 5-CSRTT is not enough to draw-out the attentional deficits observed in the NK1R-/- mice in the more demanding LITI and VITI tasks. A lack of attentiveness in the 5-CSRTT could be interpreted as a blunted motivation to complete the task, perhaps due to apathy towards the reward. However maintaining the animals at 90% of their free feeding weight along with the incentive of highly calorific condensed milk as a reinforcer, this is an unlikely explanation.

Increasing the inter-trial interval (ITI) time from 5 to 7 seconds in the long ITI test, did not impact on the accuracy, nor the latency of the responses made to the cue lights. However NK1R-/- mice committed a greater percentage of omission errors than the wildtype controls in the LITI test. When the attentional demand of the task was further increased in the variable ITI test, both genotypes displayed an increase in the percentage of omissions, with the NK1R-/- mice failed to respond to the cue light more than the

wildtype mice. Also, the increased difficulty of the test increased the average time taken to respond to the cue light in both genotypes, but more so in the NK1R-/- group, thus revealing a genotype difference in the latency to make a correct response. In addition to the deficits in acute attention (i.e. responding to each individual trial), NK1 receptor knockout mice completed fewer trials in the VITI test than the wildtype controls, suggesting they may also have a problem with sustaining attention across a long period of time. These findings point towards an inattentive phenotype of the NK1R-/- mouse.

By analysing the data from the VITI in terms of each separate ITI time, we can gain more of an insight into the attention profile of the mice. After the longest ITI period of 15s, NK1R-/- mice were less accurate with their responses thus adding to their profile of attentional deficits. Percentage omissions are greater with the longer ITI times, in both genotypes, while the NK1R-/- mice omit more than wildtypes at the 5, 10 and 15s ITI splits, but not at the shortest ITI length of 2s. This is most likely due to the fact that there is less time for the mice to get 'distracted' or carry out other behaviours such as grooming before the cue light is illuminated. The split ITI analysis for the latency to make a correct response is a little more insightful. When split, the genotype difference of NK1R-/- mice responding slower disappears. Both genotypes were slowest to respond after the 2s ITI time, possibly indicating that the mice are 'out of position' and have to cross the length of the box before responding. Also, the quickest response times are after the 5s ITI, which is the same ITI period as the baseline level which the mice are most accustomed to.

With this evidence, the most likely explanation for the increased number of omissions recorded by the NK1R-/- mice in the LITI and VITI tests is a deficit in attention caused by a lack of the NK1 receptor.

The neural circuits and computations that underlie normal and abnormal attention are not well understood. Attention is a complex cognitive faculty, and the 5-CSRTT can only measure one aspect of the multifaceted behaviour, namely top-down (voluntary) spatial attention. The main brain regions associated with attention are the prefrontal and parietal cortices as well as those associated with gaze and oculomotor function (Robbins, 2002; Moore, 2006; Noudoost et al., 2010; Noudoost and Moore, 2011).

Several neurotransmitters are associated with attention including acetylcholine (ACh), noradrenaline (NA) and dopamine (DA) but how they modulate attention is unclear.

ACh efflux in the medial frontal cortex has been shown to increase at the onset of the 5-CSRTT, implicating it as having an important role in the modulation of behaviour in the task. Furthermore, a depletion of cholinergic neurons in the nucleus basalis magnocellularis resulted in a depletion of cortical ACh efflux and corresponding impairment in accuracy (McGaughy et al., 2002). Another hypothesis for ACh is that it works via muscarinic acetylcholine receptors (mAChR), as the central muscarinic antagonist scopolamine (but not a nicotinic antagonist) reduced choice accuracy in rats, however mice carrying nicotinic acetylcholine receptor (nAChR) deletions of the β2 subunit also have impaired attentional performance in the in the 5-CSRTT (Ruotsalainen et al., 2000; Guillem et al., 2011). The mAChR theory is corroborated by work in macaque monkeys where delivery of ACh into V1 sites increased attentional modulation (Herrero et al., 2008). Alternative hypotheses postulate that the ACh governs attention via gating of information within sensory cortical areas or by mediation of prefrontal cortex (PFC) function (Xiang et al., 1998; Parikh and Sarter, 2008).

Dopamine and noradrenaline also play important roles in the function of the PFC as demonstrated in other rodent studies using the 5-CSRTT and other attention measuring paradigms such as set-shifting (Crofts et al., 2001; Dalley et al., 2001; Milstein et al., 2007; Tait et al., 2007). The striatum is also a potential component of the neural system associated with performance in the 5-CSRTT because of the input it receives from the PFC. Lesions of the medial and lateral striatum both cause considerable disruption of 5-CSRTT performance and depletion of DA in the dorsal and ventral striatum using 6-OHDA increased errors of omission and increased response latencies (Cole and Robbins, 1989; Baunez and Robbins, 1999; Rogers et al., 2001). Conversely, infusion of the DA D1 receptor agonist, SKF 38393, into the pre-limbic frontal cortex produced improvements in otherwise poor performing rats in the 5-CSRTT (Granon et al., 2000). The dopaminergic system in the core of the nucleus accumbens is also implicated in attentional accuracy via D1 receptors, since region-selective D1 antagonism impaired accuracy and increased omissions, whilst a D1 stimulations improved accuracy and decreased omissions (Pezze et al., 2007). Milstein and colleagues examined the effect

of noradrenaline depletion in the PFC and reported that accuracy was decreased in the VITI test after a selective lesion (Milstein et al., 2007).

Although basal levels of acetylcholine efflux have not been measured in the NK1R-/-, these mice do have a greater tonic release of noradrenaline in corticostriatal brain regions and reduced extracellular dopamine in the PFC which could contribute to their inattentiveness (Fisher et al., 2007; Yan et al., 2009, 2010).

5.4.3 NK1R-/- mice are impulsive in the VITI

In the 5-CSRTT the percentage of premature responses (a nose poke into a hole prior to the presentation of the cue light) provides a direct index of the impulsive nature of an animal. From the hypothesis that the NK1R-/- mouse is a model of ADHD, and would thus display traits of impulsivity, we would predict that they would commit a higher number of premature responses than the wildtype controls in the 5-CSRTT. Although this was proved to be correct in the variable ITI test, no difference was seen between genotypes during the training stages or in the long ITI test. These results clearly show that impulsivity is correlated with the difficulty of the task.

The difference in findings from the LITI and VITI provides insight into the nature of the impulsivity displayed by these animals. Both genotypes responded to the prolonged inter-trial interval in the LITI test equally, thus showing that simply elongating the 'wait-time' from 5 to 7 seconds is not a large enough increase in difficulty to draw-out any underlying differences in impulsivity. However when the inter-trial interval was varied, and delivered in a non-predictable, randomised schedule of 2, 5, 10 or 15 seconds, NK1R-/- mice committed significantly more premature responses than the wildtype controls. There are two possible reasons for this; either the knockout cannot adjust to the irregular presentation of the stimulus, or that the much longer inter-trial interval times (10 and 15s) are long enough to elicit the underlying impulsive phenotype of the NK1R-/- mice. Splitting the VITI data into the individual ITI times revealed, unsurprisingly, that the percentage of premature responses increased with longer ITI times. NK1R-/- mice performed a greater number of premature responses than wildtype controls at the 5, 10 and 15s ITI splits, that when collapsed yields the overall genotype difference in premature responding in the VITI, but when split behaviour between

genotypes is significantly different only at the 10s ITI. These data demonstrate that it is the longer ITI times that draw-out the underlying deficit in impulsive behaviour of the NK1R-/- mouse.

There is a large body of evidence to suggest that impulsivity is associated with impaired serotonergic transmission, although the specific brain region(s) affected are unknown (Stein et al., 1993; Harrison et al., 1997; Evenden, 1999a; Dalley et al., 2002, 2008). However, a 90% reduction of forebrain serotonin in the rat brain does not necessarily effect all types of impulsivity (as seen by an increase in premature responding in a visual attention task, yet no change in impulsive choice was observed in a delayed-discounting test), further highlighting the complexity of the behaviour (Winstanley et al., 2004). The evidence also is contradictory as to whether impulsivity is the result of a deficiency in 5-HT transmission (Harrison et al., 1997, 1999) or whether excessive serotonin levels contribute to impulsive behaviour (Puumala and Sirviö, 1998; Dalley et al., 2002). A number of lesion and pharmacological studies have been used to examine the relationship between serotonin function and impulsivity in the 5-CSRTT. Widespread depletion of 5-HT by the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) significantly increased premature responding, but did not impair accuracy (Harrison et al., 1997). Administration of 5-HT receptor agonists, specifically for the 1A and 2A subtypes of the receptor, also produced similar increases in premature responding (Carli and Samanin, 2000; Koskinen et al., 2000a, 2000b). NK1R-/- mice have reduced levels of 5-HT1A receptors and also respond differently to wildtype mice when given systemic paroxetine (a selective serotonin reuptake inhibitor (SSRI)) (Froger et al., 2001), thus suggesting they have a significant disruption of the serotonergic neural circuitry which may contribute to the impulsivity recorded here in the 5-CSRTT. Dopaminergic transmission also plays a role in the modulation of premature responding, namely in the nucleus accumbens, since intra-accumbens injection a D1 antagonist significantly decreased premature responding in rats (Pattij et al., 2007a).

Impulsivity is known to be a multi-faceted behaviour with many different varieties (Evenden, 1999b). To further explore the difference in impulsivity between the two genotypes the mice could be tested in an adapted version of the 5-CSRTT known as the ‘go/no-go’ test. Here, in addition to the presentation of the individual cue lights on a randomised schedule (as used in this experiment), an extra test is included where all 5

apertures are illuminated simultaneously. To obtain a reward from this stimulus the animal must refrain from responding in any of the five locations (Young et al., 2009). The go/no-go task and the standard 5-CSRTT both measure action restraint, that is, inhibition of a pre-planned motor action before the response has been initiated. Yet in the go/no-go task the animals must make the additional decision whether to respond to the cue or to inhibit the action upon the presentation of a no-go signal. It does not necessarily follow that a poor performance in one task correlates with the other. An animal that successfully withholds its response during the ITI of the 5-CSRTT could still commit a large number of 'false alarm' responses (a response to the no-go signal) in the go/no-go task. This would suggest an impairment with the decision making process of whether to respond or not, a slightly different aspect of impulsive behaviour as seen in the NK1R-/- mice in the 5-CSRTT. Disrupted serotonin neurotransmission has also previously been associated with poor performance in the Go/No-go test (Harrison et al., 1999).

5.4.4 NK1R-/- mice perseverate more than wildtype mice

One unexpected finding from this experiment was the high number of perseverative responses committed by the NK1R-/- mice in relation to the wildtype mice, which was present at all phases of the experiment. The NK1R-/- mice unnecessarily continued to nose-poke into the hole after a correct response had already been recorded, up to 4-fold more than their wildtype counterparts. This may account for the increased latency to collect reward routinely seen in the NK1R-/- mice compared to wildtypes.

Although perseveration is a symptom most commonly associated with obsessive-compulsive disorder (OCD) it is also a co-morbid feature of ADHD, however it is not a diagnostic criterion (Tannock and Schachar, 1992; Houghton et al., 1999; Lawrence et al., 2004). The neural circuitry of perseveration is thought to overlap with that associated with impulsivity, since lesions of the pre-limbic cortex and medial striatum both increased perseverative responding in the 5-CSRTT (Chudasama and Muir, 2001; Rogers et al., 2001; Chudasama et al., 2003). This is consistent with other findings from studies of reversal learning (another measure of perseveration where the subject must learn a rule, and then forget the rule and learn another) after lesions of the dorsal-medial and medial striatum (in rats and monkeys respectively) and neonatal lesions of the

medial PFC (mPFC) all increased perseveration (Schwabe et al., 2004; Clarke et al., 2008; Castañé et al., 2010). The monoaminergic systems within the corticostriatal circuit may govern perseverative responding, since both d-amphetamine and stimulation of D2 receptors in the core of the nucleus accumbens increase perseverative responding in the 5-CSRTT (Pezze et al., 2007; Paterson et al., 2011). Background strain also has a significant influence on perseveration, as it does on performance in the 5-CSRTT. In a comparison of fourteen different strains in a test of operant extinction behaviour C57BL/6 mice did not show any deficit in extinction, whereas Bxd-16, Bxd-42 and NOD/Ltj mice all displayed high levels of perseveration (Malkki et al., 2011).

Interestingly, there was a decrease in perseverative responding of the NK1R-/- mouse with increased ITI interval time. The high number of perseverative responses made by the NK1R-/- mouse at 2s and 5s ITI times is not seen at 10 or 15seconds ITI. This trend of decreased perseverative responses at longer ITI times can also be seen in the work of Paterson et al., (2011), yet was not covered in their discussion of the data. This observation is likely to stem from the fact that fewer trials are completed at the longer ITI splits (see section 5.4.1), and because perseveration is recorded as a cumulative measure rather than a percentage of the total number of trials, fewer perseverative responses are made. Additionally, (although unfortunately not recorded by our computer system), it would be of interest to look at whether the NK1R-/- mice make perseverative nose-pokes into the magazine hole upon collection of the reward (to see if the behaviour is purely driven by the cue or if it also applies to the reward aspect of the task) and whether they make perseverative responses after an incorrect or premature response during the time-out darkness period (to see if the behaviour pervades after an additional environmental cue (lights out)). These measures are rarely mentioned in 5-CSRTT literature.

Although perseverative responses are recorded in the 5-CSRTT, they have no programmable consequence for the mice. Punishing the animals for a perseverative response with a time-out period of darkness has been shown to disrupt learning of the task (Bari et al., 2008). This could suggest that the knockout mice are not extinguishing the behaviour learnt in the habituation stages, when the reward is on a continuous reinforcement schedule, and therefore advantageous to nose-poke as much as possible to gain the maximum amount of reward permitted. Alternatively the perseverative

responding could be considered a 'checking' behaviour, more associated with OCD and anxiety, although this would not fit with the early findings that NK1R knockout mice display reduced anxiety compared to wildtype animals (Kramer et al., 1998; Rupniak et al., 2000; Santarelli et al., 2001). Whether either of these theories is correct, the robust nature of the genotype difference at all stages of the 5-CSRTT certainly warrants further investigation.

5.4.5 Methodological considerations

The 5-choice serial reaction time task is an excellent behavioural paradigm for measuring impulsivity and attention, although all the recorded behaviours need to be analysed in conjunction with one another to build up a complete behavioural profile of the animals (Robbins, 2002). Impulsivity, attention and perseveration are all complex behaviours and the 5-CSRTT only measures one such aspect of each (Evenden, 1999b; Dalley et al., 2011). Therefore, even though the NK1R-/- mice display impulsivity in the 5-CSRTT (thought to be due to a deficit in disinhibition), this may not be true in other tests of impulsivity such as temporal-discounting (which measures whether an animal will choose an immediate small reward, or a larger reward after a period of time has elapsed) or the go/no-go task which measures the ability to stop an already initiated action.

The large number of variables within the 5-CSRTT schedule e.g. the ITI time, stimulus duration, and session length make it unlikely that any two protocols will be exactly the same, making comparisons between studies difficult. It must also be noted that after the completion of the study, a fault in the programming of the LITI test was discovered, which had resulted in the stimulus duration of the LITI test being just 1 second, instead of 1.8s, however this does not appear to have had a detrimental effect on the performance of the animals since accuracy remained high in both genotypes. The revelation that there is a bias for the shorter ITI times, which impacts on the interpretation of the data, is an important finding that other users of the 5-CSRTT should be aware of and take into consideration.

5.4.6 Conclusions

NK1R-/- mice display deficits in attentiveness and impulsivity when challenged with the variable inter-trial interval test in the 5-choice serial reaction time task. This supports the proposal that the NK1R-/- mouse is a model of attention-deficit hyperactivity disorder. To further investigate whether these deficits are due to a lack of functional NK1 receptor the mice in cohort 2 were treated with NK1R antagonists to see if the findings from the knock out mice could be replicated in wildtype animals. The data from this experiment are reported in the next chapter.

6 Effect of NK1 receptor antagonists on behaviour in the 5-CSRTT

6.1 Introduction

From the findings in the previous chapter we know that NK1R-/- mice display inattentiveness and impulsivity in the 5-choice serial reaction time task (5-CSRTT), which are both core symptoms of ADHD. NK1R-/- mice also committed a greater number of perseverative responses than the wildtype controls, and although it is not a diagnostic criterion, it is a co-morbid feature of the disorder (Tannock and Schachar, 1992; Houghton et al., 1999; Lawrence et al., 2004). The aim of this chapter is to look at the effect of treating wildtype mice with NK1R antagonists to see if the behavioural profile of the NK1R-/- mice can be replicated. If so, it is likely that the behavioural deficits observed in the previous chapter are caused by the lack of a functional NK1 receptor.

6.1.1 NK1 Receptor Antagonists

Antagonists are agents that bind to a channel or receptor, but do not elicit a biological response. The first antagonist produced for the NK1R, CP-96,345, was developed by Pfizer in the early 1990's (Snider et al., 1991) thereby opening up the NK1R research field and many more were developed in quick succession. NK1R antagonists generally fall into two categories: the less common peptide antagonists such as FR 113680 (a tri-peptide SP antagonist, with NK1R selectivity (Morimoto et al., 1992)) and non-peptide antagonists which include CP-96,345, RP 67580 and L-733,060 (Garret et al., 1991; Snider et al., 1991; Rupniak et al., 1996). The two antagonists used in this chapter, RP 67580 and L-733,060, both have high stereospecificity for the NK1 receptor, over the other tachykinin receptors, and their respective enantiomers (RP 67581 and L-733,061) are inactive (Garret et al., 1991; Moussaoui et al., 1993; Rupniak et al., 1996).

6.1.2 Antagonist vs. knockout

When investigating the role a receptor may play in controlling certain behaviours it is prudent to study the effect of antagonist treatment in conjunction with that of the transgenic receptor knockout mouse (if available). This allows a comparison of the antagonist treatment that creates an acute, temporary blockade of the receptor functionality vs. the long-term chronic effects that the absence of a functional receptor may cause along with any compensatory effects that may occur in a knockout animal. If findings from the two types of experiment yield the same results confidence increases that the observed differences are a direct consequence of the receptor in question. In one such example, a double knockout of the muscarinic acetylcholine receptors M2 and M4 ((M(2)/M(4)(-/-)) mice, had impaired long-term depression, but not long-term potentiation in the striatum, findings which were corroborated with an M(2)/M(4) antagonist (AF-DX384) (Bonsi et al., 2008). The coloboma mutant mouse is hyperactive and is a less well-known animal model of ADHD, but it has been used to dissociate D_2 dopamine receptors as the mediators of hyperactivity, which was confirmed using a selective D_2 receptor antagonist L-741,626 (Fan et al., 2010).

NK1R-/- display greater locomotor activity in the light-dark exploration box compared to their wildtype controls (Herpfer et al., 2005; Fisher et al., 2007). Furthermore, hyperactivity is induced in wildtype animals treated with two NK1R antagonists RP 67580 and L-733,060 (5 and 10 mg/kg, i.p.) (Yan et al., 2010), thus suggesting that the hyperactivity of NK1R-/- mice is a direct consequence of the lack of functional NK1 receptors, rather than any secondary developmental or adaptive changes of the genotype. The experiments in this chapter serve to investigate whether the cognitive deficits of the NK1R-/- mice in the 5-CSRTT, namely inattention, impulsivity and perseveration (see previous chapter), are also a consequence of the disruption of the NK1 receptor. Wildtype mice injected with the same NK1 antagonists (and doses) that were previously shown to induce hyperactivity are tested in the 5-CSRTT, while NK1R-/- mice also received antagonist treatment to control for non-NK1R-mediated effects of the antagonists. The data are discussed in two parts; firstly the effect of repeated testing in the LITI and VITI tests followed by the effect of antagonist treatment on the behaviour of wildtype and NK1R-/- mice in the 5-CSRTT.

6.2 Materials and Methods

6.2.1 Subjects

The animals used in the 5-CSRTT were from the original, homozygous bred, MF1 colonies as outlined in section 2.1.2.1. 12 wildtype (WT) and 12 knockout (KO) animals (males, aged approximately 8 weeks old at the start of the experiment) began the experiment but one wildtype mouse died during training. The mice were kept on a restricted diet to maintain body weights at 90% of the animals free-feeding weight to ensure they remained motivated for the task. Animals were weighed every day prior to the start of the experiment.

6.2.2 NK1R antagonists

The NK1R antagonists RP 67580 and L-733,060 hydrochloride (Tocris, Bristol, UK) were dissolved in a 10% Tween80 in 0.9% sterile saline solution and were administered i.p. at an injection volume of 10ml/kg. Control injections of the vehicle solution consisted of the equivalent volume. Doses for L-733,060 are expressed as the salt.

6.2.3 Testing regime

The habituation and training procedure for these mice are outlined in sections 2.4.2 and 2.4.3 respectively. After completion of training half the cohort was tested with no-injection in the long inter-trial interval test (LITI NI-1), the other with no-injection in the variable inter-trial interval test (VITI NI-1). One week later, after performing satisfactorily at baseline, the corresponding test was conducted. There was then an unscheduled 5-week break in testing due to various reasons. The mice were on ad lib food for 3 weeks, followed by a week to re-establish the 90% body weight and a final week to re-establish satisfactory baseline performances. They were then re-tested in both tests (LITI- and VITI-NI-2), before starting on a pseudo-randomised (Latin-square) treatment regime of either, i.p. injection of vehicle (saline with 10% Tween80, 10ml/kg), NK1R antagonist L-733,060 or RP 67580 (5 or 10mg/kg, (L-5, L-10, RP-5 and RP-10 respectively)) or no-injection (NI-3) in the LITI or VITI test. Animals were injected 30 minutes prior to being tested in the 5-CSRTT and each animal received each treatment once. The dose of NK1R antagonists was chosen based on previous work in

the lab where hyperactivity was induced in wildtype mice, but did not dampen the hyperactivity of the knockout (Yan et al. 2010). The same behavioural outputs were recorded as in the previous chapter.

6.2.4 Statistics

A repeated measure ANOVA with 'test' (e.g. no injection 1 or 2 (NI-1 or NI-2) or drug treatment (RP-5)) as a within subject factor and 'genotype' as the between subject factor. In tests where Mauchly's test of sphericity in the repeated measure ANOVA was significant, the Greenhouse-Geisser 'ε' correction was read instead of the 'sphericity assumed' output. A significant effect of 'test', 'genotype', or and interaction between 'test' and 'genotype' (test*genotype) was the criterion for progressing to a one-way multivariate ANOVA with 'genotype' as the between subject factor (for differences of 'genotype') and matched pair t-tests (for differences of 'test' for each genotype) (or the equivalent non-parametric tests, Kruskal-Wallis and Wilcoxon-rank, respectively). Variable ITI data were further analysed with a 3-way ANOVA ('genotype', 'ITI', and 'treatment'). Statistical significance was set at $P<0.05$.

6.3 Results

There are three main findings from this study into the effects of NK1R antagonist treatment on the behavior of wildtype and NK1R-/- mice in the 5-CSRTT: 1) Behavior of both wildtype and NK1R -/- mice changes with repeated exposure to the test sessions of the 5-CSRTT in the long and variable ITI tests. 2) A high dose of the NK1R antagonist RP 67580 (10mg/kg) has behavioral consequences for *both* genotypes, suggesting a non-NK1R mediated effect 3) Splitting the VITI data into individual ITI comparisons reveals effects of antagonist treatment on premature responding in wildtype mice.

6.3.1 Effect of repeated exposure to the test parameters

The protocol was initially designed to only incorporate two no-injection tests in the VITI and LITI, the initial treatment naïve test (NI-1) immediately after training and a

second no-injection test embedded in the Latin-square design drug-testing phase. However, the experiment was interrupted which resulted in a 5-week break in testing at the point when the animals had completed training and had completed the first LITI- and VITI tests (NI-1). The decision was then taken to re-test the animals after the break with a no-injection test (NI-2) before they commenced the drug-testing phase, where they experienced the third test (NI-3) without a prior injection as part of the treatment regimen.

6.3.1.1 Repeated testing in the LITI test

Repeated exposure to the LITI test parameters improved performance in several of the measured behaviours including accuracy, omissions, and the latency to make a correct response, thus demonstrating that this should be considered when running a long experiment in the 5-CSRTT.

Total number of trials There was no change in the total number of trials completed across the three LITI where the animals did not receive an injection. All animals (bar one knockout at NI-1) completed the maximum limit of 100 trials in the test (Figure 6.1A).

% Accuracy There was a significant interaction between 'genotype' and 'test' ($F_{(2,42)}$=3.38, P<0.05). Post-hoc analyses revealed that the behaviour of the wildtype mice did not change with repeated exposure to the LITI test, yet the NK1R-/- mice became more accurate in the latter tests (NI-1 vs. N1-3 $t_{(11)}$=4.7, P<0.001). This improvement in accuracy also resulted in a significant genotype difference at NI-3 ($t_{(21)}$=2.6, P<0.05) (Figure 6.1B).

% Omissions A main effect of 'test' ($F_{(2,42)}$=6.1, P<0.01), but no interaction with 'genotype' demonstrates that the performance of both genotypes equally improves with repeated exposure to the LITI test parameters (*c.f.* NI-1: NI-2 $t_{(22)}$2.9, P<0.01; NI-3 $t_{(22)}$=3.0, P<0.01) . However there is also a main effect of 'genotype' ($F_{(1,21)}$=14.2, P<0.001) with NK1R-/- mice performing more errors of omission across the three tests compared to WT controls, but by the time the mice experienced the third no injection

test in the LITI, the difference had resolved (NI-1 $t_{(21)}$=2.2, P<0.05; NI-2 $t_{(21)}$=2.7, P<0.05; NI-3 $t_{(13.7)}$=1.6, P=0.14) (Figure 6.1C).

% Premature responses There was no significant change in the behaviour of either genotype across the three tests, nor was there a difference in the number of premature responses made by either genotype (Figure 6.1D).

Latency to correct response A main effect of 'test' ($F_{(2,42)}$=5.4, P<0.01) but no interaction with 'genotype' signifies that both wildtype and NK1R-/- mice improved their response times with repeated exposure to the LITI test parameters (NI-3 vs. NI-3 $t_{(22)}$=2.8, P<0.01) (Figure 6.1E).

Latency to collect reward Repeated testing in the LITI had no effect on the latency to collect the reward after a correct response. There was a main effect of 'genotype' ($F_{(1,21)}$=5.8, P<0.05) with NK1R-/- mice taking longer to collect the reward at all no-injection tests, but this was significant only in the NI-1 and NI-3 tests (NI-1 $t_{(21)}$=3.0, P<0.01; NI-3 $t_{(21)}$=2.3, P<0.05) (Figure 6.1F).

Perseveration A 2-way ANOVA revealed an overall main effect of 'test' ($F_{(2,42)}$=5.3, P<0.01) and 'genotype' ($F_{(1,21)}$=5.5, P<0.05) but no interaction between the two. With repeated testing the number of perseverative responses made by both genotypes increased (NI-1 vs. NI-3 $t_{(22)}$=3.1, P<0.01), while the genotype difference was only significant in the NI-2 test ($t_{(17.3)}$=2.2, P<0.05) (Figure 6.1G).

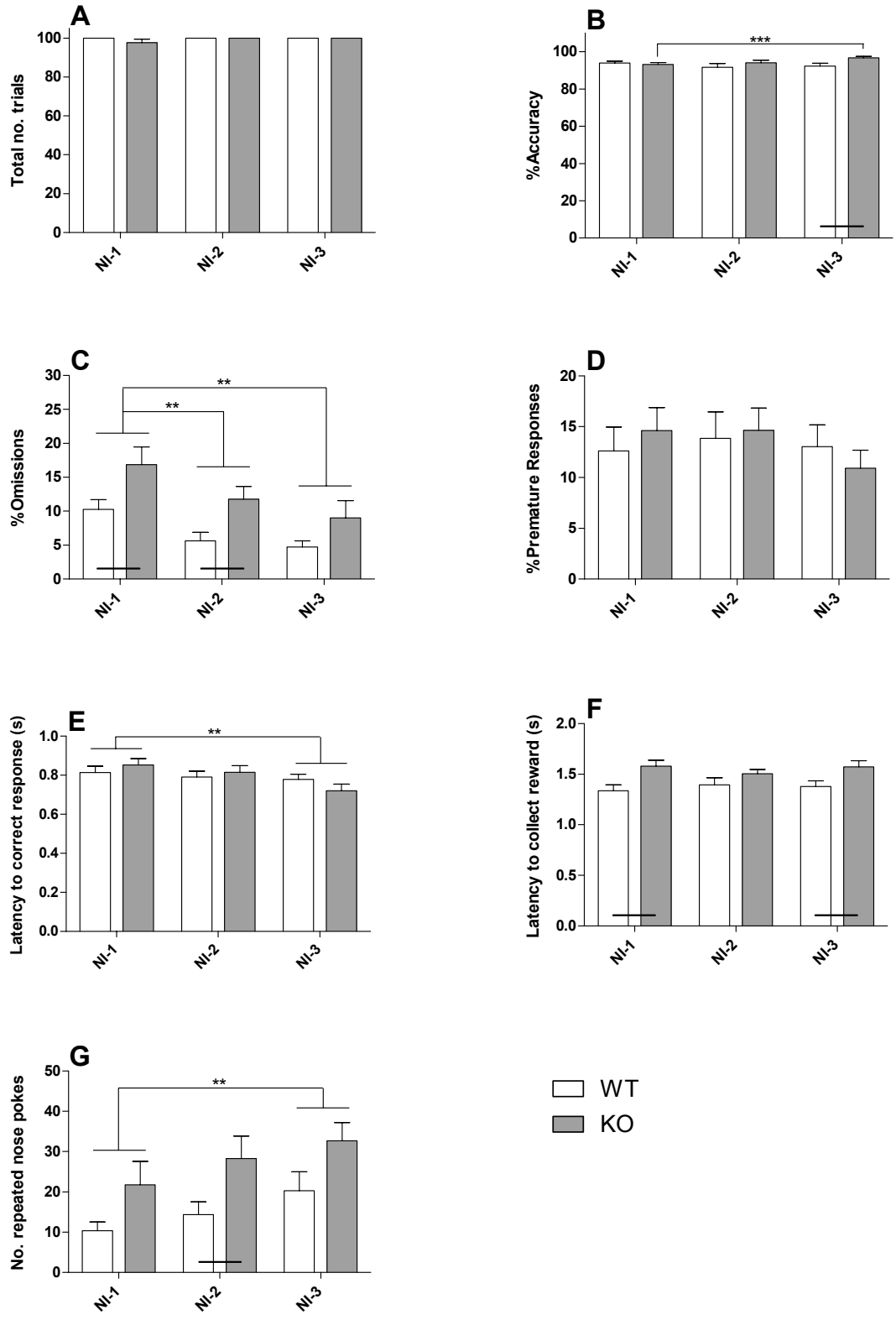


Figure 6.1 Effects of repeated exposure to the LITI test. Repeated exposure to the test parameters improved performance in terms of omissions, but not premature responding or perseveration. (A) Total number of trials completed, (B) Percentage accuracy, (C) percentage omissions, (D) percentage premature responses, (E and F) latencies to make a correct response and to collect the reward respectively and (G) perseverative nose pokes made after a correct response. * P<0.05, ** P<0.01, ***P<0.001 N=11-12. Some statistical bars are in bold for clarity only. A bar across columns represents a statistical difference in behaviour between genotype of at least P<0.05.

6.3.1.2 Repeated testing in the VITI test

As with the LITI test, both wildtype and NK1R-/- mice adjusted their behaviour with repeated exposure to the VITI test parameters as shown by improvements in several behaviours including accuracy, omissions, and the latency to make a correct response.

Total number of trials completed It is difficult to complete statistical analysis on the data for total number of trials completed because all animals from both genotypes in the second and third experience of the VITI test reached 100 trials. Therefore there is no variance in the data and an analysis of variance (ANOVA) test is not possible. Non-parametric analysis (Friedman test) revealed an overall effect of 'test' (χ^2=18.0, P<0.001). Wilcoxon signed rank tests showed no change in total number of trials completed by the wildtype animals, but the NK1R-/- increased the total number of trials completed between NI-1 and NI-2 (Z=2.5, P<0.01) (Figure 6.2A).

% Accuracy Repeated exposure to the VITI test increased accuracy in both genotypes with time (interaction between 'test' and 'genotype' $F_{(2,40)}$=4.2, P<0.05). NK1R-/- showed continual improvement with each consecutive test (NI-1 vs. NI-2 $t_{(11)}$2.8, P<0.05, NI-2 vs. NI-3 $t_{(10)}$=2.5, P<0.05), whereas wildtype mice only significantly improved between NI-1 and NI-3 ($t_{(10)}$=2.5, P<0.05) (Figure 6.2B). This shows that both genotypes adjusted their behaviour after their first experience of the variable inter-trial interval, as indicated by an increase in % accuracy (an index of attention), by NI-3.

% Omissions A main effect of 'test' ($F_{(2,40)}$=17.5, P<0.001), but no interaction with 'genotype' demonstrates that the performance of both genotypes equally improves with repeated exposure to the VITI test parameters (*c.f.* NI-1: NI-2 $t_{(22)}$=5.4, P<0.001; NI-3 $t_{(21)}$=5.3, P<0.001). There is also a main effect of 'genotype' ($F_{(1,21)}$=14.2, P<0.001) with NK1R-/- mice performing more errors of omission across the three tests compared to WT controls (NI-1 $t_{(21)}$=3.2, P<0.01; NI-2 $t_{(21)}$=3.6, P<0.01; NI-3 $t_{(20)}$=2.5, P<0.05) (Figure 6.2C).

% Premature Responses The behaviour profile of premature responses for each genotype differed with repeated exposure to the VITI test parameters (interaction between 'test' and 'genotype' $F_{(1.6,31)}$=10.7, P<0.001). At the first experience of the VITI test parameters NK1R-/- mice made more premature responses than the wildtype

controls ($t_{(21)}$=3.3, P<0.01). This apparent impulsive behaviour of the knockout mice improved with repeated exposure to the test parameters, and by the third no-injection test, no genotype difference remained (NI-1 vs. NI-3 $t_{(10)}$=4.3, P<0.01; NI-2 vs. NI-3 $t_{(10)}$=3.2, P<0.01). Interestingly there was a transient increase the number of premature responses made by wildtype mice in the NI-2 test ($t_{(10)}$=3.1, P<0.05) which returned to NI-1 levels in their third VITI no-injection test (NI-2 vs. NI-3 $t_{(10)}$=3.7, P<0.01) (Figure 6.2D).

Latency to correct response Repeated testing with the VITI parameters had a differential effect on each genotype (main interaction of 'test' and 'genotype' $F_{(2,40)}$=3.7, P<0.05). Compared to NI-1, the latency to a correct response was reduced in both genotypes at NI-2 (WT $t_{(10)}$=2.3, P<0.05; KO $t_{(11)}$=2.6, P<0.05), and further still at NI-3 in NK1R-/- mice ($t_{(10)}$=4.6, P<0.001). NK1R-/- mice were slower to respond to the stimulus light at NI-1 ($t_{(13.7)}$=2.5, P<0.05) and NI-2 ($t_{(21)}$=2.2, P<0.05), but by the third no-injection test, there was no difference in the response time of wildtype and NK1R-/- mice (Figure 6.2E).

Latency to collect reward NK1R-/- mice were robustly slower to collect the reward after a correct response compared to wildtype mice (main effect of 'genotype' $F_{(1,20)}$=11.6, P<0.01). This was despite a decrease in these animals response time at NI-2 compared to NI-1 ($t_{(11)}$=3.0, P<0.05) (Figure 6.2F).

Perseveration NK1R-/- mice made more repeated nose pokes into the cued hole after the initial correct response in all three 'no-injection' tests, although this was only significant at NI-1 ($t_{(12.5)}$=2.0, P<0.05) and NI-3 ($t_{(16)}$=2.3, P<0.05). Wildtype mice increased perseverative responding with repeated testing (*c.f.* NI-1: NI-2 $t_{(10)}$=2.5, P<0.05 and NI-3 $t_{(10)}$=2.5, P<0.05) (Figure 6.2G).

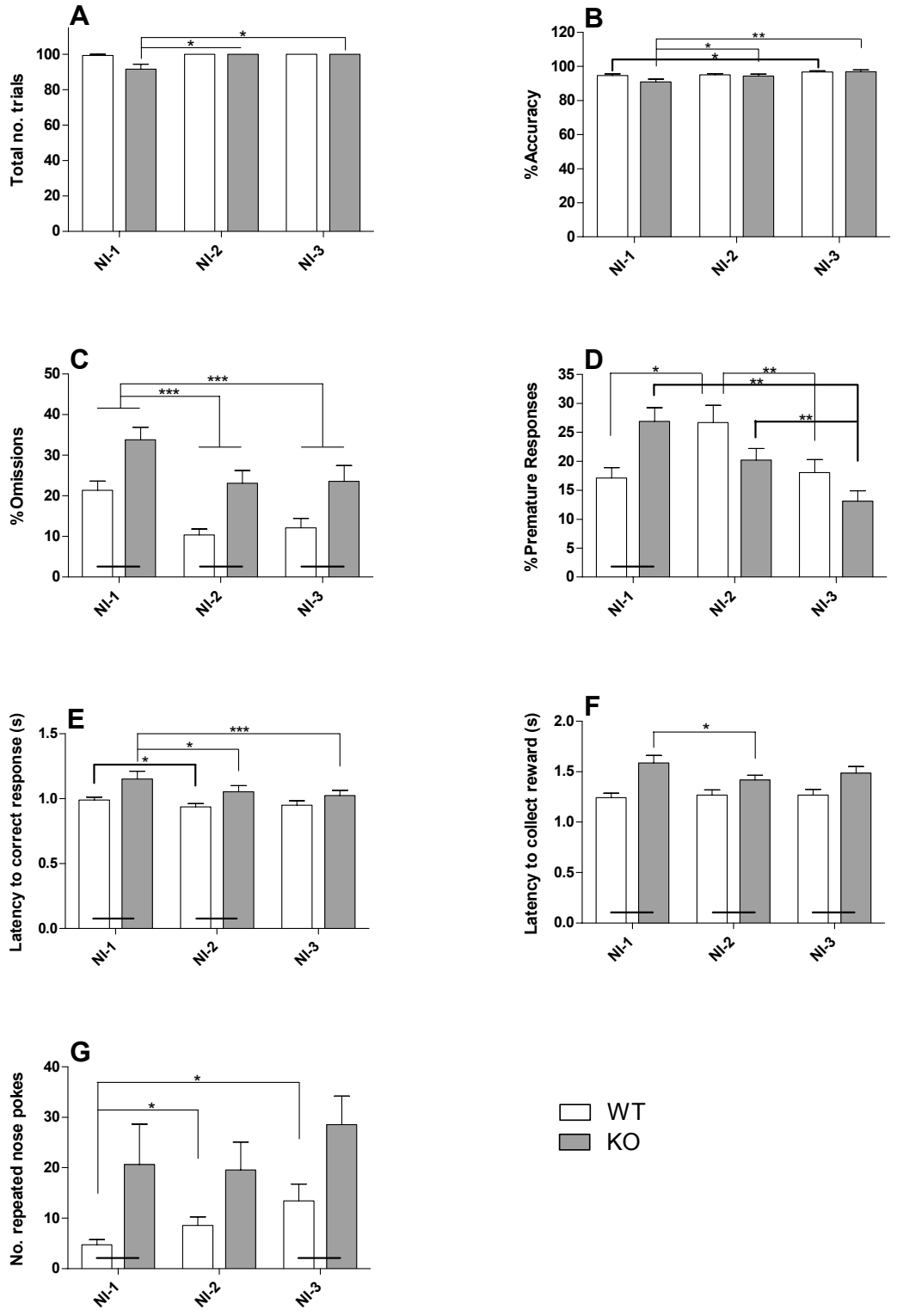


Figure 6.2 Effects of repeated exposure to the VITI test. Repeated exposure to the test parameters generally improved performance in the task, except in the case of perseveration. (A) Total number of trials completed, (B) Percentage accuracy, (C) percentage omissions, (D) percentage premature responses, (E and F) latencies to make a correct response and to collect the reward respectively and (G) perseverative nose pokes made after a correct response. * P<0.05, ** P<0.01, ***P<0.001 N=11-12. Some statistical bars are in bold for clarity only. A bar across columns represents a statistical difference in behaviour between genotype of at least P<0.05.

In summary these data show that task performance generally improves with repeated exposure to the test parameters of the long and variable inter-trial interval tests as seen in the change in the total number of trials completed, the improvement in accuracy, a decrease in omitted responses and the decrease in the latencies to make a correct response across the tests. With the exception of premature responses, the behavioural patterns of wildtype and NK1R-/- are largely comparable across the LITI and VITI tests. While the significantly higher number of omitted responses made by the NK1R-/- mice in the LITI resolves with repeated exposure to the test parameters, this is not the case in the VITI test. The same pattern is seen with perseverative responses. The higher perseverative responding is likely to explain the genotype difference observed in the latency to collect the reward, where NK1R-/- mice are robustly slower.

6.3.2 Effect of NK1 antagonists on behaviour in the 5-CSRTT

After training and initial LITI and VITI tests, the mice completed the drug testing phase of the experiment, where they were tested with two doses (5 and 10mg/kg) of NK1R antagonists RP 67580 and L-733,060. The data in this section was analysed with a repeated measures 2-way ANOVA, with the within subject factor of 'treatment' including the four conditions: no-injection (NI-3), vehicle, low dose (L5 or RP5) and high dose (L10 or RP10) antagonist, and the between-subject factor of 'genotype'.

6.3.2.1 Effect of NK1 receptor antagonist treatment in the LITI test

Total number of trials With no variance on many of the data sets the 2-way ANOVA could not be completed. Friedmans non-parametric test revealed an overall effect of 'treatment' (χ^2=19.7, P<0.001). Subsequent Wilcoxon ranked paired tests show a significant reduction in the total number of trials completed in the 45min time limit after treatment with the high-dose of RP (RP10) (Z=2.0, P<0.05) (Figure 6.3A).

% Accuracy There was a main effect of genotype ($F_{(1,20)}$=7.0, P<0.05) which revealed NK1R-/- mice were more accurate than the wildtype controls when no injection was

administered and after injection of low dose of L-733,060 (NI-3 $t_{(21)}$=2.6, P<0.05; L5 $t_{(20)}$=3.8, P<0.01). A main interaction of 'genotype' and 'treatment' allowed multiple post-hoc comparisons. The accuracy of responses made by wildtype mice increased after a vehicle injection (NI-3 vs. veh $t_{(10)}$=2.3, P<0.05) and also after treatment with the higher dose of RP 67580 (veh vs. R10 $t_{(10)}$=2.3, P<0.05). The accuracy of responses also increased in the NK1R-/- mice treated with low doses of the two antagonists (*c.f.* Veh: L5 $t_{(10)}$=3.0, P<0.05; R5 $t_{(10)}$=2.9, P<0.05) (Figure 6.3B).

% Omissions There was a main effect of 'treatment' ($F_{(3.1,61.9)}$=6.4, P<0.001), but no interaction with 'genotype'. The only treatment to elicit a change in behaviour was the high dose of RP 67580, which increased the number of omission errors made by both genotypes ($t_{(22)}$=5.2, P<0.001) (Figure 6.3C).

% Premature responses As with % omissions there was a main effect of 'treatment' ($F_{(5,100)}$=7.3, P<0.001), but no interaction with genotype. A vehicle injection significantly reduced the number of premature responses made by both wildtype and NK1R-/- mice (NI-3 vs. veh $t_{(22)}$=2.6, P<0.05). Treatment with the high dose of RP 67580 reduced premature responding in both genotypes ($t_{(22)}$=2.6, P<0.05) (Figure 6.3D).

Latency to correct response In an identical pattern to premature responses, there was a main effect of 'treatment' ($F_{(5,100)}$=12.9, P<0.001), but no interaction with 'genotype'. Injection of the vehicle fluid increased response time to the cue light in both genotypes ($t_{(22)}$=2.9, P<0.01) which was further increased by RP 67580 (10mg/kg) (veh vs. RP10 $t_{(22)}$=4.1, P<0.001) (Figure 6.3E).

Latency to collect reward A main effect of 'genotype' ($F_{(1,20)}$=14.0, P<0.001) was further analysed with post-hoc independent t-tests which revealed that NK1R-/- mice were robustly slower to collect the reward after a correct response to the cue light under every treatment condition. The post-hoc analysis of the main effect of 'treatment' ($F_{(2.4,49.0)}$=13.4, P<0.001) echoed the other behavioural profiles (NI-3 vs. veh $t_{22)}$=2.9, P<0.01 and veh vs. RP10 $t_{(22)}$=3.2, P<0.01) (Figure 6.3F).

Perseveration There was a main effect of 'genotype', but despite the NK1R-/- mice perseverating more at each treatment condition this only reached significance at low-dose of L-733,060 (L5) ($t_{(20)}$=3.1, P<0.01) (Figure 6.3G).

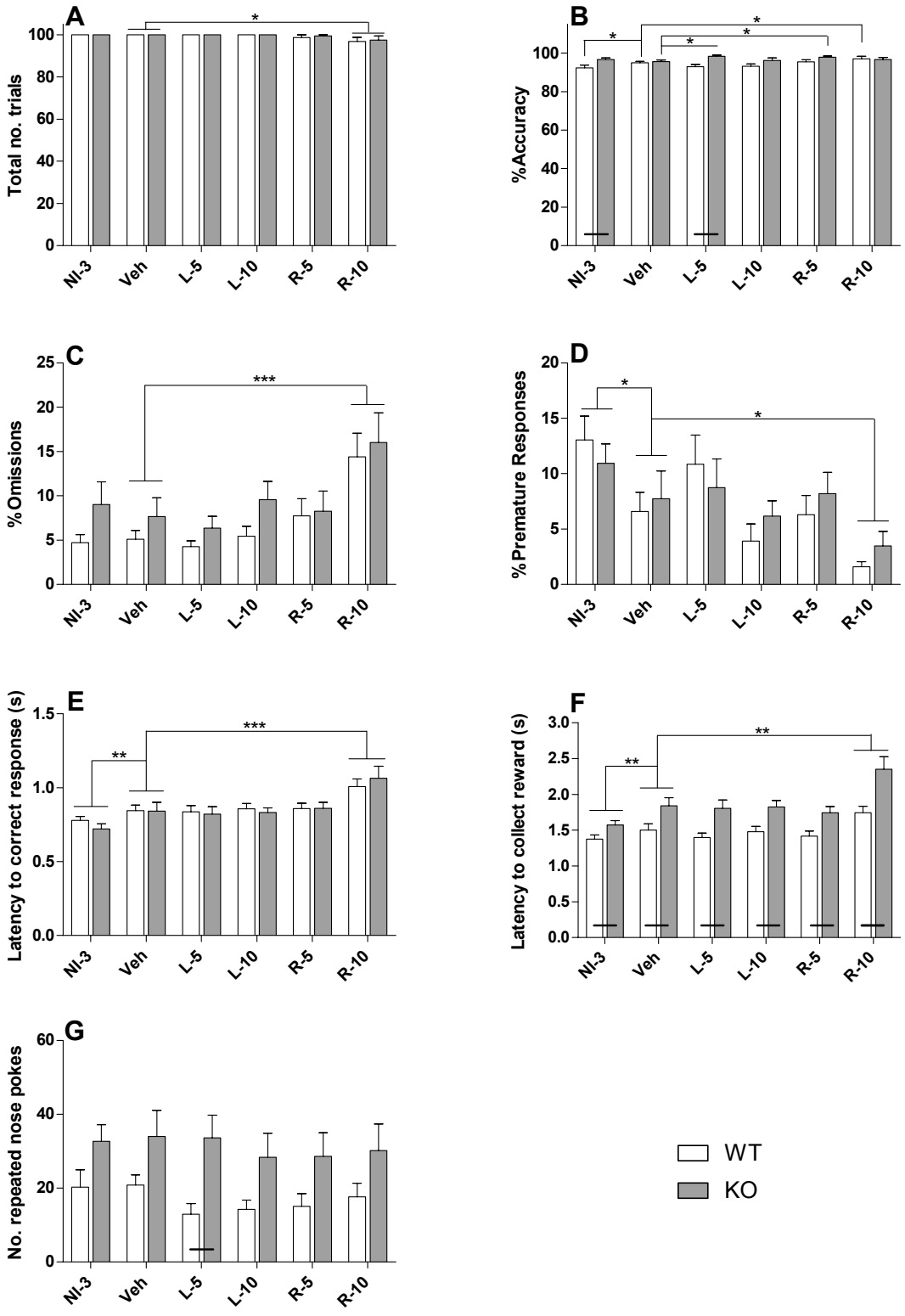


Figure 6.3 Effect of NK1R antagonists L-733,060 and RP 67580 on performance in the LITI test. High dose RP 67580 (RP-10) affected both genotypes for most behaviours except perseveration. L-733,060 treatment induced no behavioural changes (A) Total number of trials completed in the session, (B) percentage accuracy, (C) percentage omissions, (D) percentage of premature responses, (E) latency to a correct response, (F) latency to collect reward and (G) number of perseverative nose-poke responses. WT – wildtype (N=11) and KO – knockout (N=12). Lines between bars denote a significant difference between genotype of at least P<0.05.

6.3.2.2 Effect of NK1 receptor antagonist treatment in the VITI test

Total number of trials With no variance on many of the data sets the 2-way ANOVA could not be completed. Friedmans non-parametric test revealed an overall effect of 'treatment' (χ^2=12.3, P<0.05). Subsequent Wilcoxon ranked paired tests show a significant reduction in the total number of trials completed in the 45min time limit after treatment with the high-dose of RP (RP10) (Z=2.1, P<0.05) (Figure 6.4A).

% Accuracy No main effect of 'test', 'genotype', or interaction between the factors was found for %accuracy, thus showing that treatment with NK1 receptor antagonists has no effect on the accuracy of responses made in the VITI test for either genotype (Figure 6.4B).

% Omissions Statistical analysis for %omissions had to be non-parametric since the raw data violated Levene's test of variance, which could not be corrected by transformation of the data. NK1R-/- mice always scored a higher percentage of omissions compared to wildtype mice (Friedman χ^2=17.0, P<0.01). Further analysis with shows that this only reached significance in the NI-3, L5 and L10 tests (NI-3: Z=2.3, P<0.05; L5: Z=2.3, P<0.05; L10: Z=2.0, P<0.05). Wilcoxon signed rank tests revealed that more omissions were made after treatment with the higher dose of RP 67580 compared to vehicle (Z=3.0, P<0.01)(Figure 6.4C).

% Premature responses There was a main effect of 'treatment' ($F_{(5,100)}$=4.2, P<0.01), but no interaction with 'genotype'. Post-hoc analysis showed that premature responses were reduced in both genotypes after treatment with the high dose of RP 67580 (veh vs. R10 ($t_{(21)}$=2.9, P<0.01) (Figure 6.4D).

Latency to correct response Non-parametric statistical tests were required for analysis of this data set, since Lg10 or square-root transformations did not normalise the variance of the data. An overall Friedman test was significant (χ^2=30.0, P<0.01) showing that treatment had an effect on the response time to the cue light. Wilcoxon signed rank tests revealed that both genotypes were slower to respond after treatment with 10mg/kg of RP 67580 (veh vs. R10 Z=2.7, P<0.01). Although NK1R-/- mice

tended to be slightly slower to respond to the cue light, non-parametric statistical tests did not highlight this as significant (Figure 6.4E).

Latency to collect reward The 2-way ANOVA highlighted a significant effect of 'genotype' ($F_{(1,20)}$=22.5, P<0.001) because NK1R-/- mice were consistently slower to collect the reward after a correct response at all conditions in drug-testing phase. Post-hoc analysis showed this to be significant at every test. There was also an interaction between the effect of 'treatment' and 'genotype' ($F_{(2.7,53.7)}$=5.9, P<0.01). Vehicle injection increased the time taken by NK1R-/- mice to collect their reward after a correct response ($t_{(10)}$=3.4, P<0.01), which was further increased by the high dose of RP 67580 ($t_{(10)}$=4.0, P<0.01). The same dose of antagonist also increased the latency to collect the reward in wildtype mice ($t_{(10)}$=3.7, P<0.01) (Figure 6.4F).

Perseveration While antagonist treatment had no effect on the number of perseverative responses made by either genotype, NK1R-/- mice consistently made a greater number of repeated nose pokes into the cue hole after a correct response compared to wildtype mice (main effect of genotype ($F_{(1,20)}$=6.3, P<0.05), although this only reached significance at NI-3, and with low dose antagonist treatment (NI-3: $t_{(16.0)}$=2.3, P<0.05; L-5: $t_{(13.5)}$=2.9, P<0.01; R-5 $t_{(11.4)}$=2.4, P<0.05) (Figure 6.4G).

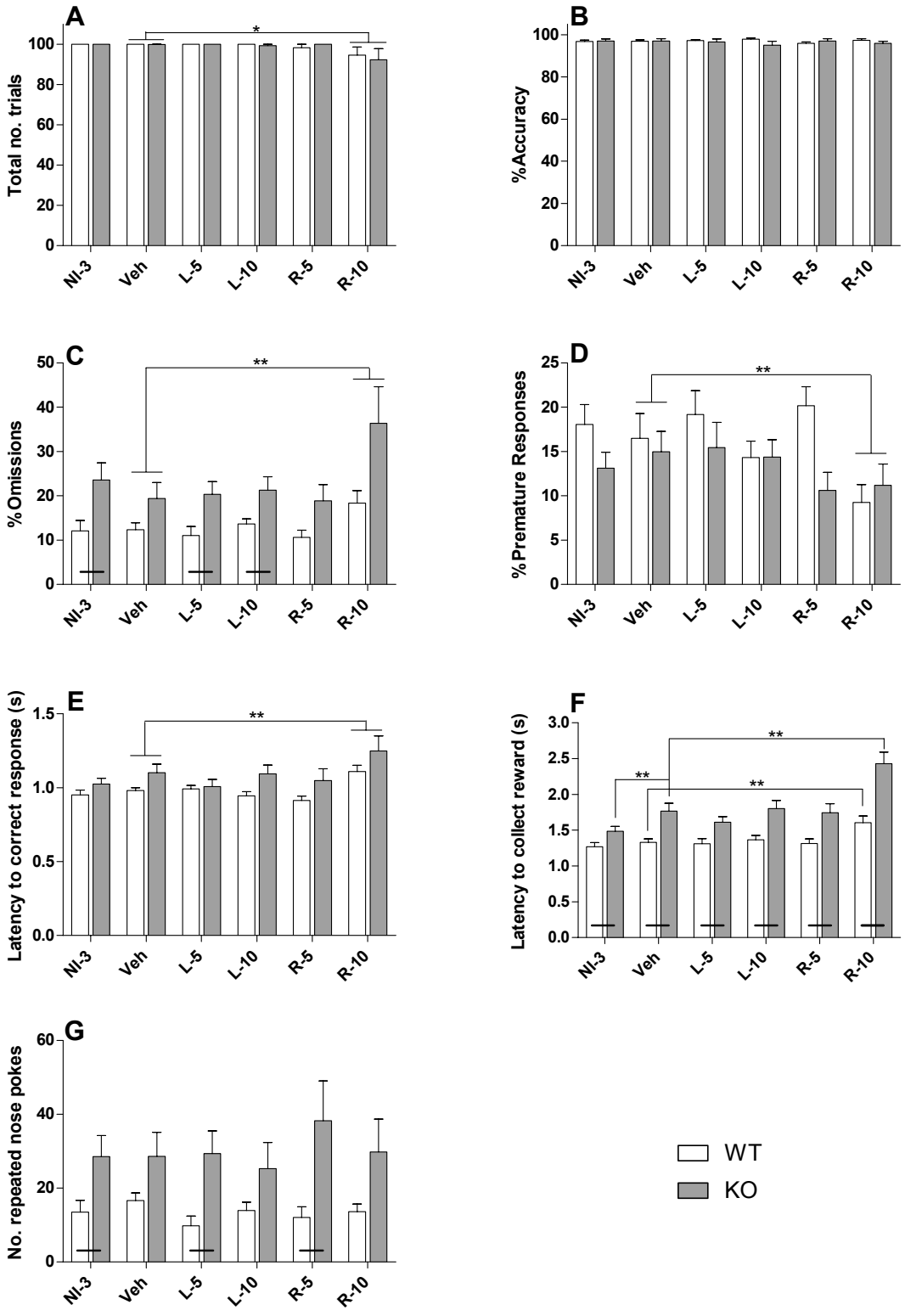


Figure 6.4 Effect of NK1R antagonists L-733,060 and RP 67580 on performance in the VITI test. High dose RP 67580 (RP-10) affected both genotypes for most behaviours except perseveration (A) Total number of trials completed in the session, (B) percentage accuracy, (C) percentage omissions, (D) percentage of premature responses, (E) latency to a correct response, (F) latency to collect reward and (G) number of perseverative nose-poke responses. WT – wildtype (N=11) and KO – knockout (N=12). Lines between bars denote a significant difference between genotype of at least P<0.05.

6.3.3 Splitting of VITI data into individual ITI times

As shown in the previous chapter (section 5.3.3) the behavioural responses of the mice in the VITI test vary with ITI duration, e.g. premature responses increase with longer ITI durations. Therefore it is important to look at the effect of antagonist treatment at each individual ITI time split as changes could be masked in the overall analysis of VITI data. A 3-way ANOVA with 'genotype' (G) as the between subject factor, and 'ITI split' (I) and 'treatment' (T) as within-subject factors was conducted for each behaviour with subsequent post-hoc analyses.

The only behaviour to reveal a different outcome from the analysis of collapsed VITI data was premature responses (G*I*T interaction $F_{(6.9,138.8)}$=2.76, P<0.01). The overall analysis only showed a decrease in premature behaviour after treatment with the high dose of RP 67580 (Figure 6.4D), which was echoed in the 10s ITI split (Figure 6.5C). But also 5mg/kg RP 67580 increased premature responding in wildtype mice at the longest ITI duration of 15s which was not seen in the overall analysis (G*T interaction $F_{(5,100)}$=3.92, WT Veh vs. R-5 $t_{(10)}$=2.3, P<0.05) (Figure 6.5D). The graphs for the other measured behaviours can be found in Appendix A4.

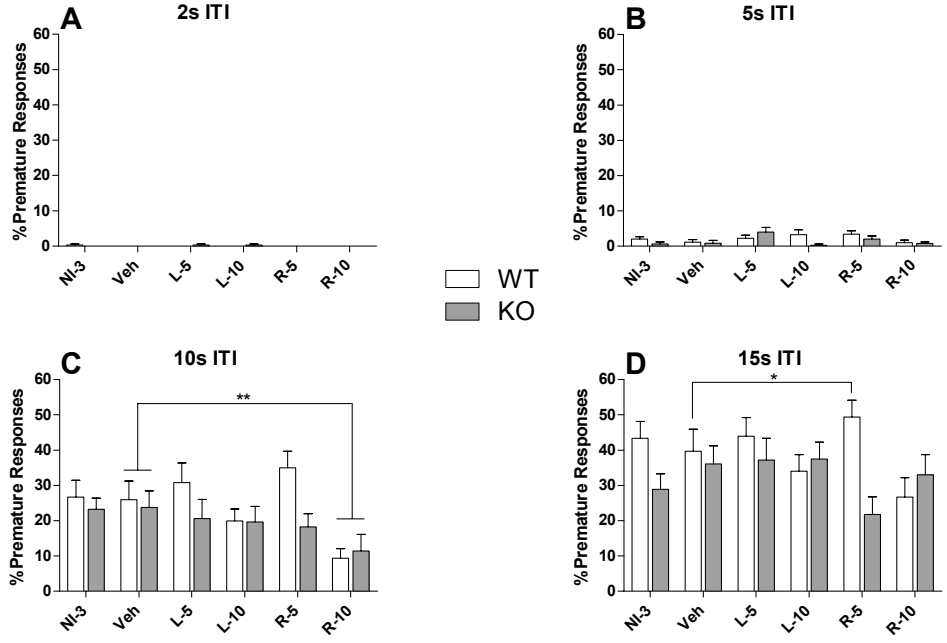


Figure 6.5 Effect of NK1R antagonist treatment on premature responses made in the VITI test split for individual ITI times of 2, 5, 10 and 15s (A,B,C and D respectively). RP-5 increases premature responding in WT mice at the 15s ITI split. WT – wildtype (N=11) and KO – knockout (N=12). *P<0.05, **<0.01.

6.4 Discussion

6.4.1 Summary of results

Repeated exposure to the LITI and VITI tests in the 5-CSRTT leads to a general improvement in performance including increased accuracy of responses, decreased omissions and a reduction in premature responding. However this sometimes resulted in a resolution of behavioural differences between NI-1 and NI-3. For example at their first experience of the LITI test the NK1R-/- mice committed more errors of omission than their wildtype counterparts, but by the embedded control test in the drug testing regimen (NI-3) the genotype difference was no longer present. This similarly occurred with premature responses in the VITI test.

NK1R antagonists were administered to both genotypes, firstly to see if the findings from Chapter 5 where NK1R-/- mice displayed impulsivity and inattentiveness in the 5-CSRTT, could be replicated in wildtype mice given NK1R antagonist and secondly to control for any effects that are non-NK1R mediated by dosing the knockout mice. The high dose of RP 67580 (10mg/kg) affected several behavioural measures in **both** genotypes, while the selective effect on wildtype mice of low-dose RP was limited to an increase in premature responses at the longest ITI split in the VITI test.

6.4.2 Effect of repeated testing in the LITI and VITI

Improvements in performance were seen with repeated exposure to the LITI and VITI tests in which the animals were not subject to the stress of an injection (no injection (NI-1, -2 and -3). In both the LITI and VITI tests %accuracy improved, errors of omission decreased and the latency to make a correct reward decreased with repeated exposure to the test parameters. This trio of behaviours can be interpreted in terms of attentiveness and/or motivation for the task. If considered in terms of motivation the findings would point towards an increased drive for the task with repeated exposure, an unlikely scenario given that the latencies to collect the reward did not differ with repeated testing. The overall improvement in the level of ‘attention’ paid to the task, could be explained by a ‘learning’ based change, that is, the mice learn that the task has

been modified, and adjust their behaviour accordingly. By NI-3 in the LITI there was no longer a genotype difference for omissions, but NK1R-/- mice continued to make more omissions than their wildtype counterparts in the VITI.

In contrast to the improvement in errors of omission, the pattern of premature responses with repeated exposure differed between the long and variable ITI tests. In the LITI test, both wildtype and NK1R-/- performed a similar number of premature responses that did not decrease with repeated exposure to the test. In the VITI test the NK1R-/- mice committed a greater number of premature responses than the wildtypes at NI-1, but by NI-3 the difference was no longer present. The wildtype mice displayed a transient increase in premature responses at NI-2 compared to NI-1, but by NI-3 this returned to the same level as NI-1, whereas the premature responses of the NK1R-/- mice decreased with each consecutive test.

Since the NI-3 test was embedded in the drug testing regime, some animals will have completed this test having previously been exposed to the VITI up to seven times (NI-1, NI-2, Veh, RP5, RP10, L5 and L10). This is an important methodological consideration since the effects of drug treatment are compared to the results from the NI-3 test. One other group have reported an improvement in premature responding with repeated testing in the VITI test (Walker et al., 2011), while high impulsive (HI) rats continue to score high numbers of premature responses over three repeated exposures to the LITI test (Dalley et al., 2008; Besson et al., 2010), yet it must be noted that the rats in the above studies are from a population of animals that are selected for their level of impulsivity (high vs. low). The neurobiological differences between these two populations have been attributed to deficits in PFC function, especially the anterior cingulate cortex (Cg1), with specific regards to DA turnover and 5-HT release (Barbelivien et al., 2001; Dalley et al., 2002). In contrast, the high impulsivity observed in the NK1R-/- mice at NI-1, was spontaneous and likely to be caused by the lack of a functional NK1 receptor. NK1 receptors are found on serotonergic neurons of the dorsal raphe nucleus that are known to project to the forebrain (Baker et al., 1991; Lacoste et al., 2006). The firing rate of DR serotonergic neurons in NK1R-/- mice, and wildtype mice acutely pre-treated with RP 67580, is significantly higher than in control animals demonstrating direct negative regulation of neuronal activity by NK1 receptors (Santarelli et al., 2001). If this system were responsible for impulse control in the

5-CSRTT, it would account for the high impulsivity of the NK1R-/- at NI-1, yet must adapt with repeated exposure to the test as shown by consecutive performance improvement in the NK1R-/- mice. Unfortunately the fact that the impulsive phenotype of the NK1R-/- mouse diminishes with repeated testing somewhat limits its use as a model of ADHD since it would be impossible to run a drug-testing regime designed to reduce impulsivity in the 5-CSRTT.

In both the LITI and VITI tests there is a general trend for an increase in perseverative responding with repeated exposure to the test parameters. In the LITI test both wildtype and NK1R-/- mice make more perseverative responses at NI-3 compared to NI-1, while in the VITI the increase is only significant for wildtype mice, but the high level of perseverative responding by NK1R-/- mice pervades throughout. This, at least in part, arises from the combination of a small increase in the total number of trials completed, along with a slight improvement in accuracy i.e. an increase in correct responses and since perseveration is a cumulative score across correct trials a positive correlation is likely. Another explanation is that the increased familiarity with the test parameters somehow manifests as an increase in perseverative responses. Increased perseverative responses with repeated exposure to the LITI test has been reported elsewhere in C57BL/6 mice (Peña-Oliver et al., 2011). Other 5-CSRTT experiments that have resulted in increased perseverative responding include lesions of the prelimbic and anterior cingulate cortical regions and medial striatum (Muir et al., 1996; Chudasama and Muir, 2001; Rogers et al., 2001). High levels of perseverative responding have been induced with d-amphetamine treatment (Paterson et al., 2011), although this is not always seen (Yan et al., 2011).

6.4.3 Effect of NK1R antagonist treatment in the 5-CSRTT

The two NK1R antagonists and doses used in this experiment were chosen based on their efficacy to induce hyperactivity in wildtype mice to levels that match those of NK1R-/- mice (Yan et al., 2010). Here we predicted that NK1R antagonist treatment in wildtype mice would induce impulsivity, impair attention and increase perseverative responding based on the previous findings from NK1R-/- mice. Yet we observed unexpected effects from the two antagonists; RP 67580 induced impulsivity in wildtype mice at the lower dose of 5mg/kg (at the longest ITI split time of 15seconds in the

VITI), but had no influence on accuracy, omissions or perseveration, and the higher dose of 10mg/kg affected the performance of both genotypes in the LITI and VITI tests of the 5-CSRTT, implicating a non-NK1 mediated effect. NK1 antagonist L-733,060 had no significant effect on the behaviour of wildtype mice in either test.

RP 67580 is a highly potent NK1 receptor antagonist (Beaujouan et al., 1993) with a higher affinity for the NK1 receptor than L-733,060 (in rats $pK_{(i)}$ RP 67580 = 7.6 (Floch et al., 1994); L-733,060 = 5.7 (Brocco et al., 2008)). Although there are well-characterised differences in binding affinities of compounds between human and rodent NK1 receptors (Fong et al., 1992), rats and mice are thought to have very high sequence homology and therefore their receptors are likely to have similar pharmacological properties. Despite the difference in binding affinities, it is still surprising that L-733,060 had no effect on any behaviour in the 5-CSRTT, since the same dose induced hyperactivity in wildtype mice in the LDEB (albeit to a lesser extent than RP 67580) (Yan et al., 2010), clearly demonstrating that it reaches the CNS at a pharmacologically relevant concentration. Therefore the doses used here may not have been high enough to induce cognitive changes that influence behaviour in the 5-CSRTT.

The increase in premature responding after 5mg/kg RP 67580 in wildtype mice adds weight to the hypothesis that the NK1 receptor is important in the regulation of impulse control via the serotonergic neurons that project from the dorsal raphe nucleus to the frontal cortex (see section 6.4.2). However neither omitted nor perseverative responses were affected by antagonist treatment, which would imply that there are differences between an acute blockade of the receptor and the long-term compensatory effects that arise from the lack of functional NK1 receptor or the level of receptor blockade was not sufficient to mimic 100% functional loss. Compensatory up- or down-regulation, or altered sensitivity of other receptors in the NK1R-/- mice in response to the dysfunctional NK1 receptor could be responsible for the robust differences in attention and perseverative responding observed in the NK1R-/- mice.

This is not the first study to report differential behavioural profiles after treatment with 2 different NK1R antagonists. RP 67580, but not CP-96,345 produced anxiolytic effects in the black-and-white box behavioural paradigm (traditionally used to assess anxiety and is similar to the light-dark exploration box on which the antagonists and doses for

this experiment were based). Interestingly, in contrast to the induction of hyperactivity found by Yan et al (2010), other groups have reported these antagonists have sedative effects (Zernig et al., 1993). This may help explain the deficit in performance seen in both genotypes after treatment with the higher dose of RP 67580 in the 5-CSRTT and is discussed below.

6.4.3.1 Non-NK1 mediated effects of high dose RP 67580

The higher dose of RP 67580 (10mg/kg) lengthened latencies, increased omissions, and decreased premature responding in both wildtype and knockout mice, implicating a non-NK1R mediated effect. Two obvious candidates for the target of off-site effects of an NK1 receptor antagonist would be the other tachykinin receptors, NK2 and NK3. This is highly unlikely since the $K_{(i)}$ RP 67580 for the NK1 receptor is 2.9nM and >10μM for NK2 and NK3 receptors (in rat) (values taken from the product Certificate of Analysis from the supplier (Tocris, Bristol, UK)). There is however evidence that in addition to the NK1 receptor, non-peptidergic tachykinin receptors also bind to L-type Ca^{2+} channels (Guard et al., 1993; Rupniak et al., 1993; Lombet and Spedding, 1994; Wang et al., 1994). L-type Ca^{2+} channels are voltage gated with a relatively **long** activation periods (hence L-type). If the higher dose of RP 67580 was binding to L-type Ca^{2+} channels it would impede neuronal transmission and could therefore contribute to the behavioural deficits observed in both genotypes. It also explains the sedative effects of NK1 receptor antagonists noted by (Zernig et al., 1992).

6.4.4 Methodological considerations and conclusions

Given the unexpected disparity in the findings from two different NK1R antagonists, despite them having similar effects in the LDEB at the same doses used here, in future experiments it would be prudent to focus on the effects of one antagonist over a wider range of doses. By the time the planned experimental conditions had been tested the mice were relatively old and the decision was made to end the experiment rather continue with higher doses of L-733,060. Pharmacologically induced manipulations in the 5-CSRTT are typically small in magnitude and often not reliably obtained at a particular dose (Koffarnus and Katz, 2011). Therefore despite the 5-CSRTT providing an excellent method for measuring attention, impulsivity, and compulsivity

(perseveration) in one behavioural paradigm, findings should ideally be followed up in complementary experiments. The effects of repeated exposure to a behavioural test need to be integrated into experimental design not only in basic science, but possibly also in human-based studies. Unfortunately the findings in this chapter bring into question the role of the NK1 receptor in the control of ADHD-like behaviours and the usefulness of the knockout as a model of the condition, since only impulsivity was induced in wildtype animals after antagonist treatment, while measures of attention were not affected. Perseverative responses from NK1R-/- mice remained almost twice as high compared to the wildtype controls, even after antagonist treatment. However the lack of effects induced by NK1 receptor antagonists on behaviour in the 5-CSRTT brings into question the role of the NK1 receptor in ADHD. Due to the unexpected and robust elevated perseverative responses made by the NK1R-/- from the very early stages of training in the 5-CSRTT through the LITI and VITI tests and even after the stress of injection, the next chapter looks at alternative measures of perseveration to further investigate this phenotype of the NK1R-/- mouse.

7 Exploring the perseverative phenotype of the NK1R-/- mouse

7.1 Introduction

The previous two chapters looked at the behaviour of NK1R-/- and wildtype mice in the 5-choice serial reaction time task (5-CSRTT), firstly in a drug naïve state (Chapter 4) and then after treatment with an NK1 receptor antagonist (Chapter 5) to investigate whether the knockout mice displayed traits of impulsivity and inattention to fit with the proposal that the NK1R-/- mouse is a model of ADHD. In addition to measures of impulsivity and attention, perseverative responses were also recorded if the mice continued to nose-poke into the cue holes after a correct response had already been made. From the early stages of training, through to the long- and variable- inter-trial interval test phases, the NK1 receptor knockout mice made significantly more perseverative responses than the wildtype controls, making it the most robust difference between genotypes in the test. However when the wildtype mice were administered an NK1 antagonist their number of perseverative responses did not increase to that of the knockout. This leads to the question as to whether it is the disruption of NK1 receptor function that is the basis for the perseverative phenotype observed in the 5-CSRTT or whether an unrelated mutation has occurred in one of the homozygous breeding colonies that either increases perseveration in the population of NK1R-/- mice, or decreases perseveration in the wildtype colony. Another explanation could be an interaction between the disruption of the NK1 receptor and the background strain that has resulted in a compensatory up- or down-regulation of other members of the substance P/NK1 receptor pathway in the NK1R-/- mice.

7.1.1 Background strain and maternal influences on animal behaviour

Differences in maternal care and background strain can lead to profound differences in both animal behaviour and physiology. There are a multitude of comparison studies into the influence of background strain across a range of behaviours including the species-

typical behavioural tests used in this chapter. Deacon et al., (2007) showed that C57BL/10 mice performed less vigorously than C57BL/6 mice in both burrowing and marble burying tests, while in larger comparisons of multiple strains CBA/J mice consistently perform well in these tasks, while 129S6/SvEv mice perform poorly (Solberg et al., 2006; Thomas et al., 2009). One study into GABA/A receptor expression encompasses the influence of both background strain and maternal upbringing (Caldji et al., 2004). This study is based on the finding that BALBc/ByJ display higher levels of anxiety than C57BL/6ByJ mice. When this phenomenon was investigated at a molecular level C57BL/6 mice showed increased mRNA levels for the α1 and γ2 subunits of the GABA/A receptor in brain regions associated with anxiety (locus coeruleus and central nucleus of the amygdala, respectively), compared to the BALB/c mice. Pups were then cross-fostered to mothers on the alternative background strain 6-hours after birth. Biological offspring of the C57BL/6 mothers, raised by BALB/c dams actually had lower levels of γ2-subunit mRNA, compared to C57 pups that were fostered to C57 mothers (and the reverse was found for the biological offspring of the BALB/c mice). The change in physiology was also accompanied by a corresponding change in anxiety-related behaviours, with BALB/c mice raised by C57 mothers showing reduced anxiety responses compared to controls. This elegant study highlights an important epigenetic effect mediated by maternal care.

7.1.2 ADHD and perseveration

Although perseveration is not one of the core diagnostic criteria for ADHD, there are many examples of the presence of co-morbid perseveration and ADHD. A response perseveration test is often used as an assessment for children diagnosed with ADHD and conduct disorder (CD). The test is a ‘door opening’ task (Daugherty and Quay, 1991) where the children are presented with option of opening a door or stop playing, knowing that there is either a ‘punishment’ or a ‘reward’ on the other side. The ratio of punished responses to rewarded responses is steadily increased and a large number of doors opened is indicative of a high response perseveration, although this could be interpreted as an aspect of impulsivity. Children with ADHD and CD show high levels of response perseveration compared to the control population (Daugherty and Quay, 1991; Matthys et al., 1998; Fischer et al., 2005). Other studies have looked at the effect of methylphenidate (Ritalin) on perseveration but findings have been varied, with

reports of both increased and decreased perseverative responses in the Wisconsin Card Sorting Task (used to assess 'set-shifting' ability (see section 7.1.3.3)) after treatment (Tannock and Schachar, 1992). Another group made an important distinction by stating that methylphenidate treatment doesn't elicit perseveration, but improves persistence (Douglas et al., 1995). Impaired stopping performance, although not a perseverative deficit per se, it is often impaired in disorders that are associated with perseverative deficits such as OCD. Family members of individuals with ADHD display deficits in tests of response inhibition, alongside those diagnosed with the condition (Schachar et al., 2005; Fisher et al., 2011). Therefore this could serve as an indicator of genetic vulnerability for the disorder. Methylphenidate treatment in patients with ADHD improves response inhibition in the stop-signal reaction test (see section 7.1.3.2) (Aron et al., 2003).

7.1.3 Behavioural paradigms used to measure perseveration

Many behavioural tests that were developed for clinical assessment of human psychological disorders have been successfully adapted for use in rodent studies. Perseveration is a broad term that is used to describe a multitude of different behaviours. Some of the tests that have been used for the measurement of perseverative behaviour are outlined below.

7.1.3.1 5-choice serial reaction time task

The 5-choice serial reaction time task used in Chapters 5 and 6 and has been widely discussed previously in this thesis. In brief, perseveration is measured in this task when an animal nose-pokes into the stimulus hole repeated times before collecting the reward. This unnecessary action has been interpreted in many ways including motor-perseveration and compulsivity (Dalley et al., 2011). Unlike premature and incorrect responses, perseverative responses are not punished by a timeout period of darkness, and tend to persist throughout all stages of the task. The number of responses also does not seem to correlate with the attentional load of the task, i.e. if an animal displays a perseverative phenotype it will pervade from the first stage of training through the long- and variable inter-trial interval tests (observation from data).

7.1.3.2 Stop-signal task

The stop-signal task is designed to measure the speed of the process of inhibition (Eagle et al., 2008), so although it is not a direct measure of a perseverative response, a deficit in this task points toward a disruption of the inhibitory brain circuits that have important roles in impulsivity and perseveration. It was first developed for use in clinical assessment of inhibitory reaction times. In short, the subject is presented with a stimulus to which they must respond; yet in some trials a stop-signal (usually an auditory tone but can also be a visual cue) is co-presented to signify that the subject should not respond. Measurements are taken for the normal reaction time to the stimulus, but also the number of trials with the stop-signal that resulted in a response, the reaction time to a stop-signal trial in which the subject failed to inhibit and the reaction time to the stop-signal (Logan et al., 1984). The test has successfully been adapted for use in rodent studies which have been used to elucidate the neural systems underlying and action cancellation.

7.1.3.3 Reversal learning and set-shifting

Reversal learning and set-shifting paradigms come in a variety of forms, all based around a central behavioural test in which subjects learn a 'rule' or action, followed by a change in the rule that requires a different response. The Wisconsin Card Sorting (WCS) test is a set-shifting paradigm used in clinical assessment of cognitive flexibility (Berg, 1948). Here the subjects are presented with a set of cards with designs that differ in colour, quantity and design image (Figure 7.1) they must assign the cards to a different pile (cards 1-4 in Figure 7.1). Participants are unaware of the rule, which can be based on colour, number of shapes or the form of the shape, but are told whether the pairing is correct or not. At various points in the test the matching rule is changed and the participant must respond accordingly. Failure to adapt to the new rule is indicative of perseverative behaviour. Non-human primates are able to perform well in a slightly simplified version of the WCS test (Buckley et al., 2009).

Figure 7.1 Example cards from the Wisconsin Card Sorting Task. The card with 2 red crosses needs to be matched to one of the cards numbered 1-4 according to a 'matching rule' that is unknown to the participant. In this example, if the matching rule was based on colour, card 1 would be a correct match. If shape was the rule then card 4 would be a match and if number of shapes on the card was the rule then card 2 would be the correct choice.

Rodent tests of cognitive flexibility and perseveration tend to be much simpler and usually involve a bifurcated choice, such as a T-maze or 2-armed lever press. In T-maze reversal learning, mice or rats are trained to collect a reward down one arm of the maze (Figure 7.2). Once they reach a specific criterion such as 8 out of 10 correct trials for a set number of consecutive days, the reward is switched to the opposite arm. The number of training days to learn the new location of the reward is recorded. Animals that require more days to adapt to the new location are seen as having a perseverative phenotype (Izquierdo and Belcher, 2012). Another measure of perseveration in the T-maze is spontaneous alternation, that is, a mouse or rat will enter alternate arms of the maze on sequential trials, a large number of alternated trials is defined as highly perseverative behaviour (Gross et al., 2011).

Figure 7.2 Example of a T-maze set-up. For reversal learning one arm is baited with a reward, while the other remains empty. When the mouse or rat has learnt which the rewarded arm is, the reward is switched to the opposite arm.

7.1.3.4 Species-typical behaviours

Species-typical behaviours encompass marble burying, digging and burrowing tests. These are simple naturalistic behaviours that provide environmental enrichment for the animals rather than a negative experimental experience. The marble burying paradigm set-up requires sawdust to a depth of 5cm and 20-25 marbles placed on top in an evenly spaced pattern (Deacon, 2006a). The animals appear to detect the depth of sawdust and begin to dig, which in turn displaces the sawdust and buries the marbles. In the burrowing test, artificial burrows made of plastic tubing are filled with a substrate, namely sawdust, food pellets or pea-shingle gravel (Deacon, 2006b). The animals remove the substrate from the burrow over time. A larger number of buried marbles or a greater percentage of substrate removed from a burrow suggests increased repetitive digging behaviour, which can be interpreted as increased perseveration. These tests are sensitive to a variety of variables including species, background strain, hippocampal lesions and pharmacological treatments (Webster et al., 1981; Dudek et al., 1983; Njung'e and Handley, 1991; Contet et al., 2001; Deacon et al., 2002; Deacon and Rawlins, 2005). Early pharmacological studies established marble burying as a model of anxiety due to its sensitivity to anxiolytics such as diazepam (Broekkamp et al., 1986). Early work carried out on the NK1R-/- mouse was associated with anxiety related

behaviours, and NK1 receptor antagonists were found to abolish marble burying behaviour (Millan et al., 2002). In more recent times marble burying has been thought of more as a model for repetitive digging and that the burying of the marbles is an indirect consequence of this behaviour (Thomas et al., 2009). The type of perseveration measured in these species-typical behavioral tests is very different to the tests above due to the amount of cognitive input required.

7.1.4 Neurobiology of perseveration

Since perseveration can be interpreted in a number of ways including stereotypy and compulsivity the study of the underlying neurobiology is difficult. However perseveration, like impulsivity, is a behavioural deficit related to the inhibition of cognitive control and the two behaviours are therefore likely to involve overlapping brain circuitry. It is therefore the connectivity from the prefrontal cortex to the structures of the basal ganglia that are thought to be disrupted in perseverative disorders such as obsessive-compulsive disorder and ADHD (Aron et al., 2007). In rats, both lesion of cholinergic cortical neurons and pharmacological blockade of glutamatergic receptors in the medial prefrontal cortex (mPFC) increase perseverative responding in the 5-CSRTT (Dalley et al., 2004; Pozzi et al., 2011). Also in the 5-CSRTT, lesions of the pre-limbic cortex and medial striatum both increase perseverative responding (Chudasama and Muir, 2001; Rogers et al., 2001). Reversal learning is also disrupted after neonatal lesions of the mPFC (Schwabe et al., 2004). Striatal lesions of the dorsal-medial and medial regions in rats and monkeys respectively, also resulted in disrupted reversal learning (Clarke et al., 2008; Castañé et al., 2010). The dopaminergic system is also implicated in perseverative responding since d-amphetamine treatment increases perseveration in a dose-dependent manner in the 5-CSRTT (Paterson et al., 2011). A more in depth overview of the interlinking neurobiology of attention, impulsivity, perseveration and the NK1 receptor can be found in the final discussion of this thesis (Chapter 7).

7.1.5 Experimental aims

In this chapter we look at measuring an alternative type of perseverative behaviour through the marble burying and burrowing paradigms, which measure ‘species-typical

behaviours'. The behaviour of wildtype and NK1 receptor knockout mice from the colonies maintained by homozygous breeding are compared with littermate pairs from heterozygous parents, which are both on a mixed background (see sections 2.1.2 and 2.1.4), but also mice from a backcrossed colony on a C57BL/6 background. This will allow an investigation into the whether the behavioural differences between genotypes are solely due to disruption of NK1 receptor function or whether genetic drift has occurred due to the maintenance of the NK1 receptor genetic mutation by homozygous breeding over an extended period of time. Also looking at the behaviour of wildtype and NK1R-/- mice on a backcrossed background will provide insight as to whether there is any interaction between the genetically modified NK1 receptor and the genes from the parental strains.

7.2 Materials and Methods

7.2.1 Subjects

For the marble-burying experiment 9 wildtype and 9 NK1R-/- mice were used from each of the homozygous bred colonies, littermate pairs from heterozygous breeders (both on the mixed background of 129/Sv x C57BL/6 x MF1) and from the backcrossed colony on a C57BL/6 background (see section 2.1.2). In the burrowing experiment, 6 wildtype and 6 NK1R-/- mice from both the homozygous- and heterozygous-bred colonies were used. After the 2 nights of habituation to the burrows the mice were then individually housed until the end of the experiment. All mice were 8-12 weeks old at the time of experiment.

7.2.2 Marble burying

The apparatus and experimental set-up is described in detail in section 2.5.1 of the Materials and Methods chapter. The protocol used is based on that of (Deacon, 2006a).

7.2.2.1 Procedure

The mice were habituated to the boxes without the marbles the day prior to the test for 15minutes. For the test the mice were placed in the boxes for 30minutes, after which the animals were returned to their home cages and the number of marbles buried by more

than two-thirds was recorded. The tests were videoed using a Panasonic SDR-100 camcorder for behavioural scoring.

7.2.2.2 Behavioural scoring

The videoed tests were going to be used to score the latency to begin digging and the number of digging bouts, defined as a coordinated movement of the front paws followed by a kick of the hind paws, performed during the 30minute tests. However another behaviour, a displacement of sawdust by the front paws and muzzle, was prevalent when the videos were viewed. As this behaviour contributes to the burying of marbles the total number of 'nose-pushes' and the latency to the first nose-push were scored alongside digging bouts. The videos were scored in 5minute time bins to allow for further analysis.

7.2.3 Burrowing

The apparatus and experimental set-up is described in detail in section 2.5.2 of Chapter 2. The protocol used is based on that of (Deacon, 2006b).

7.2.3.1 Procedure

Rodents burrow spontaneously, but their performance tends to improve with practice. To habituate the mice to the burrows and substrate, full burrows were placed in the home cages while the animals remained in group housing for two consecutive nights (burrows in at 5.00p.m and removed the next morning at 9.30a.m). The mice were then separated into individual cages for the burrowing tests, one overnight test and three 2-hour probe tests on consecutive days. Two different length tests were studied in case a ceiling effect was reached in the overnight test. Before each test the burrows were filled to approximately 1.5cm from the top and weighed before being placed in the cages. The probe tests were run from 3.00-5.00pm on three consecutive days. At the end of each test the burrows were removed and reweighed to calculate the gross weight of gravel displaced from the burrow, which was subsequently calculated as a percentage of stones removed from the burrow.

7.2.4 Statistical analysis

Firstly the data was analysed with a 2-way ANOVA with genotype and colony as between subject factors. Where a significant main-effect for either factor, or an interaction between the two was seen, independent t-tests between groups were used. The data that were split into time bins or conducted over several days (as in the burrowing experiment) were analysed with a repeated measures ANOVA with 'time-bin' or 'test' as a within-subject factor. In the repeated measures test, if Mauchly's test of sphericity was significant, the Greenhouse-Geiser output was taken. Statistical tests were carried out on raw data sets where possible, but if the variance of the groups differed (i.e. Levene's test for the equality of variance was significant) the data was transformed (square-root or Lg10) to normalise the variance. If this did not satisfactorily correct the variance, then non-parametric statistical tests were used. Statistical significance was set at $P<0.05$.

7.3 Results

Following on from the robust perseverative phenotype of the NK1R-/- mice observed in the 5-CSRTT in chapters, the behavioural paradigms used in this chapter also measure a form of perseveration, that of digging and burrowing. In addition to the comparison of perseverative behaviour across different experimental set-ups, wildtype and NK1R-/- mice from different colonies have been used to investigate the effect of background strain (pure C57BL/6 vs. a mixed 129/Sv x C57BL/6 x MF1 background) and maternal influences by studying mice from homozygous-bred wildtype and knockout colonies

versus littermate pairs from heterozygous parents both with the same mixed MF1 background.

7.3.1 Marble burying

Video recording the marble-burying test enabled the scoring of behaviours throughout the 30minute test, in addition to the traditional observation of the total number of marbles buried. Initially this was going to be number of digging bouts, defined by a coordinated movement of the front paws followed by a hind leg kick, yet some mice preferred to displace the sawdust with a push of the muzzle and front paws. Therefore, these were also scored, and recorded as 'nose-pushes'.

7.3.1.1 Total number of marbles buried

For statistical analysis of the total number of marbles buried by more than two-thirds, a 2-way ANOVA was used with colony and genotype as the between-subject factors, yet Levene's test for the equality of variance was significant for the raw data set, as well as square-rooted and lg10 transformed data. Non-parametric statistical tests were therefore used but did not yield any significant differences across the colonies or between genotype (For colony: Kruskal-Wallis χ^2=3.83, P=0.148; For genotype: Mann-Whitney Z=1.12, P-0.263) (Figure 7.3).

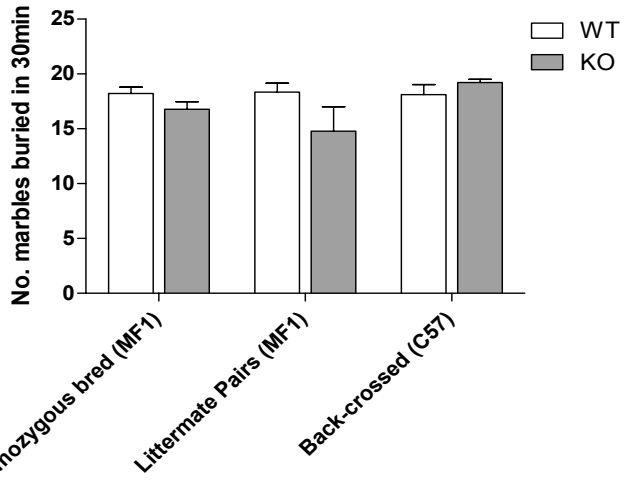


Figure 7.3 Total number of marbles buried by more than two-thirds in 30 minutes by wildtype (WT) and NK1 receptor knockout mice (KO) from homozygous-bred colonies (left), heterozygous-bred littermate pairs (middle) and backcrossed mice on a C57BL/6 background (right). N=9

Yet, just because the end point of the test was not different between groups, does not necessarily mean that it was reached in the same manner. That is, one colony may bury

the marbles in the first 5minutes and do nothing for the remainder of the test, whereas another may only bury in the final 5minutes. Therefore, using still frames from the videoed tests, the number of marbles buried was recorded at 2minute intervals (Figure 7.4) and analysed with a repeated measure ANOVA. Despite an apparent difference between littermate pairs of wildtype and NK1 receptor knockout mice, this was not enough to be pulled out in the overall ANOVA (Table 7.1).

Between-subject effects	**F-value**	**P-value**
Colony	$F_{(2,48)} = 1.42$	P = 0.253
Genotype	$F_{(1,48)} = 0.51$	P = 0.477
Colony*Genotype	$F_{(2,48)} = 1.25$	P = 0.295
Within-subject effects#		
Test	$F_{(1.6,169)} = 3.69$	**P = 0.001**
Test*Colony	$F_{(7.1,169)} = 0.90$	P = 0.505
Test*Genotype	$F_{(3.5,169)} = 1.11$	P = 0.351
Test*Colony*Genotype	$F_{(7.1,169)} = 1.44$	P = 0.193

Table 7.1 Statistical outputs from a repeated-measure ANOVA over the marble burying test with genotype and colony as between subject effects and test as the within subject factor. Significant P-values highlighted in bold. #Mauchly's test was significant therefore sphericity of the data could not be assumed and the Greenhouse-Geiser statistical output for within-subject effects was taken.

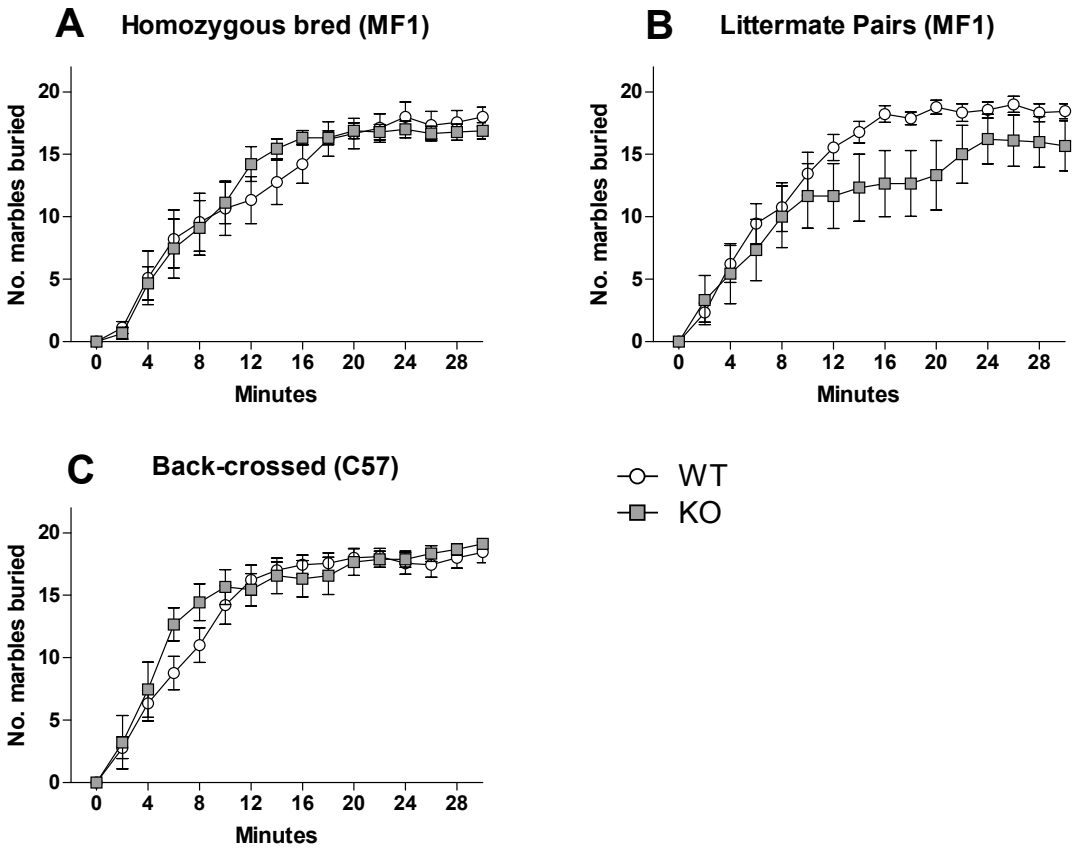


Figure 7.4 Number of marbles buried taken at two minute intervals in the marble burying test for wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies (A), littermate pairs from heterozygous parents (B) and back-crossed animals on the C57BL/6 background (C). WT data displayed with open circles, and KO data with grey squares. N=9.

7.3.1.2 Latency to first dig and nose-push

Despite there being no differences in the marble-burying profile across the different colonies it is important to consider the latency to begin digging as this measure is often analysed as an indicator of anxiety levels. The variance of the raw data violated Levene's test for the equality of variance, as did the transformed data sets (square-root and lg10) so non-parametric tests were used. There is no difference in the latency to the first dig between the three colonies, yet the NK1 receptor knockout mice were always slower to begin digging than their wildtype counterparts as seen with a Mann-Whitney test ($Z=2.33$, $P<0.05$). When further explored with independent t-tests this difference was only statistically significant for the mice from heterozygous bred parents (Littermate pairs: $t_{(16)} = 2.15$, $P<0.05$) (Figure 7.5A). The latency to the first nose-push raw data set did not have equal variance and was square-rooted for normalisation. There

was no difference in the latency to nose-push between genotype, yet there was a significant difference between colonies but with no genotype interaction (Table 7.2).

Between subject effects	F-value	P-value
Colony	$F_{(2,48)} = 3.67$	**P = 0.033**
Genotype	$F_{(1,48)} = 1.34$	P = 0.253
Colony*Genotype	$F_{(2,48)} = 1.06$	P = 0.356

Table 7.2 2-way ANOVA results for the latency to first nose-push with genotype and colony as between subject effects. Statistical test was applied to square-root transformed data. Significant P-values highlighted in bold.

A 1-way ANOVA for colony with Bonferonni post-hoc tests revealed that mice from the back-crossed colony on C57BL/6 background performed their first nose-push significantly earlier than the mice from littermate pairs on the MF1 background (P<0.05) (Figure 7.5B).

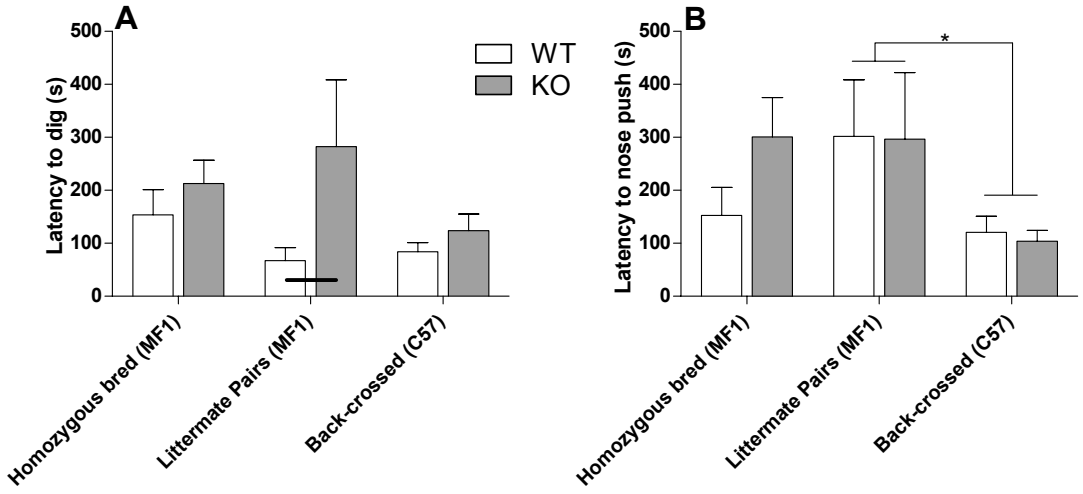


Figure 7.5 Latency to the first dig (A) and nose-push (B) performed by wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies, littermate pairs from heterozygous parents and back-crossed animals on a C57BL/6 background. A black line between bars represents significance of at least P<0.05. * P<0.05. N=9

7.3.1.3 Total number of digging bouts and nose-pushes

The total number of digging bouts and nose-pushes in the 30minute test period were scored and analysed with a 2-way ANOVA for genotype and colony as between subject-factors. Although NK1 receptor knockout mice from every colony scored a lower number of digging bouts than their respective wildtype counterparts, the difference was not significant. There was also no difference in digging behaviour across

the three colonies (Figure 7.6A). The profile of sawdust displacement by nose-pushing however does differ across the colonies. The 2-way ANOVA revealed a main effect of colony, and an interaction between colony and genotype (Table 7.3).

Between subject effects	F-value	P-value
Colony	$F_{(2,48)} = 8.62$	**P = 0.001**
Genotype	$F_{(1,48)} = 1.57$	P = 0.217
Colony*Genotype	$F_{(2,48)} = 7.12$	**P = 0.002**

Table 7.3 2-way ANOVA results for the total number of nose-pushes with genotype and colony as between subject effects. Statistical test was applied to lg10-transformed data. Significant P-values highlighted in bold.

Interestingly, NK1 receptor knockout mice from the homozygous-bred colony made significantly fewer nose-pushes than their wildtype counterparts ($t_{(11.0)}$=3.54, P<0.01), whereas in the back-crossed C57BL/6 colony, the NK1 receptor knockout mice displaced sawdust via a nose-push, than the wildtypes ($t_{(16)}$=2.18, P<0.05). There was no difference in behaviour across the wildtype mice of each colony, but the NK1 receptor knockout mice from the back-crossed colony completed more nose-pushes than those from the MF1 background strain colonies (Bonferroni post-hoc test: P<0.001) (Figure 7.6B).

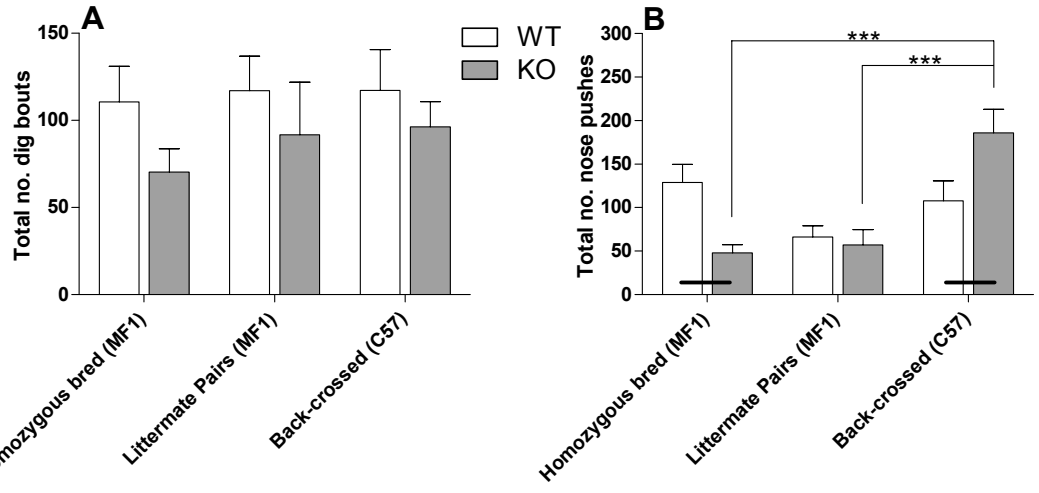


Figure 7.6 Total number of digging bouts (A) and nose-pushes (B) performed by wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies, littermate pairs from heterozygous parents and back-crossed animals on a C57BL/6 background. A black line between bars represents significance of at least P<0.05. *** P<0.001. N=9

7.3.1.4 Digging bouts and nose-pushing per 5-minute time bin

As with the total number of marbles buried, the total values only represent the end point of the 30minute test and therefore give no indication of the profile by which they were achieved. The data was recorded in 5minute time bins, and analysed with a repeated measures ANOVA with genotype and colony as between subject factors and time-bin as a within subject factor (Table 7.4).

Between-subject effects	**F-value**	**P-value**
Colony	$F_{(2,48)} = 0.35$	P = 0.706
Genotype	$F_{(1,48)} = 2.82$	P = 0.100
Colony*Genotype	$F_{(2,48)} = 0.12$	P = 0.891
Within-subject effects		
Time-bin	$F_{(5,240)} = 4.29$	**P = 0.001**
Time*Colony	$F_{(5,240)} = 1.22$	P = 0.281
Time*Genotype	$F_{(5,240)} = 2.97$	**P = 0.013**
Time*Colony*Genotype	$F_{(5,240)} = 1.61$	P = 0.106

Table 7.4 Statistical outputs from a repeated-measure ANOVA for digging bouts over 5-minute time bins of the marble burying test with genotype and colony as between subject factor and time-bin as the within subject factor. Significant P-values highlighted in bold.

There was no overall difference in the behaviour across the three colonies tested in the marble-burying paradigm and the data from each colony is presented separately in Figure 7.7. Each colony was then considered individually in a repeated measures ANOVA with only genotype as the between subject factor. There was no overall difference in digging behaviour between wildtype and NK1 receptor knockout mice in any of the three colonies tested, yet there was a significant interaction between time-bin and genotype (time*genotype) in the homozygous bred colony and an overall effect of time-bin in the back-crossed colony (Table 7.5)

	Time-bin	**Time*Genotype**
Homozygous bred (MF1)	$F_{(5,80)} = 1.79$ P=0.125	$F_{(5,80)} = 3.25$ **P=0.010**
Littermate pairs (MF1)	$F_{(2.9,46.6)} = 1.74$ P=0.173	$F_{(2.9,46.6)} = 1.34$ P=0.272
Back-crossed (C57)	$F_{(5,80)} = 3.20$ **P=0.011**	$F_{(5,80)} = 1.68$ P=0.171

Table 7.5 Statistical outputs from a repeated-measure ANOVA for dig bouts over 5-minute time bins of the marble burying test for each individual colony with genotype as the between subject factor and time-bin as the within subject factor. Significant P-values highlighted in bold.

Both wildtype and knockout mice from the homozygous-bred colony displayed an almost identical digging profile for the first 15minutes of the test, after which the wildtype mice continued to dig (time bin 16-20 vs. 26-30: $t_{(8)}$=2.47, P<0.05) whereas the NK1 receptor knockout mice decreased their digging (time bin 16-20 vs. 26-30: $t_{(8)}$=2.81, P<0.05) leading to a significant difference in the number of digging bouts completed by wildtype and NK1 receptor knockout mice in the final 5minutes of the test ($t_{(16)}$=4.07, P<0.01) (Figure 7.7A). There were no statistically significant differences in the digging profiles of the wildtype and NK1 receptor knockout littermate pairs (Figure 7.7B). The mice from the back-crossed colony displayed the same burying profile independent of genotype, where there was a significant increase in digging bouts between the first and third time bins (paired t-test: time bin 1-5 vs. 11-15: $t_{(17)}$=2.30, P<0.05) (Figure 7.7C).

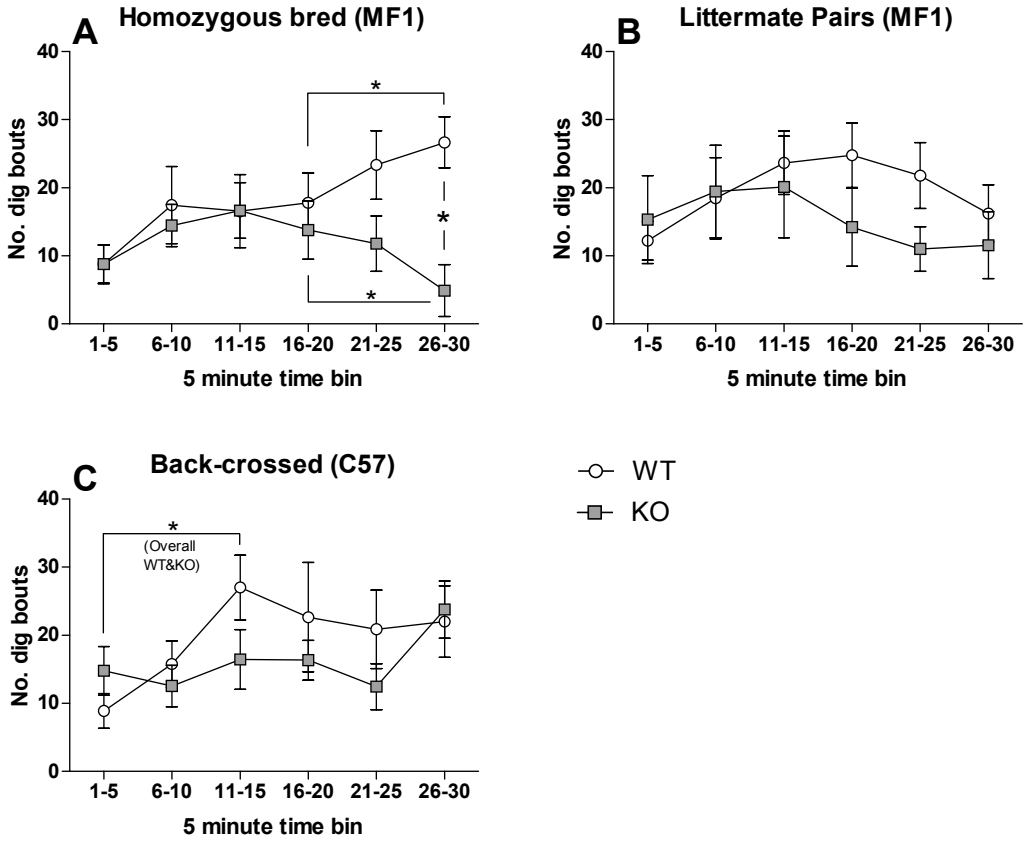


Figure 7.7 Number of digging bouts per 5-minute time bin for wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies (A), littermate pairs from heterozygous parents (B) and back-crossed mice on a C57BL/6 background (C). WT data displayed with open circles, and KO data with grey squares. N=9. * P<0.05

In the repeated measures ANOVA for nose-pushes, with genotype and colony as between subject factors and time-bin as the within subject factor significant overall differences were found across colonies, an interaction between colony and genotype and for time-bin (Table 7.6).

Between-subject effects	**F-value**	**P-value**
Colony	$F_{(2,48)} = 11.9$	**P = 0.001**
Genotype	$F_{(1,48)} = 0.61$	P = 0.438
Colony*Genotype	$F_{(2,48)} = 7.96$	**P = 0.001**
Within-subject effects#		
Time-bin	$F_{(3.7,180.6)} = 5.49$	**P = 0.001**
Time*Colony	$F_{(7.5,180.6)} = 0.71$	P = 0.675
Time*Genotype	$F_{(3.7, 180.6)} = 1.66$	P = 0.164
Time*Colony*Genotype	$F_{(7.5,180.6)} = 1.82$	P = 0.080

Table 7.6 Statistical outputs from a repeated-measure ANOVA for nose-pushes over 5-minute time bins of the marble burying test with genotype and colony as between subject factor and time-bin as the within subject factor. Square-root transformed data used for analysis to normalise the difference in variance of the raw data. Significant P-values highlighted in bold. # Mauchly's test of sphericity was significant therefore Greenhouse-Geiser outputs were taken.

The statistical difference in the behavioural profile of the colonies was independent of time, and has already been reported in section 7.3.1.3 along with overall genotype differences. Here the colony*genotype interaction is further analysed by looking at the individual colonies with a repeated measures ANOVA with genotype as the only between subject factor (Table 7.7).

	Time-bin	**Time*Genotype**
Homozygous bred (MF1)	$F_{(5,80)} = 2.47$ **P=0.040**	$F_{(5,80)} = 2.69$ **P=0.027**
Littermate pairs (MF1)	$F_{(5,80)} = 1.19$ P=0.321	$F_{(5,80)} = 1.04$ P=0.399
Back-crossed (C57)	$F_{(5,80)} = 3.82$ **P=0.004**	$F_{(5,80)} = 1.37$ P=0.246

Table 7.7 Statistical outputs from a repeated-measure ANOVA for nose-pushes over 5-minute time bins of the marble burying test for each individual colony with genotype as the between subject factor and time-bin as the within subject factor. Significant P-values highlighted in bold.

In the homozygous bred MF1 colonies, the wildtype mice performed more nose-pushes than the NK1 receptor knockout mice which increased over time and was significantly higher in the final time bin compared to the first ($t_{(8)}$=5.46, P<0.01). Conversely the homozygous bred knockout mice increased their nose-pushing sawdust displacement

over the first 15minutes, and then tailed off (time bin 1-5 vs. 11-15; $t_{(8)}$=3.21, P<0.05). These different behavioural profiles resulted in a significant genotype difference, with the NK1 receptor knockout mice performing fewer nose-pushes than the wildtype controls in the final two time bins of the 30minute test (Figure 7.8A). There was no difference in the behaviour of wildtype and knockout littermate pairs across the entire test (Figure 7.8B). Unlike the NK1 receptor knockout mice in the homozygous bred colonies, the knockout mice on the C57BL/6 background performed more nose-pushes than their wildtype counterparts although this did not reach statistical significance. Both genotypes from the back-crossed colony increased the number of nose pushes over the course of the test (*cf. time bin 1-5*; 6-10 $t_{(17)}$2.12, P<0.05; 11-15 $t_{(17)}$=3.83, P<0.01 and 26-30 $t_{(17)}$=4.11, P<0.01) (Figure 7.8C).

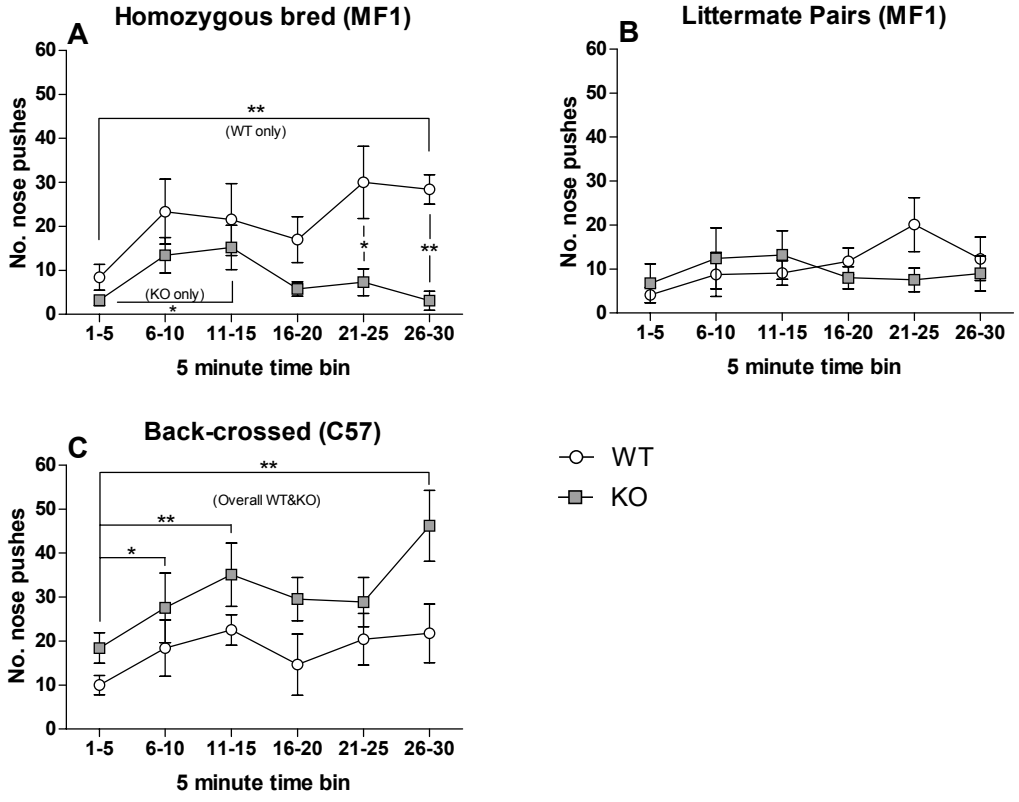


Figure 7.8 Number of nose-pushes per 5-minute time bin for wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies (A), littermate pairs from heterozygous parents (B) and back-crossed mice on a C57BL/6 background (C). WT data displayed with open circles, and KO data with grey squares. N=9. * P<0.05, ** P<0.01

7.3.2 Burrowing

In the mice's first experience of the burrows, they were filled, weighed and placed in the home-cages at 5.00 p.m., left overnight and removed the following day at 9.00 a.m. when they were again weighed. The percentage of stones removed from the burrow was calculated for analysis. The data for the overnight test was analysed with a 2-way ANOVA, with colony and genotype as the between subject factors (Table 7.8). There was a main effect of genotype but no difference in behaviour between colonies.

Between subject effects	F-value	P-value
Colony	$F_{(1,23)} = 0.82$	P = 0.377
Genotype	$F_{(1,23)} = 12.42$	**P = 0.002**
Colony*Genotype	$F_{(1,23)} = 0.71$	P = 0.408

Table 7.8 2-way ANOVA results for the overnight burrowing test with genotype and colony as between subject effects. Significant P-values highlighted in bold.

However the raw data set violated Levene's test for the equality of variance and transforming the data by square-root and lg10 did not correct for this, therefore non-parametric statistical tests were used for further analysis. Although the wildtype mice from both colonies burrowed more than their respective knockout counterparts this was only significant for the homozygous bred populations (Mann-Whitney test: $Z=2.24$, $P<0.05$) (Figure 7.9).

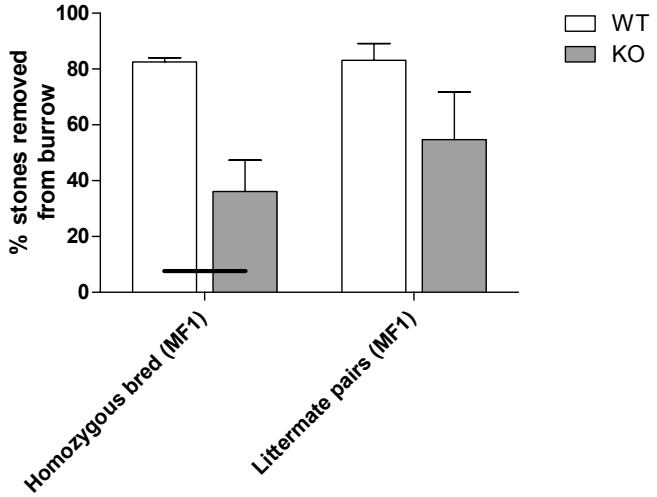


Figure 7.9 Percentage of stones removed from the burrows overnight by wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies and littermate pairs from heterozygous parents. N=6. Black line between bars represents a statistical difference of at least P<0.05.

After the initial overnight test, the mice were tested on three consecutive days with a 2-hour probe test from 3.00-5.00 p.m. A repeated-measure ANOVA with colony and genotype as between subject factors and 'test' as the within-subject factor was used for statistical analysis. Neither colony, test, nor interaction of factors were significant but there was an overall difference between genotypes (Table 7.9).

Between-subject effects	**F-value**	**P-value**
Colony	$F_{(1,20)} = 3.35$	P = 0.082
Genotype	$F_{(1,20)} = 8.98$	**P = 0.007**
Colony*Genotype	$F_{(1,20)} = 0.38$	P = 0.547
Within-subject effects		
Test	$F_{(2,40)} = 2.68$	P = 0.081
Test*Colony	$F_{(2,40)} = 0.54$	P = 0.587
Test*Genotype	$F_{(2,40)} = 0.73$	P = 0.489
Test*Colony*Genotype	$F_{(2,40)} = 1.32$	P = 0.279

Table 7.9 Statistical outputs from a repeated-measure ANOVA over the three burrowing probe tests with genotype and colony as between subject effects and test as the within subject effect. Significant P-values highlighted in bold.

Since there was no overall difference in the burrowing performance of the two colonies, they have been separated below for further genotype analysis. NK1 receptor knockout mice from both homozygous- and heterozygous-bred colonies burrowed less than their wildtype counterparts, yet this was only statistically significant in the third probe test for the homozygous bred mice (Figure 7.10A) and in the second probe test between the littermate pairs (Figure 7.10B).

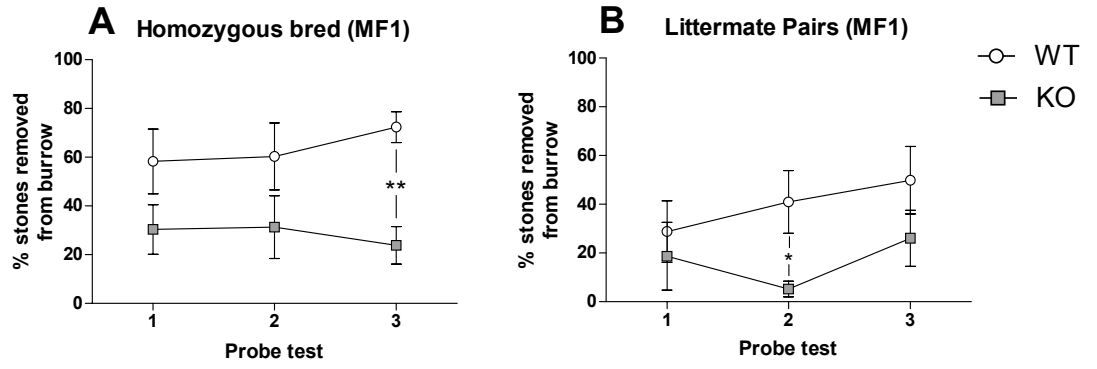


Figure 7.10 Burrowing performance across three probe tests on consecutive days for wildtype (WT) and NK1 receptor knockout (KO) mice from homozygous bred colonies (A) and littermate pairs from heterozygous parents (B). WT data displayed with open circles, and KO data with grey squares. N=6. * P<0.05, ** P<0.01

7.3.3 Summary of results

In the marble burying test there was no overall difference in the number of marbles buried by either genotype across all colonies tested, nor in the profile over time in which the marbles were buried. NK1 receptor knockout mice were in general slower to commence digging than their wildtype counterparts, although this only reached significance between wildtype and knockout littermate pairs from heterozygous parents. There was no significant statistical difference in the total number of digging bouts across genotype or colony, although knockout mice tended to dig less than the wildtype counterparts of each colony. When the data was divided into 5minute time bins a genotype difference emerged between the homozygous bred mice. For the first 15minutes the profile of wildtype and knockout digging bouts was indistinguishable, yet in the latter stages of the test the homozygous bred knockout mice decreased their digging behaviour, while the wildtype mice dug more.

The latency to the first nose-push varied across the colonies, the heterozygous littermate pairs and homozygous bred knockout mice were much slower than the back-crossed colony and the homozygous bred wildtype mice to first perform this method of sawdust displacement. This difference was also reflected in the total number of nose-pushes. While NK1R-/- mice from the homozygous bred colony performed significantly fewer nose-pushes than their respective wildtype mice, the knockout mice from the back-crossed colony nose-pushed significantly *more* than their respective wildtypes and the other knockout mice from the other colonies.

In the burrowing experiment using homozygous- and heterozygous-bred colonies, NK1 receptor knockout mice tended to burrow less than the wildtype counterparts, although this was only significant between the homozygous-bred mice in the overnight test. In the three 2-hour probe tests over consecutive days, the knockout mice always burrowed less in both colonies, yet only significantly so in probe test 3 for the homozygous-bred mice and probe test 2 for the mice from heterozygous parents.

7.4 Discussion

Marble burying and burrowing are experimental paradigms that exploit the natural tendency of rodents to dig and forage. In fact these paradigms provide the animals with a form of environmental enrichment and since they cause no 'pain, suffering, distress or lasting harm' to normal animals they are not regulated procedures as defined by the UK Animals (Scientific Procedures) Act of 1986 (Deacon, 2006a). They are very simple to run, require no specialist equipment and are therefore inexpensive to conduct, yet provide quantitative data under controlled laboratory conditions. Previous studies have shown these species-typical behaviours are sensitive to species, background strain, brain lesions and drug treatment (Webster et al., 1981; Dudek et al., 1983; Njung'e and Handley, 1991; Contet et al., 2001; Deacon et al., 2002; Deacon and Rawlins, 2005). The main premise of this chapter was to use marble burying and burrowing as a measure of perseveration after NK1R-/- mice displayed a robust perseverative phenotype in the 5-choice serial reaction time task (Chapter 4). The tests were carried out with wildtype and NK1 receptor knockout mice from three separate colonies (homozygous bred wildtype and NK1R-/- MF1 colonies, littermate pairs from heterozygous MF1 parents and the colony backcrossed onto a C57BL/6 background). Using wildtype and knockout littermate pairs from heterozygous parents negates differences in maternal bonding since NK1R-/- mice produce fewer ultrasonic vocalisations than wildtypes (Rupniak et al., 2000) and reduces the problem of genetic drift that can arise from long-term breeding of homozygous colonies. The effect of background strain on marble-burying behaviour (but not burrowing) was investigated with a comparison of the backcrossed C57BL/6 mice versus those on the mixed 129/Sv x C57BL/6 x MF1 background.

7.4.1 The role of the NK1 receptor in species-typical behaviours

Although rodents use bedding material to bury noxious stimuli such as shock probes (De Boer and Koolhaas, 2003) it immediately became apparent when viewing the videos that the mice showed no aversion to the presence of the marbles, which corroborates the findings of (Njung'e and Handley, 1991) who showed that mice did not avoid the marble-containing side of a two-compartment box. The mice rarely displayed any interest in the presence of the marbles, and any marble burying was an indirect

consequence of spontaneous digging behaviour. This is inline with previous observations from other groups that state that marble burying is simply a measure of repetitive, perseverative digging (Gyertyán, 1995; Masuda et al., 2000; Thomas et al., 2009). Interestingly, the mice showed two very distinct methods of sawdust displacement, firstly by traditional 'digging', (a coordinated movement of the front paws followed by a kick of the back paws) but also by a pushing of the sawdust with the snout and front paws. This behaviour has been reported in rat burying studies as a deliberate strategy for burying objects (Poling et al., 1981), but not in mouse marble-burying studies. The number of 'nose-pushes' was therefore scored in addition to digging bouts.

In the 5-choice serial reaction time task NK1R-/- mice displayed a high number of perseverative responses, i.e. they continued to nose poke into the cue hole after a correct response, yet a perseverative phenotype was not observed in the marble burying and burrowing tests, where there was no difference in the number of marbles buried, or total number of digging bouts performed between wildtype and knockout mice (although the NK1R-/- mice from all colonies dug less than their respective wildtype counterparts this failed to reach significance). In fact in the burrowing test, NK1R-/- mice burrowed *less* than their wildtype counterparts, the very opposite of a perseverative phenotype. Interestingly, a previous study into the effect of NK1 receptor antagonists in the marble-burying paradigm reported an absolute blockade of marble burying behaviour after antagonist treatment (Millan et al., 2002). This highlights that perseveration, like impulsivity is a multifaceted behaviour that does not necessarily translate across behavioural paradigms.

Other genotype differences in behaviour were noted in the marble-burying and burrowing tests. NK1R-/- mice tended to be slower to begin digging, although this was only significant between the wildtype and knockout littermate pairs from heterozygous parents. When marble burying is used in the context of measuring anxiety, reduced marble-burying and a long latency to commence digging is interpreted as an 'anti-depressed' phenotype (Catches et al., 2012). The marble burying profile of the NK1R-/- mouse would therefore fit into this antidepressant-like phenotype, which is in line with early work on the NK1 receptor knockout mouse, that ultimately led to NK1 receptor antagonists being trialed as anti-depressants (Rupniak et al., 2001; Santarelli et al.,

2001). Yet the use of the marble burying test is highly controversial and the consensus is that it is a better measure of repetitive perseverative behaviour more than novelty induced anxiety (Deacon, 2006a; Thomas et al., 2009).

7.4.2 Effect of homozygous vs. heterozygous breeding for colony maintenance

Although maintaining a genetic mutation through homozygous mating is probably the easiest and cheapest option, it is not good laboratory practice. The best methods for maintaining a mutation are either by breeding heterozygotes with each other or backcrossing on to an inbred strain such as C57BL/6 (Wolfer et al., 2002). This eliminates the risk of genetic drift of separate wildtype and knockout colonies over time, and also negates the possibility of differential maternal upbringing by wildtype and knockout dams (Crusio, 2004). This is especially important in this study since NK1 receptor mouse pups elicit fewer ultrasonic vocalisations when removed from their mother than wildtype pups, so maternal bonding could differ between the wildtype and knockout homozygous colonies (Rupniak et al., 2000). However, if it is only the pups, and not the mother that are effected, this would put NK1R-/- pups at a distinct disadvantage in a large litter of offspring from heterozygous parents in which there would be wildtype and heterozygous pups too that do not have this bonding problem. In this study there are no fundamental differences in the behaviour of wildtype and NK1 receptor knockout mice from the homozygous maintained colonies, compared to littermate pairs from heterozygous parents, in either the marble burying- or burrowing-test. This suggests that the previously considered differences in maternal upbringing either did not arise or they do not effect the instinctive and naturalistic behaviours measured in the marble burying and burrowing tests.

7.4.3 Effect of background strain on behaviour

Many studies have been conducted into the influence of background strain on mouse behaviour, yet it is often overlooked when planning experiments and interpreting results. It is also possible in a genetically manipulated mouse that background genes from parental strains may interact with the mutated gene, which could compromise the interpretation of the mutant phenotype (Crawley et al., 1997). In this chapter wildtype

and knockout mice on two different background strains were used, a backcrossed colony on a 'pure' C57BL/6 background (crossed for more than 10 generations so they are ~99.9% C57BL/6) and a mixed background of 129/Sv x C57BL/6 x MF1. This is unusual since most background comparisons are of single background strains, whereas the behaviour of the mixed background mouse is determined by the combination of the three different strains. Marble burying behaviour is known to differ across background strains, with AKR/J, FVB/NJ and CBA/J strains burying the most marbles in a comparison of 10 inbred strains, while 129S6/SvEv-Tac mice buried the least (Thomas et al., 2009). The greatest difference between the strains tested here was not seen in the traditional measure of 'digging' but in 'nose-pushing', the other recorded method of sawdust displacement. The NK1 receptor knockout mice from the backcrossed colony performed more nose-pushes than the knockout mice on the mixed background, whereas the behaviour of wildtype mice was comparable across all three colonies. This suggests there is an interaction between the NK1 receptor and strain of the mouse. Similar interactions were found in a comparison of corticosterone levels of wildtype and NK1R-/- mice in response to a mild-stressor (restraint) on two background strains (backcrossed C57BL/6 and a mixed 129/Sv x C57BL/6) (McCutcheon et al., 2008).

Due to limited availability of the back-crossed colony of wildtype and NK1 receptor knockout mice, these mice were not included in the burrowing experiment. However previous comparisons of mouse burrowing behaviour across different backgrounds have highlighted marked strain differences. In one, SLJ mice burrowed less than C57BL/6 or DBA/2 mice (Deacon, 2009), while in another, 129S2/SvHsd mice burrowed considerably less than C57BL/6JOlaHsd and did not improve over days, whereas the C57s did (Contet et al., 2001). Burrowing data across a larger number of strains (A/J, AKR/J, BALBc/J, CBA/J, C3H/HeJ, C57BL/6J, DBA/2J and LP/J) reported that CBA/J, DBA/2J and LP/J mice were prolific burrowers (Solberg et al., 2006). Since species specific behaviours are based on digging behaviour, it has been hypothesised that behavioural polymorphisms between strains stem from ancient habitat selection that potentially required different levels of digging depending on the location and substrate into which the burrows were dug (Dudek et al., 1983). This theory is supported by the lack of burrowing of the spiny mouse in laboratory conditions compared to that of other rodents (Deacon, 2009), as in the wild they prefer to use rock crevices for shelter, rather than dig their own burrows (Elvert et al., 1999).

7.4.4 Methodological considerations and conclusions

Tests for species-typical behaviours such as marble burying and burrowing are cheap easy to run, yet over the years they have been used as a 'measure' of many behaviours including anxiety, obsessive-compulsive disorder (OCD)-like symptoms and digging. In order to appropriately interpret behavioural differences between subjects it is imperative that researchers understand the basis of the behaviour itself. I believe that marble burying and burrowing are both a fundamental test of digging, which is a spontaneous and intrinsic natural phenomenon displayed by rodents when the opportunity arises. There is mounting evidence against marble burying being a measure of anxiety. One such study tested a range of inbred mouse strains in the marble burying test and in open-field and light/dark exploration tests, both of which are traditional tests of anxiety-related behaviours. No correlation was found between levels of marble burying and the traditional tests of anxiety across all ten tested strains (Thomas et al., 2009). Secondly, if digging were a natural phenomenon, the fact that anti-depressants reduce this behaviour would mean that normal wildtype mice are 'depressed'. Therefore the treatment is actually creating a new problem by preventing a natural instinctive behaviour rather than correcting a behavioural deficit. However to this day, papers are still being published that use marble burying as an anxiogenic test (Catches et al., 2012).

The burrowing test exploits the fact that rodents in the wild will build burrows for a variety of purposes including safety from predators, food storage, and shelter (Boice, 1977; Dudek et al., 1983). Although rodents kept under laboratory conditions do not *need* to build burrows, they will if presented with the chance. Although the species-typical behaviours used here both measure digging behaviour, the burrowing test is reported to be slightly more sensitive than marble burying (Deacon, 2006a). Although the species-typical behavioural tests used in this chapter provide invaluable data into the naturalistic behaviour of rodents, I feel that they are not a measure of perseveration in clinical terms, and that scientists should proceed with caution when relating these behaviours to conditions such as OCD and ADHD.

The findings from this chapter in which marble burying and burrowing have been used as measures of perseverative behaviour do not correlate with the previous chapters

where NK1R-/- mice display a robust perseverative phenotype in the 5-choice serial reaction time task. Here the behaviour of the NK1 receptor knockout is rarely different from that of the wildtype controls, and in cases where there are genotype differences it is more common for the NK1R-/- to show less perseveration in the species-typical behaviours. These studies show that perseveration is a multifaceted behaviour and where a perseverative phenotype may be robust in one paradigm, this does not necessarily translate across to other behavioural paradigms. The perseverative phenotype of the NK1R-/- therefore requires further investigation, perhaps with a reversal test in a T-maze set-up, as this would have more of a cognitive aspect like the 5-CSRTT, rather than the species-typical behaviours that measure repetitive motor movements. Reversal learning in the Morris Water Maze was conducted as part of a battery of learning and memory experiments for the NK1R-/- mouse, but no difference was found between wildtype and NK1R-/- mice (Gadd, 2003). We are currently testing the heterozygous-bred littermate pairs in the 5-choice serial reaction time task. This will provide clarity as to whether the difference in perseverative responding between wildtype and NK1R-/- mice is due to the functional disruption of the NK1 receptor, or whether it was a result of a spontaneous mutation and genetic drift as a result of maintaining the NK1 receptor mutation through homozygous breeding.

In the final chapter, findings from this thesis will be brought together to consider the implications of this work, including a wider discussion of 'perseveration'. There I will look at the different types of perseveration that are covered in this thesis, the overlapping neurobiology of perseveration and impulsivity and finally a look at the future directions of research into the role of the NK1 receptor.

8 General Discussion

Attention deficit hyperactivity disorder (ADHD) is a developmental disorder characterised by pervasive and impairing symptoms of inattention, hyperactivity and impulsivity (American Psychiatric Association, 1994). Prevalence estimates of childhood ADHD range from 1% to 20%, although meta-analysis of 102 studies from across the globe placed the overall figure at 5.3% (Polanczyk et al., 2007). For many years ADHD was considered to be a childhood disorder, but it is now known to pervade into adulthood in approximately 50% of cases (Schmidt and Petermann, 2009). Adoption, twin and family studies show that there is a strong heritable component of ADHD (Sprich et al., 2000; Rietveld et al., 2003; Ouellet-Morin et al., 2008), and although the candidate genes are numerous, many are related to monoamine systems (Sharp et al., 2009). Given that ADHD can be a lifelong disorder it can place major financial burden on families and society and despite many years of research into the disorder, the definitive causes are as-yet unknown. Therefore more research is needed into the aetiology of this psychiatric disorder.

There are already a number of animal models of ADHD, but none fit completely with the strict criteria for face, predictive and construct validity. The NK1 receptor knockout mouse has a hyperactive phenotype, (measured in the LDEB paradigm) that is reduced with psychostimulant treatment and underlying differences in dopamine and noradrenaline levels that are inline with proposed deficits of the condition, which led to the proposal that the NK1R-/- mouse is a model of ADHD (Yan et al., 2009, 2010). This was further supported by a genetic study of the TACR1 gene (the human equivalent of the NK1 receptor), in which four single-nucleotide polymorphisms were found to be strongly associated with ADHD (Yan et al., 2010). With this evidence, the NK1 receptor knockout mouse was a promising model of ADHD, meeting face validity with the hyperactive phenotype, predictive validity in its response to psychostimulant treatment and construct validity with the neurochemical differences, yet no studies had been done to measure impulsivity and attention, the other core symptoms of ADHD.

The aim of this thesis was to assess the characteristics of ADHD (hyperactivity, impulsivity and inattention) in the NK1R-/- mouse phenotype. Firstly by measuring activity levels of treatment naïve NK1R-/- mice as all previous recordings of activity were taken after injections had been administered. Attention and impulsivity behaviours were investigated using the 5-choice serial reaction time task (5-CSRTT). A thorough mapping of the expression pattern of the NK1 receptor, and its preferred ligand Substance P throughout the mouse brain was also conducted. With these results, the brain regions that were likely to be effected by the loss of a functional NK1 receptor and that may contribute to the behavioural phenotype of the knockout mouse in relation to ADHD could be elucidated.

8.1 Summary of key findings

The first chapter of results in this thesis set the scene by investigating the complex expression patterns of the NK1 receptor and its preferred ligand substance P throughout the mouse brain. In this immunohistochemical study, the brain regions implicated in the pathophysiology of ADHD, including the structures of the basal ganglia, were looked at in relation to the presence of the NK1 receptor and SP. Based on previous literature, dysfunction of basal ganglia circuitry, and disturbed monoamine transmission are the forerunners in the likely aetiology of ADHD and therefore will be the focus in trying to relate the underlying neurobiology of the NK1 receptor to the phenotype of the NK1 receptor knockout mouse.

Activity levels of wildtype and NK1R-/- mice were monitored over a 48-hour period in circular corridor apparatus on a 12-hour light dark cycle. Peak activity levels were found in the hours immediately following the onset of the dark phase, inline with normal circadian fluctuations. NK1R-/- mice displayed greater activity levels in the first hour after lights out, but over the entire 48-hour period there was no difference in the overall activity of wildtype and NK1R-/- mice. During the light phase, the time when experiments are ordinarily conducted, no difference in activity levels were noticeable between genotypes. Although there is elevated activity of the NK1R-/- mice for one hour time bin, these results do not indicate that the NK1R-/- mouse has a robust hyperactive phenotype.

The 5-choice serial reaction time task (used in chapters 5 and 6) was an excellent behavioural paradigm in which to further explore the proposal that the NK1R-/- mouse is a model of ADHD. Levels of attention and impulsivity can be assessed in a single test, and the difficulty of the task can be increased by elongating and varying the inter-trial interval timings (LITI and VITI respectively). These tests increase the attentional load and promote impulsive responses. NK1 receptor knockout and wildtype controls were trained over six stages of increasing difficulty to reach a stable baseline performance. During these training stages, there was no difference in the percentage accuracy, percentage of omissions, or percentage of premature responses made by the wildtype and NK1R knockout mice, nor in the latency to make a correct response. The increased number of perseverative responses made by the NK1R-/- mice at all stages of the training stages was an unexpected finding. When the attentional load of the 5-CSRTT was increased, firstly with a longer inter-trial interval time of 7 seconds, and then with a variable inter-trial interval or either 2, 5, 10 or 15 seconds more differences between the genotypes emerged. In the LITI NK1R-/- mice made more errors of omission than the wildtype controls, while in the harder VITI test, NK1R-/- mice omitted more and made more premature responses than wildtype controls, indicative of deficits in attention and impulse control respectively. The increased number of perseverative responses made by the knockout mice remained throughout every phase of the experiment. Therefore in the animals' first experience of the variable inter-trial interval test of the 5-CSRTT, the NK1 receptor knockout mouse fits well as a model for ADHD. Repeated exposure to the test parameters did lead to resolution of some of these genotype differences and thus by the imbedded no-injection test (NI-3) among the drug testing regimen the high impulsive responding of the NK1R-/- mouse was no longer present.

The aim of the second chapter of results from the 5-CSRTT (Chapter 6) was to emulate the findings from Chapter 5 by treating wildtype mice with NK1 receptor antagonists to see if the characteristics of the NK1R-/- phenotype were induced. The two antagonists (RP 67580 and L-733,060) and doses were chosen based on previous work that induced hyperactivity in wildtype mice to the level seen in the NK1R knockout mice (Yan et al., 2010). The antagonists were also tested in the NK1R-/- mice to control for any non-NK1R-mediated effects. L-733,060 had no effect on the behaviour of either genotype

and either of the two doses tested in either the LITI or VITI test, whereas RP 67580 did modify the behaviour of the wildtype mice. The lower dose (5mg/kg) of RP 67580 increased premature responding in wildtype mice compared to vehicle (but only at the 10s ITI split of the VITI data), but did not effect accuracy or omissions. The higher dose (10mg/kg) appeared to have a sedative effect as omissions and latencies increased across both genotypes. Perseverative responses remained high in the NK1 receptor knockout mice, but were not increased by antagonist treatment in the wildtype mice. The unexpected perseverative phenotype of the NK1R-/- mouse that featured so prevalently at all stages of the 5-CSRTT was the target for further exploration in the final results chapter.

Perseveration, like impulsivity, is a complex behaviour that can have different connotations depending on the paradigm used to measure it and is strongly associated with both habit formation and impulsivity. To complement the perseverative behaviour findings from the 5-CSRTT, a very different type of perseveration was measured using marble burying and burrowing tests. These two tests come under the umbrella of species-typical behaviours and both measure of digging behaviour. This is a natural instinctive behaviour that rodents perform spontaneously when presented with the opportunity (deep sawdust in the marble burying test and a substrate filled tube in the burrowing test). From the previous findings in the 5-CSRTT, we would hypothesise that the NK1R-/- mice have a perseverative phenotype and would therefore bury more marbles, and displace more stones from a burrow than wildtype mice. However, there were few genotype differences in these tests, and where differences emerged, it was the NK1 receptor knockout mice that buried and burrowed less than the wildtype controls. In this chapter the importance of background strain and breeding strategy was also investigated by testing NK1 receptor knockout mice (and the respective wildtype controls) on a backcrossed C57BL/6 background, and littermate pairs from heterozygous parents.

8.2 The role of the NK1 receptor in attention, impulsivity, and perseveration

From the experiments conducted in this thesis we can conclude that the NK1 receptor does play a role in the modulation of the symptoms associated with ADHD, yet it is impossible to ascertain a specific role for the NK1 receptor without further investigations. Below are putative suggestions as to the direct and indirect roles that the NK1 receptor could play in controlling attention, impulsivity and perseveration taking into consideration the findings from the distribution of the receptor, results from the 5-CSRTT from NK1R-/-, wildtype and wildtype mice treated with NK1 receptor antagonist and the differential findings of perseveration from the species-typical behaviours.

The high levels of expression of the NK1 receptor in the striatum and nucleus accumbens, the association of these regions with attention and impulsivity and the multiple monoaminergic innervations of these structures make them strong candidate regions for the regulation of ADHD-like symptoms via the NK1 receptor. Figure 8.1 overleaf is a schematic to summarise these interactions.

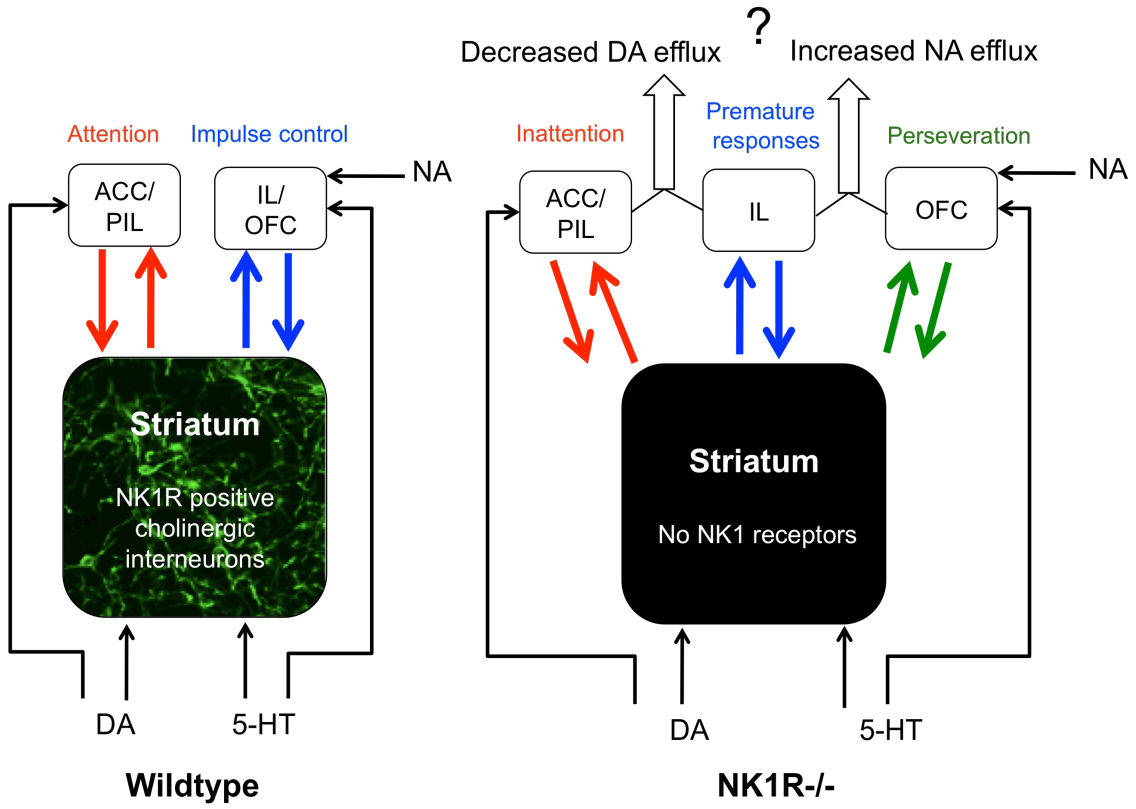


Figure 8.1 Schematic diagram to demonstrate a possible role for the NK1 receptor in the modulation of behaviours associated with ADHD. In a wildtype mouse there are multiple interactions between the monoaminergic systems and the basal ganglia, of which the striatum plays an important role in the modulation of Go/No-go signals that control attention and impulsivity. NK1 receptors are expressed on cholinergic interneurons which given their tonic activity and unique firing patterns during action selection and attentional shifts makes them prime candidates for being essential regulatory neurons of impulse control and attention. The lack of functional NK1 receptor in the NK1R-/- mouse causes ADHD like symptoms in the 5-CSRTT. This could be due to a direct consequence of the lack of NK1 receptors in the dorsal and ventral striatum, by causing an imbalance in monoaminergic control (as seen by decreased DA efflux and increased NA efflux in the frontal cortex of NK1R-/- (Herpfer et al., 2005; Fisher et al., 2007; Yan et al., 2010)).

8.2.1 Attention

The attention component of the behaviour measured in the 5-CSRTT is defined as 'top-down, spatial attention', that is, the focus is on the location of the cue light (spatial attention) and the response is driven by a voluntary behavioural goal (top-down control) (Noudoost et al., 2010). The microcircuitry of attention in the 5-CSRTT involves a loop between the anterior cingulate and prelimbic cortical regions and the dorsal striatum as revealed by excito-toxic lesion studies (Muir et al., 1996; Chudasama and Muir, 2001; Rogers et al., 2001). These cortical regions have multiple monoaminergic inputs, which would explain why drugs such as atomoxetine that increases NA levels have a positive effect on attention in the 5-CSRRT but as part of this loop, the signal must pass through the interneurons of the striatum, possibly the NK1R expressing cholinergic

interneurons. This could explain the deficit in attention of the NK1R-/- mice in the variable inter-trial interval test of the 5-CSRTT.

Cholinergic interneurons in the striatum and nucleus accumbens make up approximately 1% of the local neuronal population yet have extraordinary control over transmission of signals through the basal ganglia circuitry (Wang et al., 2006; Aosaki et al., 2010). They are of major relevance the work presented here since they co-express NK1 receptors and have strong interactions with dopaminergic transmission (Kaneko et al., 1993). Cholinergic interneurons are tonically active, with a distinct firing pattern (slow tonic firing, wide action potentials, and prominent hyperpolarisations) (Aosaki et al., 1995). Given the interlinked circuitry of the dopaminergic and cholinergic systems in the basal ganglia, it is not surprising that the activity of cholinergic interneurons has been implicated in many ADHD-like symptoms in addition to the hyperactivity mentioned above. Ding et al., (2010) conducted an elegant series of experiments in which the thalamic input to cholinergic interneurons was implicated in 'attentional-shift' and cessation of ongoing motor activity, which has direct links to the 5-CSRTT. Furthermore patients with the combined subtype of ADHD have been shown to have a higher incidence of a specific splice variant of the pre-synaptic choline transporter gene thus directly implicating the cholinergic system in the aetiology of ADHD (English et al., 2009).

However acute blockade of the NK1 receptor in wildtype mice did not impair attention in the 5-CSRTT. It could be that the chronic loss of a functional NK1 receptor as in the NK1R-/- mice has caused long-term changes in the connectivity/activity profiles of cholinergic interneurons that cannot be replicated with an acute dose of antagonist, or that the level of receptor blockade achieved in these experiments. NK1R-/- mice have a reduced number of choline acetyl transferase (ChAT) positive interneurons in the dorsal striatum compared to wildtype controls (unpublished observation), which may indicate that NK1 receptor function is necessary for either the production or maintenance of cholinergic interneurons. The disruption to cholinergic transmission in the direct and indirect pathways of the basal ganglia (Go pathway and no/go pathway respectively) could account for some of the ADHD-like symptoms observed in 5-CSRTT in the NK1 receptor knockout mouse that could not be replicated with NK1 receptor antagonist treatment.

8.2.2 Impulsivity

Dopamine and serotonin are the neurotransmitters most commonly associated with the neurological basis of impulsivity, which is covered in section 1.5.3.2 of the Introduction chapter. Although NK1R-/- mice displayed an impulsive phenotype in their first experience to the variable inter-trial interval test of the 5-CSRTT this diminished upon repeated exposure suggesting that the underlying neurobiological system has a large degree of plasticity. This mimics human data where impulsive responding of ADHD patients in the delayed-response time task is known to decrease with repeated exposure to the task (Williams and Dayan, 2005). Both hypo- and hyper-functioning dopamine systems have been reported to result in impulsivity (Sagvolden and Sergeant, 1998; Spielewoy et al., 2000; Williams and Dayan, 2005). Dopamine efflux in the PFC of NK1R-/- mice is less than 50% that of wildtype control animals (Yan et al., 2010), which could be contributing to the impulsive phenotype of the NK1 receptor knockout mouse. There is also evidence that an imbalance of serotonin levels (both increased and depleted) can lead to impulsivity (Harrison et al., 1997, 1999; Puumala and Sirviö, 1998; Dalley et al., 2002) and to complicate the picture further an interaction between the dopaminergic and serotonergic systems has been postulated to control impulsive responding (Harrison et al., 1997). NK1R-/- mice have reduced levels of the 1A subtype of the serotonin receptor (Froger et al., 2001), and substance P is abundant in the dorsal raphé nucleus (the origin of serotonergic neurons). The firing rate of serotonergic neurons in the dorsal raphé in NK1R-/- mice, and wildtype mice acutely pre-treated with RP 67580, is significantly higher than in control animals demonstrating direct negative regulation of neuronal activity by NK1 receptors (Santarelli et al., 2001).

Disruption of the cholinergic system has also been shown to effect impulsivity, with DHβE, an α4β2 nAChR antagonist decreasing impulsive responding in a simpler version of the 5-CSRTT (a 3-CSRTT) (Tsutsui-Kimura et al., 2010). Muscarinic antagonists also shift behaviour in rats from waiting longer for a larger reward, to choosing a small immediate reward in the delay-discounting test (Mendez et al., 2012). In a small clinical trial (29 adult participants) the cholinergic agonist (ABT-418) performed better than placebo in alleviating the symptoms of ADHD, especially attention (Wilens et al., 1999). Patients with Tourette syndrome, another condition that

falls under the umbrella of impulse control disorders, have decreased number of cholinergic interneurons in the striatum (Kataoka et al., 2010).

Treatment of wildtype mice with the low dose of the NK1 receptor antagonist RP 67580 increased impulsive responding in the 5-CSRTT to a comparable level of the knockout mouse. This suggests a more direct involvement of the NK1 receptor in the modulation of impulsive responding, possibly through a decrease in neuronal inhibition of the dopaminergic or serotonergic systems at the cortical level or a cholinergic basis at the level of the ventral striatum.

8.2.3 Perseveration

The neurobiology of perseveration, sometimes referred to as compulsivity, overlaps with that of attention and impulsivity, as it too involves microcircuitry loops between the pre-frontal cortex and the basal ganglia. Monoaminergic corticostriatal circuitry plays an important role since lesion of the pre-limbic cortex and medial striatum increased perseverative responding but effected no other measures in the 5-CSRTT (Chudasama and Muir, 2001; Rogers et al., 2001), while treatment with d-amphetamine increased perseveration in a dose dependent manner (Paterson et al., 2011). The serotonin system is thought to play an important role in the modulation of perseveration since agonist induced activation of 5-HT-1A receptors induced perseverative arm choices in the spontaneous alternation test, while increased expression of 5-HT_6 in the dorsolateral striatum decreases habitual lever pressing (Eskenazi and Neumaier, 2011; Rhoads et al., 2012). The NK1R-/- mice display a robust perseverative phenotype in the 5-CSRTT, which could be caused by their disrupted monoamine transmission, although it is impossible to say specifically which one without further experiments. However treatment of wildtype animals with NK1 receptor antagonists did not increase perseverative responding, making it unlikely that the action NK1 receptor is involved directly in the control of perseveration. When tested in alternative paradigms of perseverative behaviour, no difference was observed between wildtype and NK1R-/- mice, highlighting that perseveration has discrete subtypes.

8.3 Future directions

Although the results presented here have progressed our knowledge into the putative role that the NK1 receptor may play in the neurobiological basis of ADHD, there are still many unanswered questions that require further experimentation to answer. Firstly the key findings from the 5-CSRTT should be replicated either with wildtype and NK1R-/- mice from the backcrossed C57/BL6 background colony, or using littermate pairs from heterozygous pairings. This would control for the fact that the findings reported here are from wildtype and knockout mice, not only on a mixed background strain (129/Sv x C57BL/6 x MF1) in which there is a possibility of an interaction between the NK1 receptor and the parental strains, but also from colonies that were maintained for many generations by homozygous breeding, which may have resulted in genetic drift through spontaneous mutations within the separate colonies.

Further tests of impulsivity and perseveration should be conducted to try and narrow-down the specific impulsive and perseverative phenotype of the NK1 receptor knockout mouse. Given the multifaceted nature of both these behaviours, further tests such as the stop-signal task, and the Go/No-go and delay-discounting would provide insight into the nature of the impulsivity. For example, the impulsive behaviour measured in the 5-CSRTT is an inability to wait for the cue light to appear, whereas the stop-signal tasks measures ability to stop an already initiated response (Dalley et al., 2011). The above tests also incorporate measures of perseveration, while another test of perseverative behaviour can be measured in a T-maze (previously mentioned in chapter 7). This test of perseveration is highly linked with habit formation, which is attributed to the circuitry of the basal ganglia, especially the dorsal striatum (Yin and Knowlton, 2006).

Lesion studies would be a viable avenue in which to pursue this project and would provide a means to further explore the role of the NK1 receptor in causing disruption of monoaminergic transmission. By using a targeted approach to ablate NK1 receptor positive cells in discrete brain regions, rather than global disruption of the NK1 receptor as in the knockout mouse, or systemic antagonist treatment, the role of the NK1 receptor can be more precisely elucidated. Despite the all-encompassing data for attention, impulsivity and perseveration that can be obtained from the 5-CSRTT, the experiments are extremely time consuming taking approximately 6 months to complete.

For lesion studies, it would therefore be preferable to find shorter experiments that replicate the findings from the 5-CSRTT. Possible target regions for lesions given their association with ADHD the NK1 receptor would be the discrete structures of the basal ganglia including the pre-frontal cortex, striatum, nucleus accumbens, subthalamic nucleus and the substantia nigra. Ablation of NK1 receptor containing neurons in the dorsal raphe nucleus and locus coeruleus would provide insight into the role of NK1 receptor in the modulation of serotonergic and noradrenergic transmission and the role these systems play in of the aetiology of the symptoms of ADHD. An NK1 receptor specific lesion of the striatum would provide results to further the cholinergic hypothesis, although the GABAergic NOS/somatostatin/NPY-positive interneurons in the striatum also express NK1 receptors (Kaneko et al., 1994). However it must be taken into consideration that lesion studies totally ablate the cells containing the target structure, which may disrupt other systems rather than simply disrupting the function of the single receptor.

One relatively new field that could be used to further explore the cholinergic hypothesis of ADHD is that of optogenetics, where the activity of neurons can be driven or blocked by light. Selective inhibition of ChAT interneurons in the nucleus accumbens with high temporal precision has the overall effect of increasing MSN activity and blocking cocaine conditioning in freely-moving mice (Witten et al., 2010). These findings opposed those from chronic ablation of cholinergic interneurons in the NAc which reported increased sensitivity to cocaine (Hikida et al., 2001). This further highlights that chronic changes such as permanent lesions of cell populations or knockout mice can provide opposing results to temporary manipulations such as pharmacological treatment or optogenetic control of neuronal firing. (English et al., 2012) have conducted optogenetic studies of cholinergic interneurons in the dorsal striatum and report that the characteristic pause-excitation firing pattern inhibits the activity of MSNs. Simple behavioural paradigms would be required to see if manipulation of NK1 receptor positive cholinergic interneurons induced or reduced ADHD-like symptoms, of which locomotor activity would be the easiest.

8.4 Final conclusions

We must not forget that ADHD is not a monogenic disorder and perhaps by trying to study all angles of the condition in a knockout mouse of a single gene we may overlook the real worth of the model. The NK1R-/- mouse could be considered as a general model of impaired impulse control, given its impulsive and perseverative phenotype. From this angle it could be used for research into a wider range of disorders including obsessive-compulsive disorder, autism, Tourette's syndrome, addiction, as well as ADHD. Broadening the scope in which this valuable knockout mouse could be used would benefit the wider scientific community in elucidating the origin of neurological disorders that relate to deficits in impulse control.

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Detailed laboratory protocols

A1 – General laboratory solutions

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

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

810 mM Na_2HPO_4 (BDH)

Paraformaldehyde

4 % paraformaldehyde (BDH)

0.1 M phosphate buffer

Heparinised saline

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

0.9 % NaCl (Baxter, Lessines, Belgium)

Sucrose with sodium azide

30 % or 5 % sucrose (BDH)

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

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

A2 – Genotyping with PCR

PCR solutions

Alkaline Lysis Reagent

25mM NaOH

0.2mM Na_2 EDTA

Ultrapure water (18 MΩ/cm)

Neutralising Reagent

40 mM Tris-HCl

Ultrapure water (18 MΩ/cm)

dNTP mix (10 mM)

10 mM dATP (Promega)

10 mM dCTP (Promega)

10 mM dGTP (Promega)

10 mM dTTP (Promega)

PCR reaction master mix (enough for 20 samples) – keep all reagents on ice during preparation

200µl ultrapure water (18 MΩ)
51.6µl *Taq* DNA polymerase buffer (Promega)
33.3µl 25mM $MgCl_2$ (Promega)
11.1µl dNTP mix (10 mM) (see above)
27.5µl NeoF: (0.5µg/µl; Sigma Genosys, Cambridge, UK)
27.5µl NK1-F: (0.5µg/µl; Sigma Genosys)
27.5µl NK1-R: (0.5µg/µl; Sigma Genosys)
2µl *Taq* DNA polymerase (Promega) – add last

Primer Name	Primer Sequence
NeoF	5'-GCAGCGATCGCCTTCTATC-3'
NK1-F	5'-CTGTGGACTCTGATCTCTTCC-3'
NK1-R	5'-ACAGCTGTCATGGAGTAGATAC-3'

Table A1 Primer sequences used in genotyping of wildtype and NK1 receptor knockout mice

0.5x TBE buffer

30ml 10x Tris-borate-EDTA (National Diagnostics)

570ml distilled H_2O

Running buffer

500ml 0.5x TBE buffer

2µl ethidium bromide

DNA extraction from ear clipping

- Swab the ear to be clipped with ice-cold alcohol
- Take a tissue sample using a 2mm ear punch
- Place the sample in a 0.5ml Eppendorf tube and keep on ice
- Add 75µl of alkaline lysis reagent to each tube and place in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) at 95°C for 30minutes
- Allow the samples to cool to 4°C and then add 75µl of neutralising reagent to each tube, mix thoroughly
- The samples can be frozen at this point until the PCR step

NK1 receptor PCR protocol

- Add 6µl DNA sample to 19µl PCR reaction master mix and mix thoroughly
- Include extra tubes for wildtype and knockout positive controls and a negative H_2O control
- Quickly spin the tubes to draw all the contents to the bottom
- Place tubes in the PCR temperature cycler and run the NK1 programme (conditions outlined in Table A2.

Step	Temperature (°C)	Duration (min)
1	94	5
2	58	1
3	72	1
4	94	1
5	Cycle 35 times stages 2-4	
6	58	1
7	72	7
8	4	Hold

Table A2 PCR conditions for the amplification of DNA for genotyping of wildtype and NK1 receptor knockout mice

Running the gel

- Add 2g agarose to 100ml running buffer and dissolve in the microwave (do not let it boil over)
- Cool under the tap and add 8µl of ethidium bromide
- Pour slowly into the gel tray to avoid bubbles, add combs and leave to set
- Add 2µl of ethidium bromide to the remaining 500ml running buffer and pour into gel tank
- Add 4µl of 5x DNA loading buffer (Bioline, London, UK) to each sample and mix well
- Load 15µl of each sample into the wells
- Run the gel at 100-120 mV for approximately 1 hour
- Visualise and photograph under ultraviolet illumination

A3 – Immunohistochemistry

Protocol for DAB staining as used in Chapter 3

Preparation of gelatinised slides

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

Tissue preparation

Cut 40μm coronal sections on a freezing microtome and place into 5% sucrose with azide. Staining should be carried out as soon as possible after sectioning.

Solutions for immunohistochemistry

Blocking solution

0.1M phosphate buffer

3% Normal serum from the species in which the secondary antibody was raised

2% H_2O_2 to quench endogenous peroxidase activity (not required for fluorescent staining)

0.3% Triton X-100

Tris/Triton buffered saline (TTBS)

0.05M Tris base

0.3% Triton X-100

0.9% NaCl

Primary antibodies concentrations (made up in TTBS)

Rabbit anti-NK1 receptor 1:5000

Rat anti-SP 1:200

DAB staining

All steps are carried out on a rocker at room temperature unless otherwise stated

- Select relevant sections and rinse in phosphate buffer to remove residual sucrose solution
- Place the sections in blocking solution for one hour (with normal goat serum for the NK1 receptor stain and normal donkey serum for SP stain)
- Place the sections in primary antibody solutions and either incubate overnight at room temperature or for 3 days at 4°C
- 3 x 10 min washes in 0.1M PB
- Incubate the sections in biotinylated antibodies (goat anti-rabbit for NK1R and donkey anti-rat for SP) at 1:250 made up in TTBS for 90 mins
- Meanwhile, make up the avidin-biotin complex (ABC) solution using the Vectastain kit (Vector laboratories)
    - Mix 1 µl/ml Reagent A (avidin) and 1 µl/ml Reagent B (biotinylated horseradish peroxidase, HRP) with 0.1M phosphate buffer and incubate for at least 30 min to allow reaction to take place
- 3 x 10 min washes in 0.1M PB
- Incubate the sections with ABC solution for 30 mins
- 3 x 10 min washes in 0.1M PB
- Make up DAB solution using the DAB Vectastain kit (Vector Laboratories)
    - Add 2 drops buffer, 4 drops DAB, 2 drops peroxidase and 2 drops nickel to 5 ml dH2O
- Incubate sections in DAB until a suitable level of staining is achieved, normally within 3-10 min
- Stop the reaction with dH2O and transfer to 0.1M phosphate buffer for 5 min
- Rinse in 0.01M phosphate buffer before mounting onto gelatinised slides and leave to air-dry overnight.
- Dehydrate sections through dH2O, 70% ethanol (twice), 95% ethanol (twice) and 100% ethanol (twice), dipping the slides for 1 min at each stage
- Clear in Histoclear (National Diagnostics, Hull, UK) twice for 1 min
- Coverslip immediately using DPX mounting medium (BDH)

A4 – Split VITI data from antagonist 5-CSRTT experiment

Although many significant interactions were found by the 3-way ANOVA analysis (G-'genotype', I-'ITI split' and T-'time') of the data from the split VITI tests from the antagonist experiment in the 5-CSRTT, no new observations (except for a small effect of RP 67580 5mg/kg which is discussed in the main results chapter) were drawn out that had not yet been previously discussed (such as the dependence of behaviour on ITI split (Chapter 5, Section 5.3.3) or the effect of antagonist treatment on behaviour in the VITI test (Chapter 6, Section 6.3.2.2).

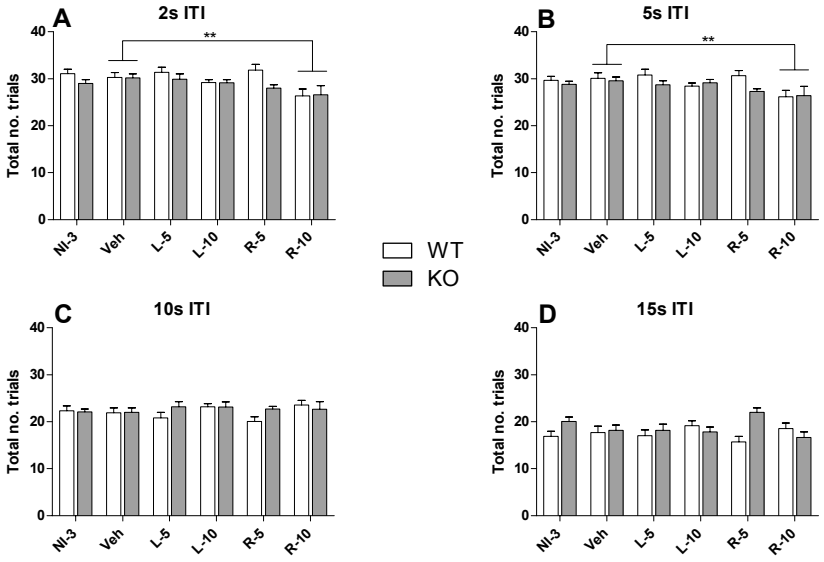

Figure A.1 Total number of trials. Main G*I*T interaction ($F_{(6.6,131.2)}$=2.88, P<0.01). RP-10 reduced the number of trials completed at 2 and 5s ITI splits. Fewer trials completed at the longer ITI splits than for shorter ITI times. This is discussed in section 5.3.3 of the thesis. WT – wildtype, KO – NK1R-/-. **P<0.01

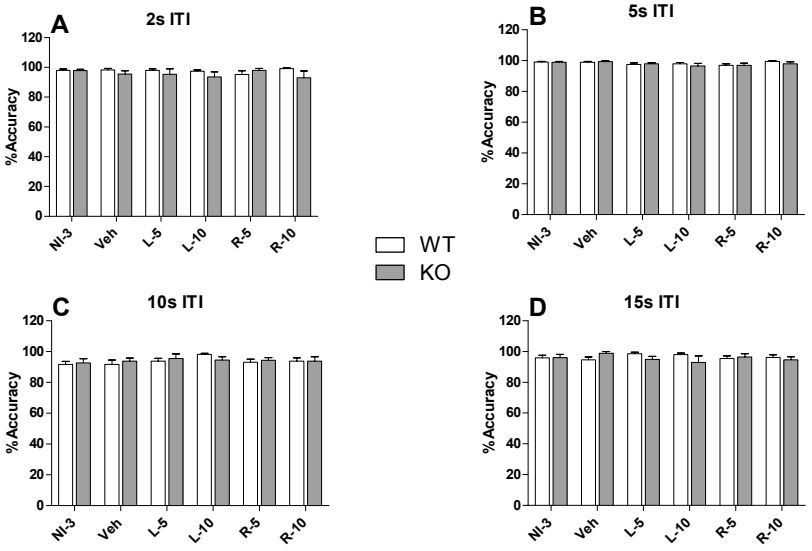

Figure A.2 Percentage accuracy. Treatment nor genotype had any effect on percentage accuracy. A main effect of ITI split revealed both genotypes were more accurate at the 5s ITI than 15s.

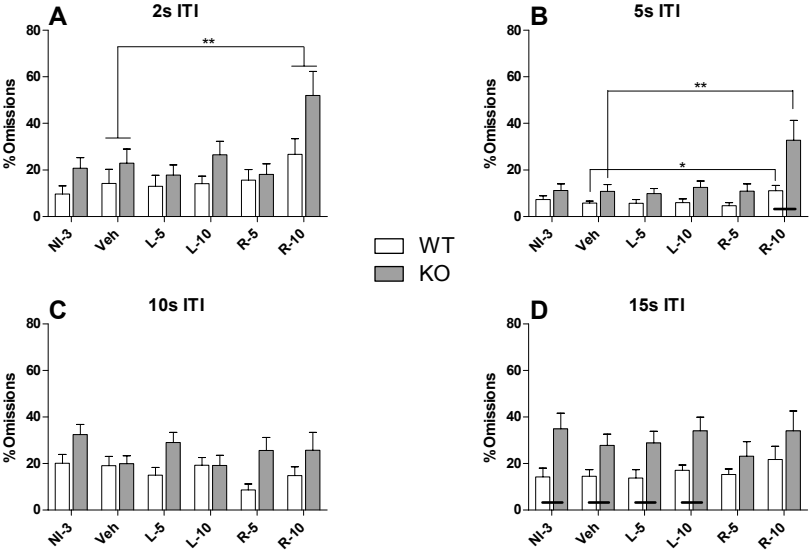

Figure A.3 Percentage omissions. A significant G*I*T interaction ($F_{(6.0,120.2)}$=2.51, P<0.05). RP-10 increased omissions in both wildtype and knockout mice at the shorter ITI splits but not in the longer ITI splits. More genotype differences were observed at the longest ITI split of 15s. WT – wildtype, KO – NK1R-/-. *P<0.05, **P<0.01. Black bar between columns represents a significant P value of <0.05 at least.

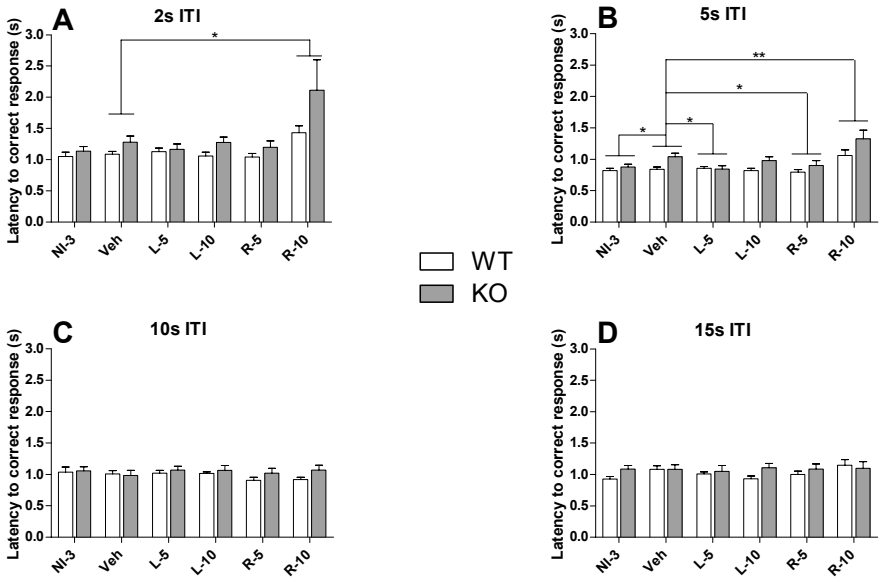

Figure A.4 Latency to correct response. Main I*T interaction ($F_{(2.1,40.4)}$=4.3, P<0.05). Antagonist treatment only had significant effects at the shorter ITI splits. WT – wildtype, KO – NK1R-/-. *P<0.05, **P<0.01.

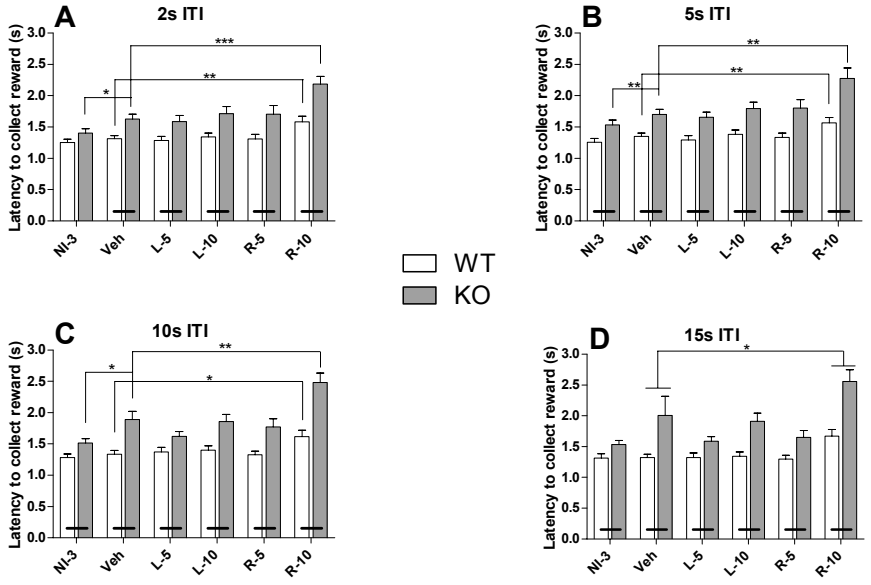

Figure A.5 Latency to magazine. Main effect of ITI ($F_{(1.7,32.2)}$=8.2, P<0.01) and a G*T interaction ($F_{(3.0,56.2)}$=4.2, P<0.01) reveal that RP-10 increased the latency to collect the reward at all ITI splits. WT – wildtype, KO – NK1R-/-. *P<0.05, **P<0.01, ***P<0.001. Black bar between columns represents a significant P value of <0.05 at least.

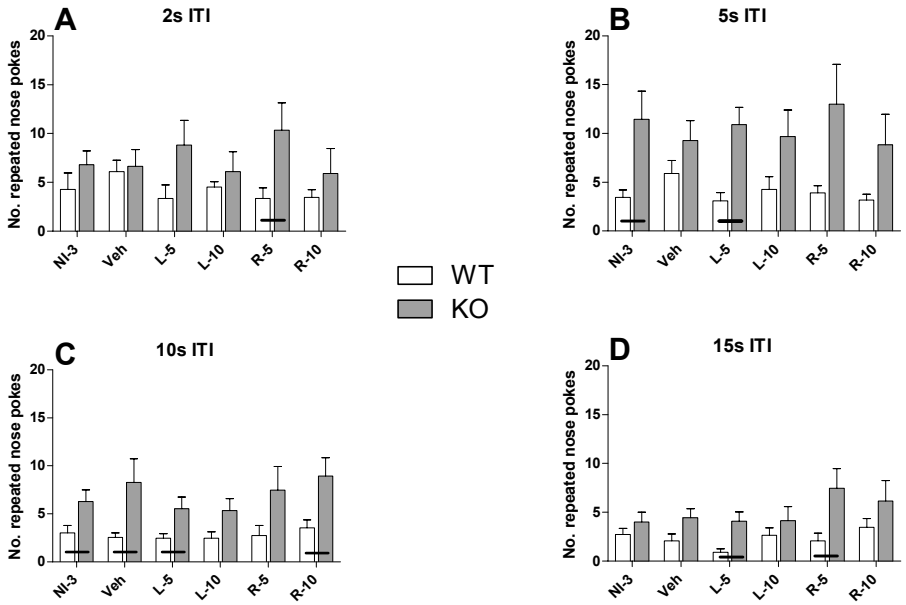

Figure A.6 Perseverative responses. Main G*I interaction ($F_{(1.8,36.5)}$=13.5, P<0.05). More perseverative responses are made at the shorter ITI splits because more trials are completed at these splits (see section 5.3.3). Black bar between columns represents a significant P value of <0.05 at least.