Predictive validity of the neurokinin-1 knockout mouse in research into Attention Deficit Hyperactivity Disorder

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I, Katharine Margaret Pillidge, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The neurokinin-1 receptor 'knockout' (NK1R-/-) mouse has been proposed as a preclinical model of Attention Deficit Hyperactivity Disorder (ADHD). Previous work from our group demonstrated that these mice are hyperactive, and display inattentiveness and impulsivity: the core signs of ADHD. The main aim of this thesis was to examine the predictive validity of the NK1R-/- mouse, by testing the effects of the ADHD treatments, guanfacine, atomoxetine and methylphenidate on ADHD-like behaviour, primarily in the 5-Choice Serial Reaction-Time Task (5-CSRTT).

Guanfacine (0.1 mg/kg) improved attention in NK1R-/- mice, but not wildtypes, and did not affect anxiety-like behaviour in either genotype in the Elevated Plus Maze. This drug also reduced impulsivity in both genotypes at a higher dose (1 mg/kg), but this was likely secondary to its locomotor suppressant effect. Further studies revealed that the low dose of guanfacine improved spatial memory in NK1R-/- mice, but not wildtypes, in an object recognition task, but this effect was not mirrored by another alpha2-adrenoceptor agonist (medetomidine, at 1 – 10 μg/kg). In contrast, atomoxetine had no effect on attention, but selectively improved hyperactivity and impulsivity in NK1R-/- mice, only, at doses of 3 and 10 mg/kg, respectively. Similarly, methylphenidate (10 mg/kg) reduced two types of impulsivity (motor impulsivity and behavioural disinhibition) in NK1R-/- mice, only, but had negligible effects on attention in either genotype, when tested in an extension of the 5-CSRTT, the 5-Choice Continuous Performance Task (5C-CPT).

Based on literature suggesting ADHD and obesity can be comorbid, and the finding that NK1R-/- mice were smaller, but ate more than wildtypes during the 5-CSRTT and 5C-CPT tasks, the second aim of this thesis was to determine whether NK1R-/- mice had underlying differences in body composition. Carcass composition analyses revealed that the fat content of mice depended on an interaction between genotype and gender, but bone density was increased in NK1R-/- mice compared with wildtypes. Furthermore, a comparison between two types of carcass composition analyses revealed that dual energy X-ray absorptiometry (DEXA) may over-estimate body fat by approximately 10%. Overall these studies consolidate the predictive validity of the NK1R-/- mouse model of ADHD, and highlight a role for NK1Rs in growth and body composition.

Publications arising from this work

Pillidge, K., A. J. Porter, J. A. Dudley, Y. C. Tsai, D. J. Heal and S. C. Stanford (2014). "The behavioural response of mice lacking NK1 receptors to guanfacine resembles its clinical profile in treatment of ADHD." ***Br J Pharmacol*** **171**(20): 4785-4796.

Pillidge, K., A. J. Porter, T. Vasili, D. J. Heal and S. C. Stanford (2014). "Atomoxetine reduces hyperactive/impulsive behaviors in neurokinin-1 receptor 'knockout' mice." ***Pharmacol Biochem Behav*** epub ahead of print.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

5-CSRTT	5-Choice serial reaction-time task
5-HT	Serotonin
6-OHDA	6-hydroxydopamine
ADHD	Attention deficit hyperactivity disorder
ADRA2A	Alpha 2A-adrenoceptor
Alpha2-AR	Alpha2-adrenoceptor
AM	Ante meridiem
AMP	Amphetamine
ANOVA	Analysis of variance
ATX	Atomoxetine
BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BSU	Biological services unit
°C	Degrees Celsius
cAMP	Cyclic adenosine monophosphate
Cm	Coloboma
COMT	Catecho-O-methyl transferase
CPP	Conditioned place preference
CPu	Caudate putamen
CV	Cardiovascular
DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine-b-hydroxylase
DEXA	Dual energy X-ray absorptiometry
DFMO	Alpha-difluormethylornithine
DNA	Deoxyribonucleic acid
DNAB	Dorsal ascending noradrenergic bundle

DOPAC	Dihydroxyphenylacetic acid
DRD	Dopamine receptor D4
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EPM	Elevated Plus Maze
ESC	Embryonic stem cell
FDA	Food and Drug Administration
FM	Fat mass
g	Gram(s)
GFC	Guanfacine
GIT-1	G protein–coupled receptor kinase–interacting protein-1
GPCR	G-protein coupled receptor
h	Hour(s)
HTR	5-Hydroxytrypamine receptor
HVA	Homovanillic acid
i.p.	Intraperitoneal
ITI	Inter-trial interval
kb	Kilobase(s)
KD	Knock-down
kg	Kilogram(s)
KO	Knockout
kV	Kilovolt(s)
L	Litre(s)
LC	Locus coeruleus
LDEB	Light dark exploration box
LH	Limited hold
LITI	Long inter-trial interval
m	Meter(s)
MAO	Monoamine oxidase
MAT	Monoamine transporter
MDD	Manic depressive disorder
mg	Milligram(s)

ml	Millilitre(s)
MPH	Methylphenidate
MRC	Medical Research Council
n	Number
NA	Noradrenaline/norepinephrine
NAcc	Nucleus accumbens
NAT	Noradrenaline transporter
Neo	Neomycin
NHE	Naples High Excitability
NI	No injection
NK1R-/-	Neurokinin-1 receptor knockout
NKA	Neurokinin A
NKB	Neurokinin B
NLE	Naples Low Excitability
NOL	Novel object location
NOR	Novel object recognition
OCD	Obsessive compulsive disorder
OROS	Osmotic release oral system
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFC	Prefrontal cortex
PM	Post meridiem
PPT-B	Preprotachykinin-B
PTSD	Post-traumatic stress disorder
R^2	Correlation coefficient, R squared
RM	Repeated measures
RMM	Relative molecular mass
ROI	Region of interest
SAD	Seasonal affective disorder
SD	Stimulus duration; Sprague-Dawley rat; standard deviation
SERT	Serotonin transporter

SHR	Spontaneously hypertensive rat
SHS	Septo-hippocampal system
SLC	Solute carrier
SNAP-25	Synaptosomal associated protein 25
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
SWM	Spatial working memory
Syn-CAM1	Synaptic cell adhesion molecule-1
TA	Tissue area
TACR1	Tachykinin receptor-1
TMD	Transmembrane domain
TTM	Total tissue mass
μA	Microamp(s)
μg	Microgram(s)
UCL	University College London
UCSD	University of California, San Diego
VEH	Vehicle
VITI	Variable inter-trial interval
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
Wi	Wistar
WKY	Wistar-Kyoto
XR	Extended release

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Chapter 1

Introduction

Chapter 1. Introduction

1.1 Attention Deficit Hyperactivity Disorder

Attention Deficit Hyperactivity Disorder (ADHD) is characterized by three core behaviours: hyperactivity, inattention and impulsivity, as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V). The signs of ADHD have been described since the 18th Century under various terminology, including 'minimal brain damage', 'hyperactivity' and 'hyperkinetic disorder' (Lange *et al.* 2010). ADHD, as it is defined today, is one of the most commonly diagnosed childhood psychiatric disorders. Moreover, the number of diagnoses is increasing: rates increased by approximately 5.5% per year between 2003 and 2007 in the USA, and this rise has been reflected in increasing prescription rates of ADHD treatment medication (Pastor and Reuben 2008). ADHD is estimated to affect between 3-5% of children worldwide, although some studies estimate up to 16% in some communities, and it is about 2-3 times more common in males than females (Willcutt 2012). The highly variable, but increasing, prevalence rates have led to some controversy surrounding ADHD, in particular over whether it is being over- or under-diagnosed. Interestingly, approximately 65% of children carry their ADHD through to adulthood, suggesting either neurodevelopmental changes occur, or there are issues with diagnosis of children in the first place (Wender *et al.* 2001; Kessler *et al.* 2005; Polanczyk *et al.* 2007). A recent study showed that approximately 2.5% of the adult population could have ADHD (Simon *et al.* 2009). This is projected to lead to a loss of approximately 144 million days of productivity per year, across 10 countries (de Graaf *et al.* 2008).

Despite the controversy surrounding ADHD, there has been a general movement towards acceptance of the phenotype as a treatable disorder. This may in part be because of the physical differences that have been identified in the brains of children with ADHD (for review see Krain and Castellanos 2006). Children with ADHD have a reduced total brain volume, compared with age- and sex-matched controlls (Castellanos and Acosta 2004). The prefrontal cortex (PFC) has been shown to be significantly smaller in children with ADHD compared with

controls and their unaffected siblings (Durston *et al.* 2004), and there have also been reports of reductions in the size of the caudate putamen, cerebellum and corpus callosum (Castellanos *et al*. 2002; Durston *et al.* 2004; Hill *et al.* 2003; Hynd *et al.* 1991). Differences in the ratios of grey to white matter have also been reported, although these have not always been consistent (Filipek *et al.* 1997; Mostofsky *et al.* 2002, but see Sowell *et al.* 2003).

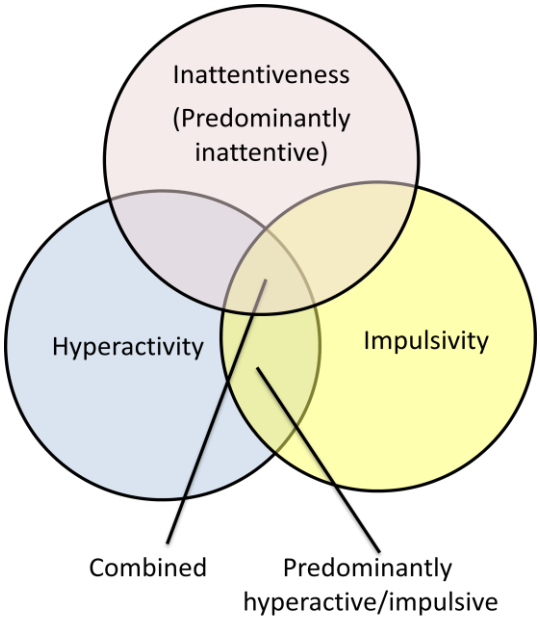

Figure 1.1 – The subtypes of ADHD, as defined by their primary characteristic.

Although significant brain abnormalities have been identified, these differences are not yet used diagnostically. Diagnosis centres around assessment of the key behaviours, hyperactivity, inattentiveness and impulsivity, and subtypes of ADHD can be specified according to the primary characteristic (see Figure 1.1) (van der Kooij and Glennon 2007). Currently, treatment medication is not determined by subtype, mostly because these subtypes are not 'fixed' and can change within an individual over time, but also because of a lack of evidence for differing treatment efficacy in different subtypes. The psychostimulant drugs, amphetamine and methylphenidate, are first-line treatments for the disorder, the latter being the most commonly prescribed medication for children with ADHD (Coghill *et al.* 2007). The prescription of psychostimulants to children provides some further controversy, although methylphenidate has a well-established safety profile and proven efficacy in reducing hyperactivity and

enhancing cognitive performance (Gibson *et al.* 2006). Methylphenidate cannot be prescribed to children under the age of 6, and has a surprisingly low abuse potential in patients it is prescribed to, given its pharmacology and action in brain areas involved in reward (Nutt *et al.* 2007). In fact, patients treated with psychostimulants are around 6 times less likely to develop an addiction to any illicit substance (Nutt *et al.* 2007). However, the potential for patients to sell their medications on to others remains. There is evidence that methylphenidate can improve cognitive performance in control subjects, particularly during fatigue or repetitive tasks (Tomasi *et al.* 2011), and thus the misuse of prescribed medication may present a real problem (reviewed by Wilens *et al.* 2008). There is also an array of literature describing cases where stimulant medication is not optimally effective in ADHD patients, leaving some cognitive deficits, or being ill-tolerated in 20-30% of children (Coghill *et al.* 2007; Heal *et al.* 2009). There remains a need to find a non-stimulant alternative, with a low abuse potential and high efficacy in treating ADHD.

There are currently three non-psychostimulant treatments approved for ADHD in the USA: atomoxetine (Strateera®), a preferential noradrenaline reuptake inhibitor, and the α2-adrenoceptor agonists, guanfacine (Intuniv®) and clonidine (Kapvay®). Although these drugs are all safe, they do not quite match up to the psychostimulants in terms of response rates (Heal *et al.* 2009). A wide range of drugs have also reportedly been used off-label to treat ADHD, including other noradrenaline reuptake inhibitors, antidepressants, Alzheimer's disease medications, monoamine oxidase inhibitors and modafinil (De Sousa and Kalra 2012). Interestingly, the mode of action of the latter is yet to be established; although it has been shown that modafinil has the ability to increase synaptic noradrenaline, serotonin, dopamine and histamine concentrations in the brain (de Saint Hilaire *et al.* 2001; Ferraro *et al.* 2002; Madras *et al.* 2006), some of these effects are almost certainly produced via an indirect mechanism or an upstream site of action, and other neurotransmitters (including GABA and glutamate) may also be involved (see Gerrard and Malcolm 2007).

1.2 The pharmacology of ADHD treatments

The psychostimulant, and non-stimulant, treatments for ADHD all act on the monoamingeric systems in the brain: amphetamine, methylphenidate and atomoxetine all inhibit the function of monoamine transporters, leading to increases in synaptic monoamine concentrations (see Table 1.1). The exception is guanfacine, which is an alpha2A-adrenoceptor agonist (for more information on guanfacine see Chapter 3).

	DAT (nM)	NAT (nM)	SERT (nM)	VMAT (μM)	Reference
d-Amphetamine (IC_{50})	400^	59$^{\#}$	>1000$^{@}$	2.1$^{\$}$	(Andersen 1989)
Cocaine (IC_{50})	690^	367$^{\#}$	389$^{@}$	-	(Andersen 1989)
Methylphenidate (K_i)	34*	339*	>10,000*	-	(Bymaster *et al.* 2002)
Atomoxetine (K_i)	1451*	5*	77*	N/A	(Bymaster *et al.* 2002)

Table 1.1 – Inhibition constant (K_i) and IC_{50} values for psychostimulants and atomoxetine at monoamine transporters. * Data from cell lines expressing recombinant DAT, NAT and SERT. Data from ^ rat striatal, # cortical and @ whole brain homogenates. Data from $ synaptic vesicles from rat striatum.

Amphetamine and methylphenidate act as competitive inhibitors of the noradrenaline and dopamine transporters (NAT and DAT, respectively; for IC_{50}/K_i values see Table 1.1). Unlike cocaine, these psychostimulants have relatively low affinities for the serotonin transporter (SERT). Atomoxetine preferentially inhibits the NAT, but has a higher affinity for the SERT than either psychostimulant.

The three aforementioned ADHD treatments prevent the normal function of monoamine transporters. Normally, according to one hypothesis, endogenous monoamines are taken up by transporters via an "alternating access" mechanism, in which a conformational change in the protein flips the transporter from 'outward facing' to 'inward facing', and transfers the substrate from the extracellular- to the intracellular matrix (Forrest *et al.* 2008). This movement is dependent on co-transport of 2 Na^+ ions and 1 Cl^- ion (Rudnick and Clark 1993; Gu *et al.* 1994).

The co-transport of substrate with inorganic ions is a mechanism which is common to a group of approximately 300 structurally related transporters, known as solute carrier (SLC) proteins

(Amara and Kuhar 1993; Hediger *et al.* 2004). The SLC6 family comprises 16 neurotransmitter transporters including the DAT, NAT and SERT (Hediger *et al.* 2004). Within these 12 transmembrane domain (TMD) proteins, amino acids in TMDs 1–3 and 9–11 are important in determining substrate affinity, whereas substrate translocation is thought to be determined by sequences in TMDs 5-8 (Amara and Sonders 1998).

Action at the translocation site of the catecholamine transporters is one way in which amphetamine differs from methylphenidate and atomoxetine. Amphetamine is a substrate for the translocation site and competes with catecholamines for reuptake: hence it acts as a competitive reuptake inhibitor. However, unlike methylphenidate and atomoxetine, amphetamine is taken up by the transporters, and once in the cytosol, causes reverse transport (retrotransport) of the catecholamines across their transporters. This increases synaptic concentrations in an impulse-independent manner: hence amphetamine also acts as a catecholamine releasing agent (for review see Sulzer *et al.* 2005; Fleckenstein *et al.* 2007).

> Amphetamine is one of a group of chemically similar compounds known as arylalkylamines (see Table 1.2). Amphetamine also belongs to a class of 'amphetamines', which includes other psychostimulants such as methamphetamine and MDMA (3,4-methylenedioxy-methamphetamine) (see Figure 1.2; this thesis refers to the compound, not the group).

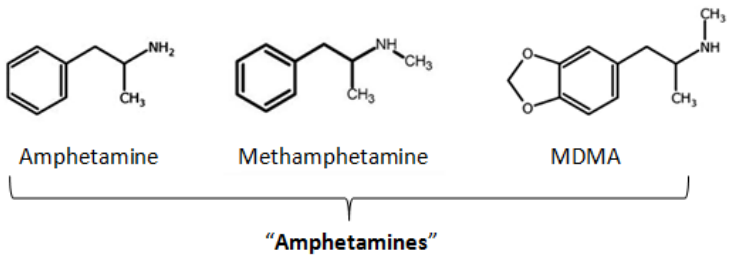

Figure 1.2 – Chemical structures of amphetamine-like compounds

Arylalkylamines				
Indolealkylamines			*Phenylalkylamines*	
N-substituted tryptamines e.g. psilocin	α-alkyltryptamines e.g. α-methyltryptamine (α-MeT)	Ergolines e.g. lysergic acid diethylamide (LSD)	Phenylethylamines e.g. mescaline	Phenylisopropylamines e.g. amphetamine

Table 1.2 – The classification of amine-containing compounds (Glennon 1999)

In addition, amphetamine is a substrate for the vesicular monoamine transporter, VMAT2. According to the 'weak base hypothesis', the basic properties of amphetamine reduce the intravesicular pH gradient caused by an excess of protons (H^+ ions) inside synaptic vesicles (Sulzer *et al.* 1992). VMAT2 relies on this acidic environment to actively transport catecholamines into the vesicles, in exchange for H^+ ions (Rudnick and Clark 1993). Therefore, vesicles storing 10^4-10^5 times the concentration of cytosolic catecholamines (Sulzer and Edwards 2000) release their contents, increasing available cytosolic neurotransmitter for transport into the synapse (Sulzer *et al.* 1995).

A third mechanism by which amphetamine can prolong increased catecholamine concentrations is by preventing their break down: amphetamine is a weak competitive inhibitor of the monoamine oxidase (MAO) enzymes, but has a higher affinity for MAO_A than MAO_B (see Sulzer *et al.* 2005). There have been proposals that, in addition to the three aforementioned established mechanisms, amphetamine also promotes dopamine synthesis and reduces DAT cell surface expression (Saunders *et al.* 2000; Larsen *et al.* 2002; Sorkina *et al.* 2003).

Despite differences in mechanism between amphetamine and methylphenidate, it is interesting to note that both psychostimulants increase catecholamine efflux *in vivo* in a similar manner: although methylphenidate is markedly less potent, there is no 'ceiling effect' with increasing doses (Kuczenski and Segal 1997; Heal *et al.* 2008). On the other hand, the profile of atomoxetine does resemble a classic reuptake inhibitor: noradrenaline efflux is increased gradually and sustained, but a maximum is reached which cannot be overcome by increasing doses (Bymaster *et al.* 2002; Swanson *et al.* 2006). It should also be noted that although atomoxetine is relatively selective for the noradrenaline transporter (Bymaster *et al.* 2002), this drug has also been shown to act as an NMDA receptor antagonist at concentrations which may

be clinically relevant (Ludolph *et al.* 2010). All three of the aforementioned drugs increase extracellular catecholamines in the prefrontal cortex (PFC), but unlike the psychostimulants, atomoxetine causes no increase in extracellular dopamine in mesolimbic brain areas of rats (Bymaster *et al.* 2002; Carboni *et al.* 2003).

Interestingly, the route of administration of these drugs may impact on their potency and behavioural effects; for example, it has been reported that orally administered methylphenidate increases locomotor activity and dopamine efflux in the nucleus accumbens of rats, to a lesser extent than the same dose administered intraperitoneally (Gerasimov *et al.* 2000). It has also been reported that methylphenidate affects reinstatement of drug-seeking in different ways depending on the route of administration (Botly *et al.* 2008).

1.3 The monoamines in ADHD

Although the cause(s) of ADHD are still not understood, all currently approved treatments for the disorder target one or more of the monoamines (Cortese 2012), indicating either that these neurotransmitters are critically involved in the aetiology of ADHD, or that they influence the characteristic behavioural signs of the condition. As discussed below, drug studies, candidate gene studies and genetic manipulations in rodents have helped to elucidate how monoamines function abnormally in ADHD.

1.3.1 Dopamine

There is good evidence that dopamine plays a role in ADHD (for review see Sonuga-Barke 2005; Swanson *et al.* 2000). There are four dopaminergic pathways in the brain; the mesolimbic pathway projects from the ventral tegmental area to the nucleus accumbens (NAcc), the mesocortical pathway projects from the NAcc to the cerebral cortex, the nigrostriatal pathway projects from the substantia nigra to the striatum, and the hypothalamic-tubero infundibular pathway projects from the hypothalamus to the pituitary gland (van der Kooij and Glennon 2007). The former two pathways, collectively known as the mesolimbocortical pathway, are thought to be involved in ADHD (Cardinal *et al.* 2004; van der Kooij and Glennon 2007).

However, there is still debate as to whether there is hypo- or hyperfunction of such pathways (Levy 1991; van der Kooij and Glennon 2007), as animal research has provided evidence for both. For example, one study demonstrated that hypofunction of mesolimbocortical circuits in rats is associated with increased impulsivity and susceptibility to drug addiction, which is noteworthy given the high level of comorbidity between unmedicated ADHD and substance abuse disorders (Dalley *et al.* 2007). On the other hand, another study found that impulsive behaviour was dose-dependently increased by infusions of amphetamine into the nucleus accumbens (Cole and Robbins 1987). These apparently opposed views could be explained by the 'dual-pathway model', which assumes differential involvement of cortical and subcortical brain regions in the expression of ADHD-like behaviour (Sonuga-Barke 2002).

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Figure 1.3 – Illustrations of the dopaminergic, noradrenergic and serotonergic pathways in the brain. Taken from Schatzberg and Nemeroff 2009.

Molecular genetics studies have corroborated the theory that dopamine function is abnormal in ADHD, by highlighting that gene mutations in the dopamine transporter (SLC6A3, aka DAT1), dopamine D_4 (DRD4) and D_5 (DRD5) receptor and catechol-o-methyltransferase (COMT) genes are associated with ADHD (Zhang *et al.* 2012). Although each of these gene associations carries small effect-sizes, this is supported by evidence that mice null for such genes display ADHD-like behaviour (Giros *et al.* 1996; van der Kooij and Glennon 2007; Gizer *et al.* 2009).

On the whole, research investigating dopaminergic agents reports changes in impulsive, reward-driven behaviour, rather than in inattentive behaviour (Robbins 2002). Furthermore, drugs which selectively target dopaminergic pathways are less effective in alleviating the signs of ADHD than those affecting all three monoamine systems. For example, the dopamine reuptake inhibitor GBR-12909 and the D_1 receptor antagonist, *cis*-flupenthixol, both had no

effect on reaction times of rats in the stop-signal reaction time test (a measure of response inhibition) (Eagle *et al.* 2007; Bari *et al.* 2009). This might imply the involvement of serotonin and noradrenaline in impulsivity to some extent.

1.3.2 Noradrenaline

There is convincing evidence to support the involvement of noradrenaline in the attentional aspects of this disorder, not least because every current treatment for ADHD directly affects extracellular noradrenaline concentrations. The main origin of noradrenergic neurones in the forebrain is the locus coeruleus (LC), which projects to a number of areas including the prefrontal cortical (PFC) regions mediating attention and working memory (Arnsten and Li 2005; Sara 2009). As such, it is this neocortical projection which is thought to be dysfunctional in ADHD. One hypothesis is that a moderate concentration of extracellular noradrenaline is concordant with optimal cognition, whereas concentrations at either end of the classic bell-shaped curve lead to worsened cognition (Aston-Jones and Cohen 2005).

Furthermore, LC neurones exhibit both tonic and phasic patterns of activity: Aston-Jones and Cohen's adaptive gain theory suggests that phasic LC activation facilitates attentive behaviour in complex, decision-based tasks, whereas tonic activation produces an increase in 'gain' (i.e. responsivity of target neurones) which underlies distraction on such tasks (Aston-Jones and Cohen 2005). In this theory, an increased release of noradrenaline at baseline increases gain, thus rendering the individual more distractible. Therefore it follows that ADHD treatments could increase acute release of noradrenaline, but lower baseline synaptic concentrations (Pliszka 2005).

Additionally, candidate gene studies have highlighted associations between single nucleotide polymorphisms (SNPs) in the noradrenaline transporter gene (SLC6A2), and gene variants of the dopamine-β-hydroxylase (DBH) and alpha2A-adrenoceptor (ADRA2A) genes, and ADHD (Bobb *et al.* 2005; Gizer *et al.* 2009).

1.3.3 Serotonin

The involvement of serotonin in ADHD is somewhat complicated by a large number of different subtypes of 5-HT receptor. Studies have highlighted the possibility that different constructs of impulsivity may be regulated by different subtypes of 5-HT receptor (Cardinal *et al.* 2004). Although there seems to be some role for serotonin in inhibitory control in particular, on the whole selective serotonin reuptake inhibitors (SSRIs) worsen the symptoms of ADHD (Verbeeck *et al.* 2009). It has been suggested that SSRI-induced increases in synaptic 5-HT levels feed forward to have a detrimental effect on dopamine function, which furthers the disruption already present in the ADHD brain (Damsa *et al.* 2004).

Nevertheless, candidate gene studies have found associations between variants of the 5-HT transporter (SLC6A4), and the 5-HT receptor genes, HTR1B and HTR2A, and ADHD. Mice null for the HTR1B gene display increased aggressive and impulsive behaviours, and do not display hyperactivity upon amphetamine treatment (Saudou *et al.* 1994; Brunner and Hen 1997). Together, these studies provide some evidence to implicate abnormal serotonergic transmission in ADHD.

1.4 Neurokinin-1 knockout mice

Much of what is known about the aetiology of ADHD comes from studies of animals which exhibit ADHD-like behaviour. Mice lacking functional NK1 receptors (NK1R-/-) have recently been proposed as one such 'model' of ADHD. Before this proposal, the NK1 receptor had been identified as a possible therapeutic target for anxiolytic and antidepressant pharmaceuticals (Rupniak *et al.* 2001; Santarelli *et al.* 2001). However, NK1R antagonists were trialled for the treatment of major depressive disorder with little success: the NK1R antagonist MK-869 (aprepitant) was effective in preliminary placebo-controlled trials, but later failed in phase-III due to lack of efficacy (Keller *et al.* 2006; Ratti *et al.* 2011). Other NK1 (L-759274, R-673, casopitant) and NK2 (M274773, saredutant) receptor antagonists were trialled but similarly fell at stages up to and including phase-III (see Quartara *et al.* 2009). Aprepitant was later

successfully developed as an antiemetic: to date it, and its prodrug (fosaprepitant), are the only NK1R antagonists in use in the clinic (Martin *et al.* 2003).

The theory that NK1R could be involved in ADHD was prompted by the finding that NK1R-/- mice exhibit locomotor hyperactivity, to a level around two-fold higher than the normal activity of wildtypes (Herpfer *et al.* 2005; Fisher *et al.* 2007; Yan *et al.* 2009). This hyperactivity is paradoxically reduced by the psychostimulants, amphetamine and methylphenidate (Yan *et al.* 2009). Furthermore, wildtype mice treated with NK1R antagonists (RP67580 and L733060) exhibit the same hyperactivity, which is also prevented by amphetamine (Yan *et al.* 2009).

Investigations into the cognitive performance and response control of NK1R-/- mice revealed that they also exhibit a higher incidence of inattentive and impulsive behaviours than wildtypes when tested in the 5-Choice Serial Reaction-Time Task (Yan *et al.* 2011). These findings together support the use of NK1R-/- mice to study the behavioural characteristics of ADHD *in vivo*. This thesis is centred on the aim of further characterising the translational aspects of the NK1R-/- mouse with respect to ADHD.

1.5 Monoamine function in NK1R-/- mice

Although the underlying changes in monoamines in ADHD are still not fully understood, rodent models of the disorder are thought to have better construct validity if they express monoaminergic abnormalities (Sontag *et al.* 2010). Neurokinin-1 receptor function influences monoaminergic neurotransmission, as evidenced by alterations in extracellular concentrations of all three monoamines in NK1R-/- mice (Herpfer *et al.* 2005; Fisher *et al.* 2007; Froger *et al.* 2001; Yan *et al.* 2009).

1.5.1 Dopamine

NK1R-/- mice have reduced extracellular concentrations of dopamine in the PFC (Yan *et al.* 2009). Evidence that systemic administration of the NK1R antagonist, RP67580, causes a reduction in dopamine efflux in the PFC of wildtype mice suggests that the hypodopaminergic state of NK1R-/- is a direct consequence of a lack of NK1R (Yan *et al.* 2009). However, in

another study the NK1R antagonist, GR205171, had no effect on dopamine efflux in this brain area (Zocchi *et al.* 2003). Moreover, dopamine efflux in the dorsal striatum of NK1R-/- mice is unchanged and systemic administration of amphetamine does not cause an increase in striatal dopamine in this brain area in NK1R-/- mice as it does in wildtypes (Yan *et al.* 2009). This is concordant with a report which suggested that infusion of an NK1R antagonist (L733060) into the striatum prevents a cocaine-induced increase in dopamine efflux in rats (Loonam *et al.* 2003).

1.5.2 Noradrenaline

NK1R-/- mice have elevated concentrations of extracellular noradrenaline in the PFC during anaesthesia compared to wildtypes, but there is no such difference in awake animals (Herpfer *et al.* 2005; Fisher *et al.* 2007). This is in accordance with findings that treatment with an NK1R antagonist (GR205171) did not induce increases in extracellular noradrenaline in the PFC of awake mice (Zocchi *et al.* 2003), rats or gerbils (Renoldi and Invernizzi 2006).

NK1R-/- mice do have a 70% reduction in [35S]GTPgammaS binding to activated α2A-ARs in the locus coeruleus, but the amount of noradrenaline transporter (NAT) protein in the PFC and LC is unchanged in these mice (Fisher *et al.* 2007). This is corroborated by the finding that the noradrenaline reuptake inhibitor, desipramine, had the same effect on noradrenaline efflux in wildtype and NK1R-/- mice (Herpfer *et al.* 2005). However, an increase in noradrenaline efflux induced by administration of RX821002 (an α2-AR antagonist), was attenuated by pre-treatment with desipramine in NK1R-/- mice, only (Herpfer *et al.* 2005; Fisher *et al.* 2007). These findings suggest that somatodendritic α2A-ARs could be functionally desensitized in the locus coeruleus of NK1R-/- mice, as a cause or consequence of increased noradrenaline efflux. Moreover, Yan *et al* (2009) suggest that a lack of functional NK1R causes excessive noradrenergic transmission during low states of arousal (i.e. under anaesthesia), but a reduction in noradrenergic transmission during high arousal states (i.e. in response to stress).

1.5.3 Serotonin

Serotonin efflux in the PFC of NK1R-/- mice does not differ compared to wildtypes (Froger *et al.* 2001), and systemic administration of an NK1R antagonist does not increase extracellular serotonin in the PFC of rats (Lejeune *et al.* 2002). However, upon treatment with paroxetine (a selective serotonin reuptake inhibitor (SSRI)), serotonin efflux is increased in NK1R-/- mice to a greater extent than in wildtypes (Froger *et al.* 2001). Similarly, paroxetine treatment caused serotonin efflux that was twice as high in NK1R antagonist (GR205171) treated mice compared to vehicle controls (Guiard *et al.* 2005).

Interestingly, treatment with the 5-HT_{1A} antagonist, WAY100635, coupled with paroxetine treatment, increased prefrontal serotonin efflux in both genotypes to the same extent (Froger *et al.* 2001). In addition, radioligand binding studies revealed that the density of 5-HT_{1A} receptor-binding was reduced in the dorsal raphe nucleus (DRN) of NK1R-/- mice (Froger *et al.* 2001). These findings suggest that 5-HT_{1A} receptors are functionally desensitized or downregulated in NK1R-/- mice, resembling the effects of chronic SSRI treatment. They further suggest that any increase in synaptic serotonin concentration is usually cleared by the serotonin transporter (SERT). Santarelli *et al* came to similar conclusions when testing NK1R-/- mice on a different background strain, suggesting the abnormal serotonergic transmission in these mice is a direct consequence of a lack of functional NK1R (Santarelli *et al.* 2001).

1.6 Substance P and neurokinin receptors

The neurokinin-1 receptor is the preferred receptor of substance P, a neuropeptide neurotransmitter which is found throughout the central and peripheral nervous system (Otsuka and Yoshioka 1993). The 11 amino acid peptide (see Figure 1.4) was sequenced in 1971, long after it was discovered in the 1930s by Von Euler and Gaddum (Von Euler and Gaddum 1931; Chang *et al.* 1971). It was originally suggested to be a sensory neurotransmitter when Lembeck discovered it was present in higher concentrations in dorsal than ventral roots of the spinal cord (Lembeck 1953). This theory was later confirmed by Otsuka and Konishi in 1975 (Otsuka *et al.* 1975).

Figure 1.4 – The amino acid sequence of the peptide, substance P.

It is now acknowledged that substance P belongs to a family of neuropeptides called tachykinins, including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). The former two peptides are translated from alternative splicing of the preprotachykinin A gene (*TAC1*), and the latter is encoded by preprotachykinin B gene (*PPT-B*) (Otsuka and Yoshioka 1993). These three tachykinins preferentially bind to three receptors, which were discovered in the 1980s (Iversen *et al.* 1982; Lee *et al.* 1982; Buck *et al.* 1984; Laufer *et al.* 1986). The order of potency of the tachykinins (see Table 1.3) was the main criterion used to define the three receptors; substance P preferentially binds to neurokinin 1, NKA to neurokinin 2, and NKB to neurokinin 3, although each also has a degree of affinity for the others (Maggi and Schwartz 1997).

Neurokinin Receptor	Preferred ligand
NK1	SP>NKA>NKB
NK2	NKA>NKB>SP
NK3	NKB>NKA>SP

Table 1.3 – Relative affinities of the tachykinin peptides for their receptors

The NK1R gene (TACR1 in humans) is 45-60 kb and is translated from five exons located in band 2, region 1, on the short arm of chromosome 2 (i.e. 2p12) in humans (Gerard *et al.* 1991). The translated protein produces a G-protein (guanine nucleotide binding) coupled receptor (GPCR) formed from 7 membrane-spanning domains (Yokota *et al.* 1989; Hershey and Krause 1990). Activation of the receptor causes G-protein subunits to trigger three independent second messenger pathways, which have a range of effects on the cell including increases in intracellular calcium, arachidonic acid mobilization and increases in cyclic AMP (see Figure 1.5) (Quartara and Maggi 1997; Roush and Kwatra 1998).

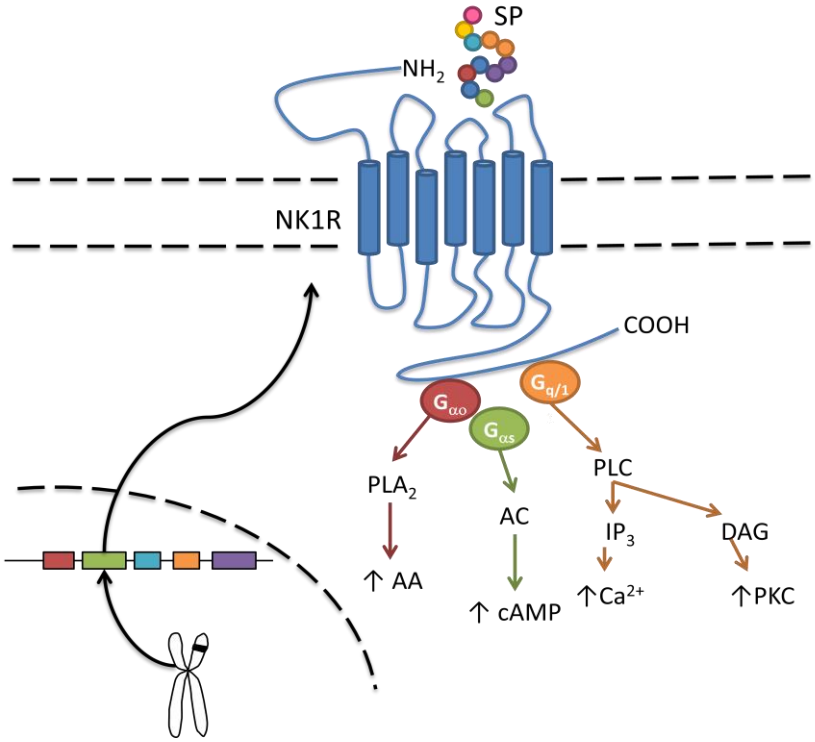

Figure 1.5 – Schematic of NK1R-G-protein coupled second messenger signalling. PLA_2: phospholipase A_2; AA: arachidonic acid; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PLC: phospholipase C; IP_3: inositol 1,4,5-trisphosphate; Ca^{2+}: calcium: DAG: diacyl glycerol; PKC: protein kinase C.

1.6.1 NK1R distribution

The location of substance P and NK1Rs in the brain is telling of their potential role in various psychiatric disorders. In the rodent brain, NK1Rs are most densely distributed in subcortical areas: the cortex and hippocampus contain relatively low receptor densities.

NK1Rs and substance P are densely expressed in the striatum, in both the caudate putamen (CPu) and the nucleus accumbens (NAcc). Both ligand and receptor are also highly expressed in the habenula, amygdala and locus coeruleus (Nakaya *et al.* 1994). However, there is a discrepancy between ligand and receptor abundance in some brain regions. For example, the substantia nigra has a high concentration of substance P, but a low density of NK1R. This mismatch could suggest that substance P diffuses through the extracellular fluid to reach areas in which NK1Rs are located, or that substance P binds to NK2/3Rs in areas lacking NK1Rs (see Herkenham 1987).

Structure	Density		Implications
	SP	NK1R	
Cerebral cortex	-	+	Higher function/cognition
Striatum	+++	++++	Habit, action selection/suppression/reward
Amygdala	+	++	Fear/anxiety
Habenula	+++	++	Affective processes
Locus coeruleus	++	+++	Arousal/attention
Substantia nigra	++++	-	Movement

Table 1.4 – SP (substance P) and NK1R densities in rat brain (modified from Otsuka and Yoshioka 1993; Nakaya *et al.* 1994).

1.7 Involvement of substance P and NK1Rs in behaviour

The behaviour of NK1R-/- mice is consistent with the localization of substance P and NK1Rs in key brain areas involved in mood, anxiety, stress and reward processing (Commons 2010; Mantyh 2002; Otsuka and Yoshioka 1993).

1.7.1 Depression and anxiety

The NK1R-/- mouse has been investigated in preclinical studies of depression (see Blier *et al.* 2004; McLean 2005), following the primary finding that NK1R-/- mice exhibit behaviour similar to wildtype mice given an antidepressant (Santarelli *et al.* 2001). This is unsurprising, given that NK1Rs are located in areas such as the habenula, amygdala and other structures in the limbic system (Otsuka and Yoshioka 1993). On a similar axis, the role of NK1Rs in anxiety has also been extensively studied. NK1Rs are particularly prominent in brain areas involved in anxiety and the stress response, including the caudate putamen, nucleus accumbens, amygdala, habenula and locus coeruleus (Otsuka and Yoshioka 1993; Nakaya *et al.* 1994). There is a substantial amount of evidence to implicate NK1Rs in emotionality, which is summarized in Table 1.5. Despite large species differences in their affinity for NK1Rs (at least 70 times lower affinity in rat/mouse compared with guinea pig/gerbil) many of these studies have utilized NK1R antagonists (Rodgers *et al.* 2004; Rupniak *et al.* 2000). However, other methods have also

been used; for example, substance P concentrations in the PFC, and NK1R expression in the amygdala and hippocampus, are increased in olfactory bulbectomised mice, which show depressive-like behaviours (Roche *et al.* 2012).

Reference	Test	Species/strain	Compound	Outcome
Teixeira *et al.* 1996	EPM	Mouse	FK888	Anxiolytic
Santarelli *et al.* 2001			RP67580	Anxiolytic
Rodgers *et al.* 2004			NKP608	No effect
Rupniak *et al.* 2001		Rat	GR205171	No effect
		Guinea pig	L-760735	No effect
Varty *et al.* 2002		Gerbil	MK-869	Anxiolytic
			L-742694	Anxiolytic
			CP-122721	Anxiolytic
			CP-99,994	Anxiolytic
File 2000; Vassout *et al.* 2000	Social interaction test	Rat	NKP608	Anxiolytic
File 1997			CGP 49823	Anxiolytic
Gentsch *et al.* 2002		Gerbil	NKP608	Anxiolytic
Cheeta *et al.* 2001			L-760735	Anxiolytic
Santarelli *et al.* 2001	Separation-induced vocalization	Mouse	RP67580	Anxiolytic/ antidepressant
Rupniak *et al.* 2001			GR205171	Anxiolytic/ Antidepressant
Rupniak *et al.* 2003		Rat	GR205171	No enantiomer selectivity
Kramer *et al.* 1998		Guinea pig	L-733060	Anxiolytic/ Antidepressant
Kramer *et al.* 1998			L-760735	Anxiolytic/ Antidepressant
Steinberg *et al.* 2002			SSR240600	Anxiolytic/ Antidepressant
Rupniak *et al.* 2001	Tail suspension test	Mouse	GR205171	No effect
		Gerbil	MK-869	Antidepressant
			L-733060	Antidepressant
			CP-122721	Antidepressant
			CP-99,994	Antidepressant

Table 1.5 – Evidence for the role of the substance P–NK1R system in anxiety and depression

Despite these seemly consistent findings, NK1R antagonists have not yet been proven to be successful antidepressants in the clinic. This is noteworthy, given that preclinical tests of 'depressive-like' behaviour have been widely criticized, and may more accurately measure

behavioural despair and the behavioural response to a change in body temperature, in the case of the tail suspension and maternal separation tests, respectively (see Rupniak 2003).

The case for NK1R antagonists having anxiolytic properties is also mixed; NK1R-/- mice display an anxiolytic phenotype on one background strain on the elevated plus maze (EPM) (Santarelli *et al.* 2001) but no such phenotype on others (Rupniak *et al.* 2001; Thorsell *et al.* 2010). Similarly, NK1R antagonists did have an anxiolytic effect in some rodent studies (Rupniak *et al.* 2001; Santarelli *et al.* 2001), but not others (Rodgers *et al.* 2004; Rupniak *et al.* 2001; see Table 1.5). Conversely, NK1R agonists (i.e. substance P analogues) had an anxiogenic effect in the EPM (Bassi *et al.* 2007) and induced a conditioned place avoidance in rats (Elliott 1988), when infused in to the dorsal periaqueductal gray and ventricles of rats, respectively. It is difficult to separate the effects of NK1R antagonists on emotionality from the effects on 'stress', as substance P has also been linked to the stress response (for review see Ebner and Singewald 2006). Swim stress and immobilization stress increase substance P release in the amygdala (Ebner *et al.* 2004), and cold stress increases substance P in the periaqueductal gray matter of rats (Xin *et al.* 1997).

1.7.2 Reward processing/addiction

Substance P and NK1Rs are expressed in the reward pathways of the brain, including in the NAcc, amygdala and VTA (Otsuka and Yoshioka 1993; Commons 2010), indicating their potential role in addiction (Schank 2014). NK1R-/- mice show a decreased preference for, and self-administer less, morphine and ethanol than wildtypes in conditioned place preference (CPP) and self-administration paradigms, respectively (Murtra *et al.* 2000; Ripley *et al.* 2002; Gadd *et al.* 2003; George *et al.* 2008; Thorsell *et al.* 2010). This effect on the rewarding properties of morphine is thought to be mediated by the amygdala: neurotoxin-induced loss of NK1R from the amygdala results in the same loss of place preference for morphine (Gadd *et al.* 2003). Interestingly, polymorphisms in the *TACR1* gene are associated with increased risk of alcoholism and sensitivity to alcohol-related cues (Seneviratne *et al.* 2009; Blaine *et al.* 2013; Sharp *et al.* 2014).

1.7.3 Cognition

The lack of NK1Rs in cortical areas of the brain is consistent with reports suggesting NK1R-/- mice have no deficit in learning and memory: the rate of learning in NK1R-/- mice does not differ from wildtypes in the Morris water maze or fear conditioning paradigms (Morcuende *et al.* 2003), and NK1R-/- mice learn complex tasks such as the 5-Choice Serial Reaction-Time Task at the same rate as wildtypes (Yan *et al.* 2011).

1.7.4 Social/sexual behaviour

NK1Rs may also be involved in social and sexual behaviours, given the distribution of substance P and NK1Rs in the hypothalamus (Otsuka and Yoshioka 1993). NK1R-/- mice of both genders display decreased preference for the other gender's pheromones, compared with wildtypes (Berger *et al.* 2012). Moreover, NK1R antagonism decreases investigation of female urine in male wildtype mice (Berger *et al.* 2012). Likewise, infusion of substance P into the medial preoptic-anterior-hypothalamic area facilitated copulatory behaviour in male rats (Dornan and Malsbury 1989). Despite these findings, de Felipe *et al* (1998) reported that NK1R-/- mice breed normally and do not show impaired maternal behaviour.

1.7.5 ADHD

Most recently, dysfunction of NK1Rs has been implicated in ADHD. NK1Rs are densely expressed in the striatum, a brain area involved in impulse and motor control (Otsuka and Yoshioka 1993). Moreover, NK1R-/- mice display hyperactivity, inattentiveness and impulsivity: the three diagnostic features of the disorder (Yan *et al.* 2010; Yan *et al.* 2011). The latter two behaviours can be tested in the 5-Choice Serial Reaction-Time Task (5-CSRTT). The 5-CSRTT was originally developed based on Leonard's choice reaction time task (Leonard 1959), and is considered an analogue of the continuous performance task used in humans (Beck *et al.* 1956). John Evenden, Mirjana Carli and Trevor Robbins sought to develop an assay of different types of attentional processes, useful for quantifying the effects of pharmacological manipulations in rodents (Carli *et al.* 1983; Robbins 2002). Although the 5-CSRTT was initially developed for the rat, versions of the task are now widely used with mice (Humby *et al.* 1999) and even zebrafish

(Parker *et al.* 2013). The 5-CSRTT (see section 2.4.1) is one of the tests used most frequently in translational research into ADHD, and is an important tool in investigating ADHD-like behaviours in rodents.

1.8 Other Rodent Models of ADHD

There are a large number of existing rodent models of ADHD (Table 1.6). The more successful animal models are valid in terms of three different aspects; face validity, construct validity and predictive validity. Good face validity describes a similar phenotype in both the animal and human condition, construct validity describes the same underlying cause and predictive validity describes a similar response to therapeutics and therefore a prediction of what new therapeutics might do in the human condition (van der Kooij and Glennon 2007).

1.8.1 Physical methods

ADHD was originally termed “minimal brain damage”, and the first model of ADHD mimicked just that: intra-cisternal administration of 6-hydroxydopamine (6-OHDA) to neonatal rat pups was used to lesion dopaminergic and noradrenergic neurones, leading to hyperactivity in postnatal days 12-22 (Shaywitz *et al.* 1978). This hyperactivity could be augmented by simultaneous lesioning of serotonergic neurones by 5,7-dihidroxytryptamine (Brus *et al.* 2004). 6-OHDA lesioned rats also show learning and memory deficits (Archer *et al.* 1988), mimicking the human condition. Although this model was the first to be proposed, it still stands as a suitable representation of human ADHD, particularly in terms of predictive validity. Methylphenidate, amphetamine and atomoxetine all attenuate the hyperactivity seen in the young rats (Shaywitz *et al.* 1978; Luthman *et al.* 1989; Moran-Gates *et al.* 2005).

A number of other neurotoxins have been tested, with little success. So-called “cerebellar stunting” by means of methylazoxymethanol, alpha-difluormethylornithine (DFMO) or dexamethasone administration, has various outcomes, including the induction of hyper- or hypoactivity in rat pups, but on the whole does not produce the attentional or impulsive characteristics seen in ADHD (Ferguson 1996). Similarly, hippocampal damage induced by X-ray

irradiation can result in hyperactivity and learning deficits in rats, but whether impulsivity is also a characteristic is yet unknown (Diaz-Granados *et al.* 1994). Administration of amphetamine improves memory function in this model, but other ADHD treatments have not been tested (Highfield *et al.* 1998).

1.8.2 Mouse Models

Coloboma mouse

One of the earliest genetically modified mouse models of ADHD to be created was the "coloboma" (Cm) mouse. This mouse has a mutation of the coloboma locus on chromosome 2, resulting in mutations in a number of genes including those coding for synaptosomal-associated protein 25 (SNAP-25) and phospholipidase C (Hess *et al.* 1994). Heterozygote (Cm+/-) mice display hyperactivity and characteristic head bobbing, together with slowed development of normal reflexes (Heyser *et al.* 1995). In terms of construct validity, Cm mice have altered DA, 5-HT and NA function: striatal 5-HT, DA (and DA the metabolites, DOPAC and HVA) concentrations are diminished, whereas striatal NA concentration is increased. Furthermore, depletion of excess NA by a specific noradrenergic neurotoxin (DSP-4) reduces the prominent hyperactivity, as does amphetamine (Jones and Hess 2003). However, methylphenidate has the opposite effect, dose dependently increasing hyperactivity (Hess *et al.* 1996). It should also be noted that this mouse has significant dysmorphology of the eye, making it difficult to utilize any task which requires visual attention. Therefore experiments demonstrating impulsivity or inattention of Cm mice, of which there are few (but for example Bruno *et al.* 2007), should be carefully assessed.

Dopamine transporter knockout/knockdown mouse

Perhaps a more relevant genetic model is the dopamine transporter knock out mouse. Since alterations to the DAT gene have been associated with ADHD, and both methylphenidate and amphetamine primarily target the DAT, it seems appropriate to study the effects of knocking down or knocking out this gene (Swanson *et al.* 1998). One major downfall of the DAT KO mouse is that testing novel therapeutics may prove inconclusive given the lack of a possible

target protein. However, this has not prevented a large body of research into the DAT knockdown (DAT KD) and knockout (DAT KO) mouse. Homozygous DAT KO mice exhibit locomotor hyperactivity at around 5-6 times that of heterozygote or wildtype activity levels: this appears to be novelty driven since KO mice are approximately 12-fold more active in a novel environment (Gainetdinov *et al.* 1999). DAT KO mice also exhibit decreased spatial memory in an 8-arm radial maze, and interestingly, also showed another (albeit not diagnostic) trait of ADHD known as perseveration (Gainetdinov *et al.* 1999).

DAT KO mice unsurprisingly show altered dopaminergic transmission: extracellular striatal DA concentrations are increased by approximately 5-fold, an increase which has a clearance rate 300 times slower than in wildtype controls (Jones *et al.* 1998). Furthermore, both amphetamine and methylphenidate attenuate the hyperactivity exhibited by these mice: this is surprising given the lack of a primary functional target of both drugs (Jones *et al.* 1998; Gainetdinov and Caron 2001). However, possible explanations are that most of the DA in the PFC is cleared by the noradrenaline transporter (NAT), and amphetamine has as some affinity for all three monoamine transporters (Moron *et al.* 2002). Blockade of the 5-HT transporter, or agonist action at 5-HT receptors, also results in a reduction of the hyperactivity, suggesting the serotonergic system may also be important in mediating the effects of psychostimulants in this mouse (Gainetdinov *et al.* 1999). However, SSRIs have limited clinical efficacy in ADHD (Verbeeck *et al.* 2009), and so this aspect of the DAT KO mouse may not be predictive of human drug treatment outcomes.

One disadvantage is that DAT KO mice exhibit growth retardation, and are more likely to die prematurely (only 68% survive past 10 weeks of age) (Giros *et al.* 1996). To overcome this problem, the DAT knock-down (KD) mouse was developed. These mice express DAT protein at about 10% of normal wildtype levels and have none of the developmental problems associated with complete DAT KO (Zhuang *et al.* 2001). However, they retain the hyperactivity in response to novelty and impaired response habituation of KO mice (Zhuang *et al.* 2001). DAT KD mice also exhibit perseverative motor patterns (Ralph-Williams *et al.* 2003) and more "risk taking" behaviour than wildtypes (Young *et al.* 2011), both of which are concordant with ADHD behaviour. Zhuang and colleagues found that both apomorphine (a D_1/D_2 receptor agonist) and

quinpirole (a D_2 receptor agonist) attenuated the hyperactivity of the mice, as did amphetamine and methylphenidate (Zhuang *et al.* 2001). This, together with microdialysis and fast-scan cyclic voltammetry data showing increased extracellular DA and reduced DA clearance, respectively, suggests that these mice are hyperdopaminergic (Zhuang *et al.* 2001). To date, DAT KO and KD mice remain the most well-established mouse models of ADHD.

New mouse models

Other mouse models have been proposed, such as the intermittent REM (rapid eye movement) sleep deprived mouse (RSD), which shows explosive jumping behaviour, relieved in part by atomoxetine, and altered noradrenergic and dopaminergic transmission (Niijima *et al.* 2010). The proposal that G protein–coupled receptor kinase–interacting protein-1 (GIT1) knockout mice (GIT1-/-) model aspects of ADHD is supported by an association between single nucleotide polymorphisms (SNPs) within the gene and ADHD (Won *et al.* 2011). These mice display hyperactivity and learning deficits, both of which are relieved by amphetamine (Won *et al.* 2011). This model is relatively new, and as such no data is available on the neurochemistry of GIT1-/- mice. One downfall of the GIT1-/- mouse is reminiscent of the DAT KO: only 50% of GIT1-/- mice survive postnatally, and surviving mice weigh 60-70% of normal wildtype mouse weight (Menon *et al.* 2010; Won *et al.* 2011). Nevertheless, preliminary data for this model look promising.

A similar proposal has been made for Syn-CAM1-/- mice. Knockout of the SynCAM1 gene (a molecule involved in cell adhesion and synaptic differentiation) results in hyperactivity and disrupted activity in the sleep-wake cycle of mice, which is attenuated by amphetamine (Sandau *et al.* 2012). The neurochemistry and other aspects of the phenotype are yet to be published but, like the GIT1-/- mouse, this model approaches ADHD from a perspective other than disrupting monoamine function, which could give further insight into the disorder.

1.8.3 Rat Models

Spontaneously Hypertensive Rat

Perhaps the most well studied and documented rodent model of ADHD is the spontaneously hypertensive rat (SHR). The original strain of Wistar-Kyoto (WKY) rats was purposefully inbred, by selecting for high systolic blood pressure (Okamoto and Aoki 1963), the result being animals in which hypertension develops at around 10-12 weeks of age. Although there are, strictly speaking, no control animals, normotensive Wistar (Wi), Wistar-Kyoto (WKY) or Sprague-Dawley (SD) rats are used as a "reference strain" in most studies of the SHR. However, studies have shown that using different reference strains can produce different results, and moreover, WKY rats themselves could display behavioural abnormalities (e.g. an abnormal stress response see Diana 2002), which limits their use as a control strain. Sagvolden and colleagues have developed this idea further, and proposed that WKY rats could model the predominantly inattentive subtype of ADHD (Sagvolden *et al.* 2009).

Interestingly, SHRs exhibit traits of ADHD before they develop hypertension. Many studies have demonstrated that SHRs are hyperactive and impulsive (Sagvolden 2000), and show learning deficits in a range of tests including the radial arm maze (Wyss *et al.* 1992) and Morris water maze (Gattu *et al.* 1997; Gattu *et al.* 1997). Far fewer studies have examined the neurochemistry. One dual-probe microdialysis study showed that SHRs had reduced noradrenaline and increased dopamine efflux in the prefrontal cortex and striatum, respectively, compared with SD rats (Heal *et al.* 2008). The same study found that amphetamine induced a greater increase in extracellular noradrenaline concentration in the PFC of SHRs than SD rats, but the same increase in synaptic dopamine concentration in both strains (Heal *et al.* 2008).

The behavioural effects of stimulants in the SHR are somewhat inconsistent: one study found that amphetamine attenuated the hyperactivity of SHRs (Myers *et al.* 1982), whereas methylphenidate is reported to be ineffective (van den Bergh *et al.* 2006), to worsen hyperactivity (Wultz *et al.* 1990; Amini *et al.* 2004; Barron *et al.* 2009) or to improve hyperactivity (Umehara *et al.* 2013). Methylphenidate was also ineffective in one test of

impulsivity (Bizot *et al.* 2007), but effective in improving attention in an attentional set-shifting paradigm (Kantak *et al.* 2008). To date, only two studies have examined the effects of methylphenidate on SHRs performing the 5-CSRTT (van den Bergh *et al.* 2006; Dommett 2014): both reported methylphenidate to be ineffective, but Dommett (2014) reported that such studies are limited by difficulties in training this strain in this task.

Other rat models

Another rat strain proposed to model ADHD-like behaviour is the Naples High Excitability (NHE) rat. NHE rats were selectively bred based on exploratory behaviour in the Làt maze (a square maze). They display greater novelty-induced activity than their counterparts, Naples Low Excitability (NLE) rats (Sadile *et al.* 1993). Both NHE and NLE rats display attentional deficits compared to Naples random-bred rats (Gonzalez-Lima and Sadile 2000), but to date there have been no reports of impulsivity in these strains. One study suggested that sub-chronic methylphenidate may attenuate the hyperactivity, but not the attentional deficits, expressed by NHE rats in the Làt maze (Ruocco *et al.* 2010). Further exploration of this phenotype in other tests of cognition may strengthen the use of this model in ADHD research.

Another method which has been used to investigate ADHD-like behaviour is to separate populations of animals into subgroups once their behaviour has been established, e.g. on a paradigm such as the 5-CSRTT. For example, rats can be selected for behaviour such as a high level of impulsivity, which is relevant not only to ADHD, but to many disorders involving impaired impulse-control. Studies have shown that methylphenidate and atomoxetine reduce impulsivity in high impulsive (HI) rats (Tomlinson *et al.* 2014), and atomoxetine improves response accuracy in poor performing but not high performing animals in the 5-CSRTT (Robinson 2012). This method of splitting animals in to different populations may be a useful tool in research, and could better represent ADHD as a heterogeneous disorder.

Model	Face Validity				Construct Validity			Predictive Validity: Amphetamine			Predictive Validity: Methylphenidate		
	Hyperactivity	**Inattention**	**Impulsivity**	**Perseveration**	**DA function**	**5-HT function**	**NA function**	**Hyperactivity**	**Impulsivity**	**Inattention**	**Hyperactivity**	**Impulsivity**	**Inattention**
NK1R-/-	✓	✓	✓	✓	**A**	**A**	**A**	✓	**x**	✓	✓	**---**	**---**
6OHDA	✓	✓	---	---	A	---	A	✓	---	---	✓	---	---
Cerebellar stunting	✓	×	×	---	---	---	---	×	---	---	---	---	---
Hippocampal lesioning	✓	✓	---	---	---	---	---	×	---	✓	---	---	---
Coloboma mouse	✓	✓	✓	---	A	A	A	✓	---	---	×	---	---
DAT KO mouse	✓	✓	✓	✓	A	A	A	✓	---	✓	✓	---	✓
DAT KD mouse	✓	---	✓	✓	A	---	---	✓	---	✓	---	---	---
RSD mouse	✓	---	---	---	A	---	A	---	---	---	---	---	---
GIT1-/-	✓	✓	---	---	---	---	---	✓	✓	---	---	---	---
SynCAM1-/-	✓	---	---	---	---	---	---	✓	---	---	---	---	---
SHR	✓	✓	✓	---	A	---	A	✓	---	---	✓	×	×
NHE/NLE rats	✓	✓	---	---	A	---	---	---	---	---	✓	---	×

Table 1.6 – A summary of rodent models of ADHD. ✓ = mimics human condition, × = contrasts with human condition, --- = unknown/not reported, A = altered neurochemistry.

1.9 ADHD comorbidities

It is estimated that two thirds of children with ADHD also have a comorbid condition: ADHD is frequently comorbid with conditions such as substance abuse, mood, anxiety and bipolar disorders and Tourette's syndrome (Madras *et al.* 2005). Figure 1.6 summarizes some of the key areas of overlap between ADHD and other conditions. These comorbidities can not only complicate diagnosis, but can influence treatment indications: for example, stimulants can worsen tics, whereas guanfacine can alleviate them (Pringsheim and Steeves 2011). Moreover, medication may prevent comorbid disorders developing: numerous studies have shown that untreated ADHD is a risk factor for substance abuse disorders in adulthood (for review see Wilens *et al.* 2003). This thesis will focus on ADHD with comorbid obesity.

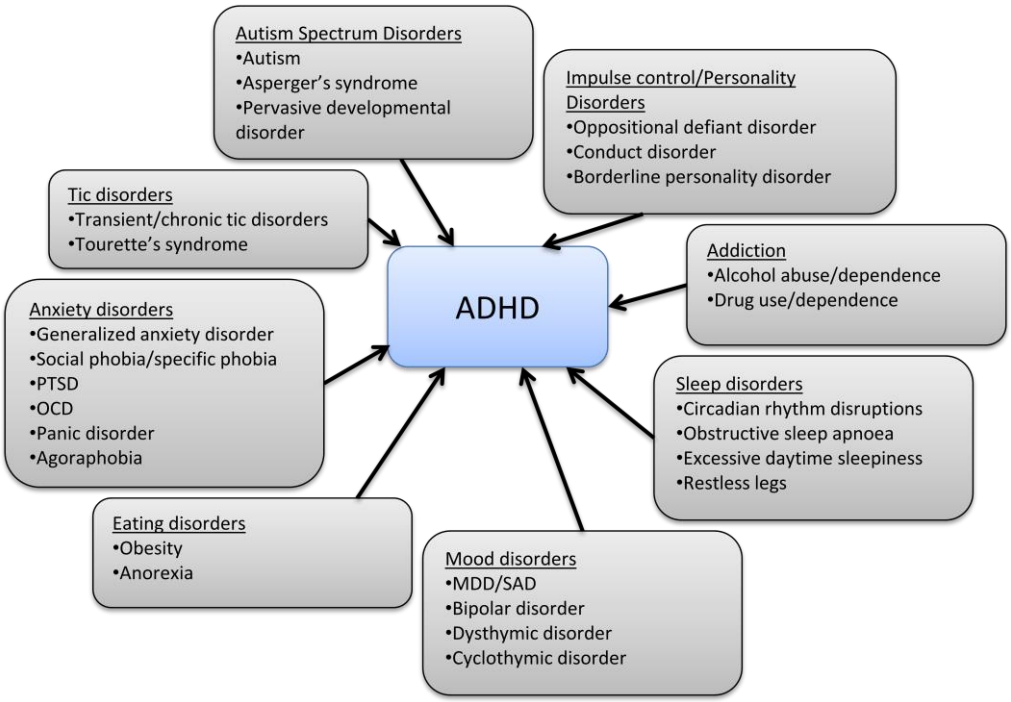

Figure 1.6 – ADHD comorbidities: adapted from Kooij *et al.* 2012. MDD; manic depressive disorder, SAD; seasonal affective disorder, PTSD; post-traumatic stress disorder, OCD; obsessive compulsive disorder.

1.9.1 ADHD and obesity

There are strong, albeit counterintuitive, links between ADHD and obesity. Findings have not always been consistent, yet in the majority of studies in which the weight status of unmedicated ADHD patients was monitored, the results suggest that ADHD patients have a higher incidence of being overweight or obese. There is also a strong treatment effect, which is to be expected, as psychostimulants are renowned for decreasing appetite. In one study of 98 children with ADHD, 23% of unmedicated patients were overweight, compared with just 6.3% of patients using pharmacotherapy (Curtin *et al.* 2005). Other studies on unmedicated patients have reported similar findings: Hubel *et al* found a higher BMI-SDS (body mass index standard deviation scores, or *z*-scores) in ADHD than in the control group of 8-14 year old boys, and Anderson *et al* found ADHD patients had higher BMI *z*-scores than controls (Anderson *et al.* 2006; Hubel *et al.* 2006). Vice versa, studies have also found that obese patients have a higher incidence of ADHD (Altfas 2002; Erermis *et al.* 2004; Agranat-Meged *et al.* 2005; Fleming *et al.* 2005). This bidirectional comorbidity could be explained by a number of theories;

1) The characteristics of ADHD (impulsivity/inattention) contribute to a dysregulated eating pattern, i.e. ADHD leads to obesity

2) Factors associated with obesity contribute to ADHD-like symptoms, i.e. obesity leads to ADHD

3) ADHD and obesity share common biological underpinnings, i.e. both are caused by the same dysfunction of the brain

4) ADHD and obesity coincide (i.e. occur in the same individuals), but are not related by aetiology.

The neurobiology of obesity is complicated, as large numbers of hormones, gut peptides and neurotransmitters are involved in the regulation of food intake and body composition. However, of particular relevance to this thesis is the involvement of the monoamines.

There is plenty of evidence to implicate the monoamines in the regulation of food intake and energy homeostasis. Historically, the use of anti-obesity agents targeting monoaminergic

systems began decades ago: the anorectic properties of benzedrine were first reported in the 1930s, and drugs including amphetamine, methamphetamine, phentermine and benzphetamine were approved for the treatment of obesity before the 1960s (Nelson and Gehlert 2006). More recently, sibutramine (a selective combined serotonin and noradrenaline reuptake inhibitor) has been used as an effective anti-obesity drug. Sibutramine was withdrawn in 2011, due to its propensity to increase the risk of heart attack and stroke in patients with cardiovascular (CV) disease (James *et al.* 2010): an important consideration given the association between CV disease and obesity, and one main reason most monoamine uptake inhibitors are not successful in the clinic (see Nelson and Gehlert 2006).

The relative contributions of serotonin and noradrenaline to the effects of anorectic drugs such as sibutramine are still unclear. Fluoxetine (a selective serotonin reuptake inhibitor) can decrease feeding and weight gain in rodents (Wong *et al.* 1988; Gamaro *et al.* 2008), but has little effect when used chronically in the clinical treatment of obesity (Ward *et al.* 1999; Suplicy *et al.* 2014). Phentermine (a preferential noradrenaline reuptake inhibitor) has been used as an anti-obesity drug for many years, and does appear to be effective (Kim *et al.* 2006; Kang *et al.* 2010). However, the combination of uptake inhibition of both serotonin and noradrenaline could be key to the anorectic effects of stimulant/antidepressant drugs. One study of food intake in rats found that preventing reuptake of serotonin or noradrenaline alone, with fluoxetine or nisoxetine, respectively, did not reproduce the anorectic effects of sibutramine, but administration of both drugs together, did (Jackson *et al.* 1997).

The role of dopamine has also been the subject of some interest. It has been suggested that dopamine is critical in mediating the rewarding properties of food: there are multiple lines of evidence which support a hypothesis that obesity is a result of disrupted dopamine-regulated reward circuits. In studies using positron-emission topography (PET) scanning techniques, DAT availability in the striatum of healthy volunteers is negatively correlated with BMI (Chen *et al*., 2008), and D_2 receptor density is decreased in obese individuals compared with healthy controls (Wang *et al.* 2001): a change which also correlates with increased BMI, and is reminiscent of a similar decrease observed in drug-addicted individuals (see Wang *et al.* 2004). Likewise, striatal D_2 receptor availability is lower in obese Zucker rats than in lean controls

(Hamdi *et al.* 1992; Thanos *et al.* 2008) and $D_{2/3}$ receptor availability negatively correlates with body weight and cocaine preference in outbred Sprague-Dawley rats (Michaelides *et al.* 2012). This, and evidence that basal and electrically evoked striatal dopamine release from brain slices of obese *ob/ob* mice is lower than that from wildtypes (Fulton *et al.* 2006), suggest that striatal dopaminergic brain circuits could be critically involved in the regulation of food intake and the development of obesity.

Interestingly, it has also been suggested that the NK1R/substance P system also plays a role in energy homeostasis: for instance, a peripheral-acting NK1R antagonist (CJ-12,255) reduced weight gain, insulin and leptin levels in mice fed a high fat diet (Karagiannides *et al.* 2008). Given the comorbidity between ADHD and obesity, and the possible involvement of NK1Rs, investigations into the weight and body composition of NK1R-/- mice could be useful in determining whether the *TACR1* polymorphism is important in this disorder with respect to obesity.

1.10 Aims of the thesis

The aims of this thesis were broadly divided into two parts: the first aim was to test the predictive validity of the NK1R-/- mouse as preclinical 'model' of ADHD-like behaviour, and the second aim was to investigate whether the weight and body composition of NK1R-/- mice could give insight into the overlap between ADHD and obesity.

The specific aims were;

1. To investigate whether the ADHD treatments, guanfacine, atomoxetine and methylphenidate, alleviate the three core ADHD behaviours displayed by NK1R-/- mice, in the light-dark exploration box and the 5-Choice Serial Reaction-Time Task
2. To investigate whether any changes in behaviour induced by guanfacine, atomoxetine and methylphenidate in these tests could be secondary to changes in emotionality or arousal/motor activity
3. To determine whether the weight and body composition of NK1R-/- mice differs from wildtypes, on a normal diet and on a high fat ('Western'-style) diet.

Chapter 2

General materials and methods

Chapter 2. General materials and methods

2.1 Introduction

This project examined the behaviour of wildtype and NK1R-/- mice in paradigms designed to study their cognitive and emotional responses to pharmacological manipulations. Two of the core behaviours (inattention and impulsivity) crucial for diagnosing ADHD are examined in the 5-Choice Serial Reaction-Time Task (5-CSRTT), a method which was designed as a translational tool for studying human ADHD behaviour in rodents. Other behavioural tests which did not require extensive training of animals were used to assess hyperactivity, emotionality and cognition.

In the second half of this project, the body composition of NK1R-/- and wildtype mice was compared in mice maintained on a normal diet, and those fed a high fat diet. The food intake of wildtype and NK1R-/- mice over the duration of the 5-CSRTT experiments was also examined.

2.2 Animals

All experimental procedures complied with the Animals (Scientific Procedures) Act, 1986 (UK) [2010/63/EU], and received local ethical approval at University College London. Experiments with animals were conducted by a person licenced and trained to carry out these procedures (Katharine Pillidge; PIL: 70/23865), under the appropriate project licence (PPL: 70/6886).

Only mice were used in this project. Separate cohorts of animals were used for each experiment, unless otherwise specified. All behavioural experiments were carried out in dimly lit, sound-attenuated rooms. Cages of animals were brought to the experimental room and allowed to habituate to the new environment before the experiment began (see section 2.4). Animals were kept in the experimental room for the duration of testing.

2.2.1 Husbandry

Only mice bred at University College London were used in this project. Mouse colonies were held in a facility at 21 ± 2°C, 45 ± 5% humidity, with a 12 h light/dark cycle (lighting increased gradually from 07.00 - 8.00 AM). Mice were group housed (cages of 2-5) and were given *ad libitum* access to food (2018 global Rodent Diet, Harlan) and water throughout, except where specified. The home cages incorporated environmental enrichment (cardboard tunnels, and nesting material (Aston-Pharma, London)) and were cleaned twice weekly (bedding: Litaspen Premium (Lillico)). Mice were used at 6 – 14 weeks of age at the start of all experiments, unless otherwise specified. Male mice were used in the behavioural tests, but males and females were used in the body composition analyses. This was because hormonal changes can influence the behaviour of females (for example, see Marcondes *et al.* 2001), and so larger numbers of mice would be needed to counteract the variability induced by these fluctuations. This was avoided in keeping with the aims of the NC3Rs.

2.2.2 Generation of NK1R-/- mice

The NK1R-/- mouse line was originally generated by homologous recombination of genetically modified 129/Sv mouse embryonic stem cells (ESC) into C57Bl6 blastocysts (de Felipe *et al.* 1998). The NK1R gene was disrupted by insertion of a cassette containing an internal ribosome entry site, *lac*Z coding sequence, and a neomycin resistance gene expressed from its own promoter, into the *Stu*I site in exon 1 of the gene (see Figure 2.1). Two targeted ESC clones were injected into C57Bl6 blastocysts, and chimeric male offspring were mated with C57Bl6 females. The resulting heterozygous (NK1R+/-) C57Bl6 mice were then crossed to produce homozygous offspring (see Figure 2.2). NK1R-/- mice were then backcrossed with C57Bl6 mice for 10 generations.

Figure 2.1 – Disruption of the NK1R gene, taken from (de Felipe *et al.* 1998).

NK1R-/- mice were then backcrossed once on to an outbred MF1 strain (Harlan OLAC, Bicester, UK) resulting in mice with a genomic make-up of 25% 129/Sv, 25% C57Bl6 and 50% MF1. Homozygous wildtype and NK1R-/- mice from this colony have been bred separately for many generations. Recently, wildtype and NK1R-/- mice were re-crossed, to produce heterozygous offspring. Heterozygous mice were mated to create wildtype and NK1R-/- littermates, which have been used to study the effects of interactions between NK1R and early life environment.

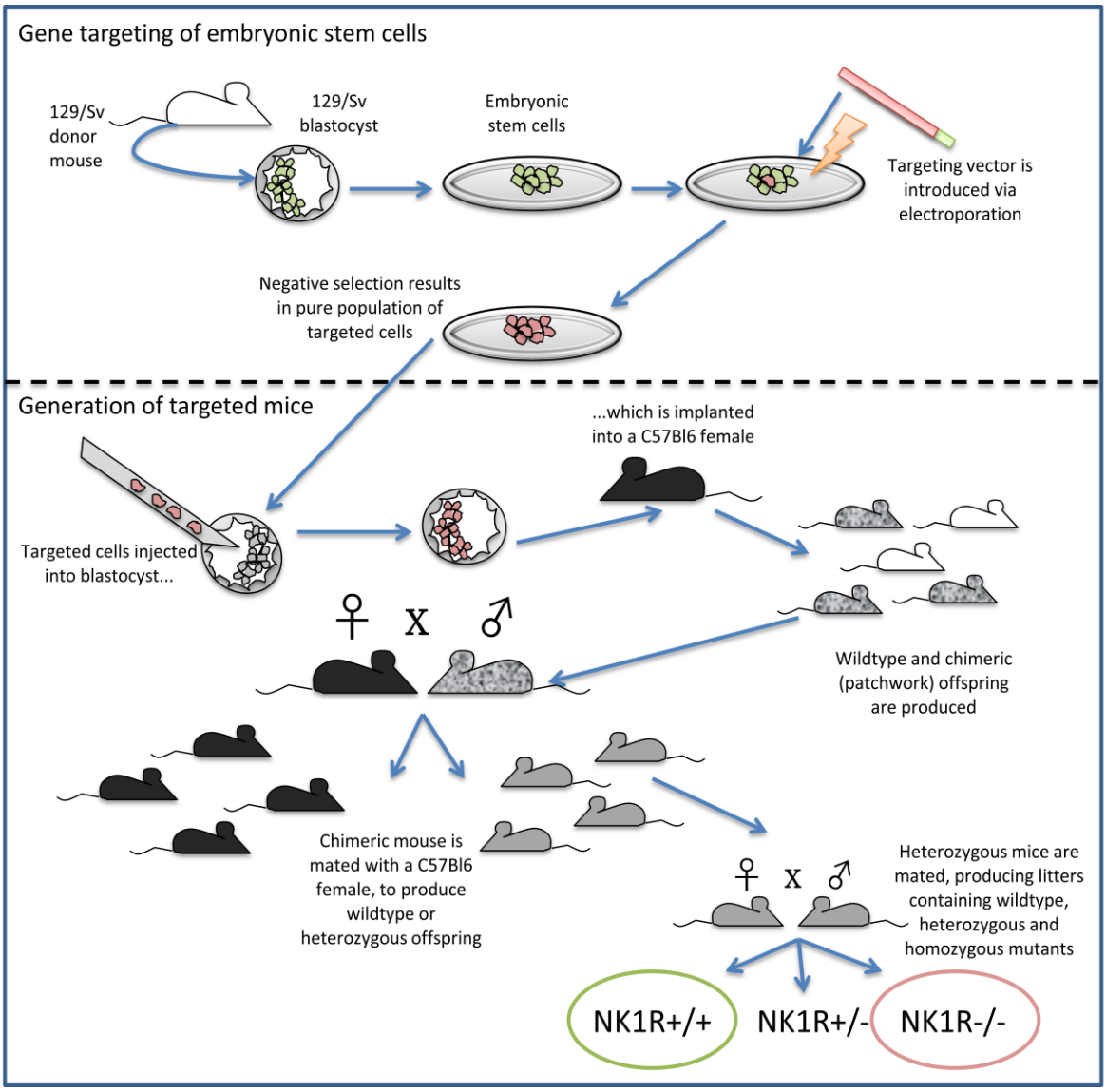

Figure 2.2 – Generation of genetically modified NK1R-/- mice.

In summary, three colonies (all with a mixed 129/Sv/ C57Bl6/ MF1 background) have been used in this project;

1. Homozygous wildtype (NK1R+/+) mice from NK1R+/+ parents

2. Homozygous 'knockout' (NK1R-/-) mice from NK1R-/- parents

3. Wildtype (NK1R+/+) and 'knockout' (NK1R-/-) littermates from heterozygous (NK1R+/-) parents

Mice bred from homozygous breeding pairs were used in all experiments. The use of NK1R-/- and wildtype littermates from heterozygote breeders was limited to the 5-CSRTT experiments, and is detailed in the corresponding chapters. Sires and dams were mated between the age of 2 and 8 months in all colonies, and allowed to have a maximum of 7 litters. Litter offspring were weaned at 3 weeks.

2.2.3 Genotyping

Mice from heterozygous parents were routinely genotyped at weaning. DNA from ear notches was used with a standardised polymerase chain reaction (PCR) protocol. Primers were used to amplify both the NK1R gene (NK1-F (forward) and NK1-R (reverse)) and the neomycin gene inserted via the cassette (NeoF and NK1-R), giving bands of 350 and 260 bases, respectively. For full genotyping methods see Appendix 1.

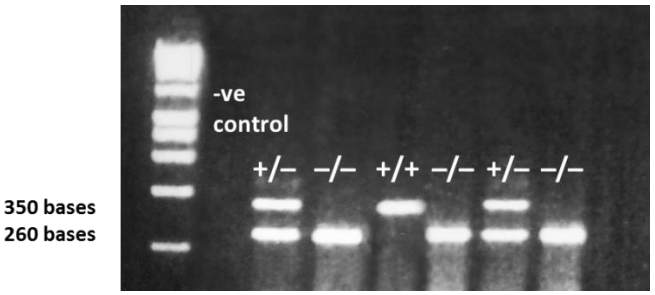

Figure 2.3 – Example of a gel showing bands at 260 bases for NK1R-/-, 350 bases for wildtype (+/+) and both for heterozygous (+/-) mice. The negative (-ve) control was a mix of primers without any DNA, to control for contamination.

2.3 Drugs

Drugs used in experiments described in Chapters 3-6 were guanfacine hydrochloride (Sigma Aldrich, Dorset, UK), atomoxetine hydrochloride (Tocris Bioscience, Bristol, UK), medetomidine hydrochloride (Domitor 1mg/ml, Pfizer Limited, UK) and methylphenidate hydrochloride (Sigma Aldrich, Dorset, UK). All of these drugs were dissolved in sterile saline (0.9% NaCl solution) and injected in a volume of 10ml/kg throughout.

2.4 Behaviour

2.4.1 5-Choice Serial Reaction Time Task

Development of the 5-CSRTT

Robbins and colleagues originally developed the 5-CSRTT using a 'nine hole box' version of the same test, with different configurations to find one which could quantify different aspects of cognition (Carli *et al.* 1983; Robbins 2002). The 5-CSRTT is now used to measure sustained attention, though it can be modified to test for selective or focused attention by the addition of interfering stimuli.

Food deprivation

The 5-CSRTT is an operant procedure: mice are given positive reinforcement in the form of a food reward when they respond correctly. In order to motivate mice to respond for this food reward, animals used in the 5-CSRTT were food restricted to 90% of their free-feeding weights. This restriction was induced gradually, and maintained carefully over the duration of the experiment.

Previous measurements by the lab found that mice eat an average of 5.2 g of food per 24 h. This was used to estimate how much a mouse eats per hour, and so formed the basis of food deprivation.

On day 1 of the experiment, mice were weighed to determine their free-feeding weight, and food was removed from the cages. During the first 4 days, mice were given a pre-determined mass of food to induce the deprivation gradually (see Table 2.1). Mice were not trained in the 5-CSRTT during this first week. From the second week and throughout the study, mice were weighed every weekday morning before training/testing in the 5-CSRTT, and fed daily at 16:00 h.

Day	Food given (g per mouse)
1	4.75
2	3.90
3	3.45
4	3.00

Table 2.1 – Food deprivation over the first week in the 5-CSRTT.

After the first 4 days, feeding was adjusted and tailored to each cage of mice, in order to maintain mice at 90% of their original body weights. If a mouse's weight dropped to below 90%, the mouse was allowed *ad lib* feeding for 30 min for every 0.5 g under its target weight. This food was given after the mouse had completed its 5-CSRTT session for the day. If a mouse was underweight and deemed to be unwell, it was culled immediately. This was a rare occurrence, but details of mice that did not complete the study are given in the relevant chapters.

Apparatus

The apparatus, supplied by Med Associates (St. Albans, VT, USA) comprises four sound attenuated operant chambers with five equally-spaced apertures, incorporated in the left wall (see Figure 2.4). These holes can be illuminated independently, and interruption of an infrared beam across the hole scores 'nose-pokes' into each one. A magazine in the right wall, where rewards are delivered, can also be illuminated independently. Interruption of an infrared beam across the magazine initiates a 'trial'. The procedure was controlled by a Smart Ctrl Package 8IN/16OUT with an additional interface by MED-PC for Windows (Med Associates, St. Albans, VT, USA).

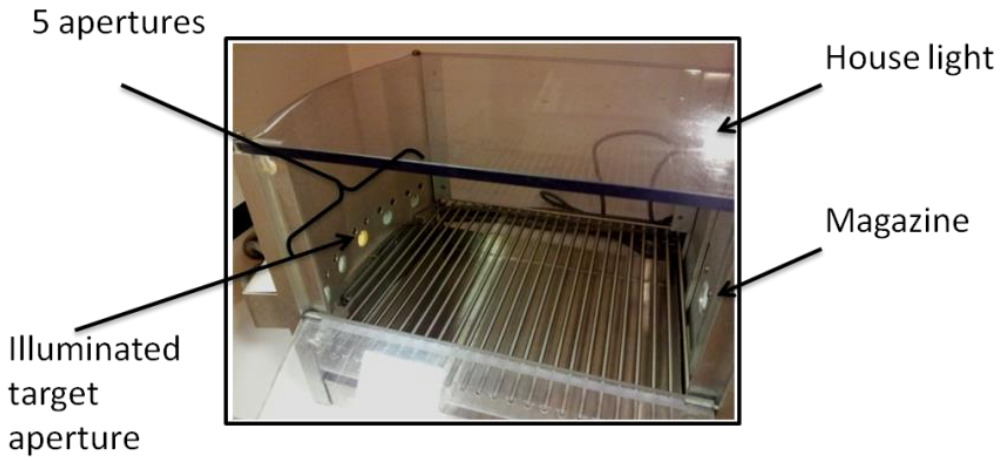

Figure 2.4 – A 5-CSRTT operant chamber.

Behaviour in the 5-CSRTT

A trial begins when the mouse nose-pokes into the magazine. One of five lights is then illuminated, signalling that the mouse should nose-poke into the illuminated aperture. There are 3 possible behavioural responses to the presentation of the cue light (see Figure 2.5). Mice can nose-poke into the illuminated target hole (correct), one of the other 4 un-illuminated holes (incorrect), or not nose-poke at all (omission). Mice may also nose-poke into one of the 5 holes before any light stimulus is presented (premature response), which does not count towards the number of trials in the session. After an incorrect, omitted or premature response, the mouse is 'punished' with a 10 s timeout period (house light off), during which nose-pokes into the magazine have no effect. The mouse must then initiate the next trial by nose-poking into the magazine when the house light is turned back on. After a correct response, the mouse is rewarded with the delivery of 0.01 ml of 30% sweetened condensed milk solution at the magazine, which in itself initiates the next trial. The sequence continues until 100 trials have taken place, or the allocated time for that session is up.

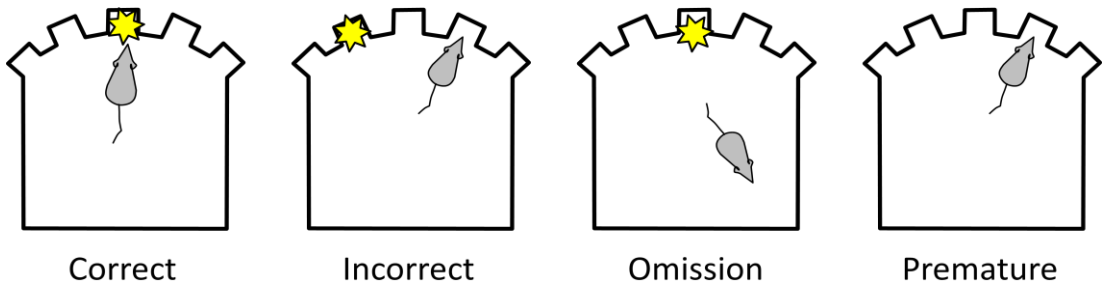

Figure 2.5 – Behavioural responses to the presentation of a light cue in the 5-CSRTT

After the session, the number of correct, incorrect, omitted and premature responses are used to calculate the 7 key variables (listed in Table 2.2).

Behavioural Outcome	Method of calculation
Total number of trials completed	Total correct responses + total incorrect responses + total omissions
% Accuracy	[correct responses/(correct + incorrect responses)]×100.
% Omissions	[total omissions/total number of trials]×100
Premature responses	Premature responses per 100 trials
Latency to correct response	Duration between onset of stimulus and a nose-poke in the correct hole
Latency to collect the reward	Duration between a nose-poke in the correct hole and collection of reward from the magazine
Perseverative responses	Number of unnecessary responses into the correct hole after the initial correct response, before collection of reward, per 100 trials

Table 2.2 – Behavioural outcomes in the 5-CSRTT

Training/testing

For mice to learn how to navigate the 5-CSRTT correctly, substantial training is required. Mice were first habituated to the apparatus for three days (for details see Table 2.3). Thereafter, training began, and mice graduated through Stages 1 – 6 after fulfilling the criteria for the given stage. The parameters of the test (below) were changed to make the stages increasingly challenging;

Stimulus duration (SD) - the length of time the aperture is illuminated for – *decreases.*

Limited hold (LH) – the time after the target light is extinguished in which the mouse can still nose-poke to achieve a correct response – *decreases.*

Inter-trial interval (ITI) – the time between initiation of a trial by magazine head-entry, to illumination of the target aperture – *increases*.

Each training session lasted 30 min, unless the animal reached a total of 100 trials plus *premature responses* before that time, at which point the session was terminated. Mice were assigned to one of the four test chambers in a pseudo Latin-square design, and were run in the same box throughout. After reaching the criteria for a stable baseline on Stage 6 for at least 3 consecutive days, mice were eligible for testing.

A variable inter-trial interval (VITI; 2, 5, 10 or 15 s (delivered on a random schedule)) was used to test the mice. Mice were tested once weekly using the VITI (on Fridays) only if a stable baseline had been achieved on the 3 preceding consecutive days (Tues – Thurs). The first week of testing was with treatment-naive mice ('NI-1') only. In the following 5 weeks, mice were subject to VITI tests, 30 min after treatment with an intraperitoneal (i.p.) injection of drug or vehicle, or a second un-treated session ('NI-2'). The drug/vehicle/ NI-2 sessions were counterbalanced across subjects, using a pseudo Latin-square. Each test session lasted 45 min, unless the animal reached a total of 100 trials plus *premature responses* before that time, at which point the session was terminated. Performance variables (listed in Table 2.2) were scored by the MED-PC program and stored online.

Stage	Conditions			Criteria for Passing	
Habituation	30% condensed milk solution available on nose-poke into magazine.100% condensed milk placed inside all 5 holes on day 1 & 2. Magazine and hole-lights illuminated throughout			None	Mice advanced to next stage after 3 days
Habyol (a non-spatial version of 5-CSRTT)	All 5 holes illuminated constantly Liquid reward available from magazine upon nose-poke into any of the 5 holes			>50 rewards	For 2 consecutive days Maximum of 10 days before progression regardless of performance
	SD	**LH**	**ITI**		
Stage 1	30s	30s	2s	>30 correct responses	For 2 consecutive days
Stage 2	20s	20s	2s	>30 correct responses	For 2 consecutive days
Stage 3	10s	10s	5s	>50 correct responses	For 2 consecutive days
Stage 4	5s	5s	5s	>50 correct responses >75% accuracy <25% omissions total trials – premature >100	For 2 consecutive days
Stage 5	2.5s	5s	5s	>50 correct responses >75% accuracy <25% omissions total trials – premature >100	For 2 consecutive days
Stage 6	1.8s	5s	5s	>50 correct >75% accuracy <25% omissions total trials – premature >100	For 3 consecutive days and minimum of 7 days
Variable ITI	1.8s	5s	2, 5, 10 or 15s	N/A	

Table 2.3 – Training procedure used in the 5-CSRTT

2.4.2 5-Choice Continuous Performance Task

It has been suggested that the 5-CSRTT represents a rodent version of the continuous performance task (CPT) which quantifies attention in humans by measuring their ability to determine whether or not a target letter had been presented in a sequence (Rosvold *et al.* 1956). However, one key difference between the 5-CSRTT and the human CPT is that the CPT incorporates stimuli which require subjects to withhold their response ("non-signal" or "no-go" stimuli). Young and colleagues recently developed the 5-CSRTT to include trials in which all five apertures are simultaneously illuminated, and as such, the animal is required to withhold nose-poking into any aperture in order to receive the reward (Young *et al.* 2009). This allows the assessment of response inhibition to irrelevant stimuli, as in the human CPT.

The 5-Choice Continuous Performance Task (5C-CPT) followed the same general method as the 5-CSRTT. However, key differences were in the training, and to some extent, the testing method; stimulus parameters and trial types were modified to resemble the methodology used by the Young group (Young *et al.* 2004; Young *et al.* 2009). These are discussed in detail below.

The 5C-CPT used the same apparatus as the 5-CSRTT, but the MED-PC files used to run the training and testing computer programs were kindly donated by Jared Young (UCSD), and adapted to suit the equipment used here.

Training

Mice were food restricted to 90% of their free-feeding body weight, in the same way as detailed in Section 2.4.1. Habituation also followed the same protocol as in the 5-CSRTT, except mice had to reach 70 reinforcers on the 'Habyol' stage compared with 50 in the 5-CSRTT.

Mice were then trained in the 5C-CPT according to the protocol detailed in Table 2.4. Training differed from that used in the 5-CSRTT in terms of success criteria; in the 5C-CPT mice were required to react increasingly quickly to the stimulus light, whereas in the 5-CSRTT, mice were required to meet certain response criteria, such as a high %accuracy and low %omissions.

Another key difference between the 5C-CPT and the 5-CSRTT is the addition of 'no go' trials. These were trials in which all 5 holes were illuminated, and the mouse was required to withhold its response by not nose-poking into any hole. This resulted in two more behavioural outcomes (Figure 2.6), in addition to the ones shown in Figure 2.5. The no go trials were introduced after the mice had successfully learned how to respond in the 5-CSRTT (i.e. one stimulus light illuminated at a time).

Other minor differences in the 5C-CPT compared to the 5-CSRTT were that the house light was continually off, with a 10 s timeout accompanied by the house light being illuminated in the 5C-CPT (the opposite was true in the 5-CSRTT), and the milk reward was available for only 4 s, compared with no time limit in the 5-CSRTT.

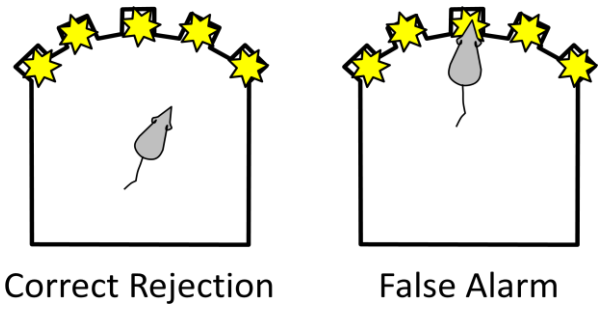

Figure 2.6 – Additional behavioural responses in the 5C-CPT.

Stage	Description	Conditions			Criteria for Passing	
Habituation		30% condensed milk solution available on nose-poke into magazine 100% condensed milk placed inside all 5 holes on day 1 & 2 Hole-lights illuminated throughout			None	Mice advanced to next stage after 3 days
Habyol	A non-spatial version of the task	All 5 holes illuminated constantly Liquid reward available from magazine upon nose-poke into any of the 5 holes			>70 rewards	For 2 consecutive days Maximum of 10 days before progression regardless of performance
		SD	**LH**	**ITI**		
Fixed ITI 5-CSRTT	SD gradually decreased from 20 to 2s, with a fixed inter-trial interval	20, 10, 8, 4 or 2s	SD + 2s, to minimum of 5s	5s	Mean correct latency half that of the SD 10 correct responses	For 3 consecutive days at each SD
VITI 5-CSRTT	SD remains at 2s, while the inter-trial interval becomes variable	2s	5s	3, 4, 5, 6 or 7s	>80% accuracy <40% omissions	For 3 consecutive days
VITI, 2:1 5C-CPT	'No go' trials are added in a 2:1 ratio of go: no go trials	2s	5s	3, 4, 5, 6 or 7s	>50% correct rejection	For 2 consecutive days
VITI, 5:1 5C-CPT	Ratio is increased to 5:1 go: no go trials	2s	5s	3, 4, 5, 6 or 7s	Sensitivity index > 0	For 3 consecutive days
Testing	An extended (60 min) session with extended inter-trial intervals	2s	5s	7, 8, 9, 10 or 11s	N/A	

Table 2.4 – The training procedure used in the 5C-CPT

Testing

Once mice had reached a stable baseline of performance in the 5C-CPT, mice were eligible for testing. Testing also differed from that used in the 5-CSRTT. In the 5C-CPT mice had already experienced variable inter-trial intervals (3 – 7 s). Therefore to challenge mice, an extended session was used (60 min/250 trials), with lengthened, variable inter-trial intervals (7 – 11 s).

Behaviours in the 5C-CPT

Behavioural measures were used to calculate the same variables as in the 5-CSRTT, with the addition of those listed in Table 2.5.

Behavioural Outcome	Method of calculation
%False alarm (PFA)	[Number of correct rejections/ (number of correct rejections + number of false alarms)] * 100
% Hits (PH)	[Number of correct responses/ number of correct responses + number of omissions)] * 100
False alarm latency	Latency to nose-poke on a no-go trial
Sensitivity index	$PH - PFA/(2(PH+PFA))-(PH+PFA)^2$

Table 2.5 – Additional behavioural outcomes in the 5C-CPT

The difference between a 'correct rejection' and a 'hit' is determined by the type of trial (see Table 2.6 below). The sensitivity index takes both of these behaviours into account, with a positive sensitivity index being a high signal to noise detection rate, and a negative sensitivity index being a low signal to noise detection rate. For example, a mouse would need to have a high level of 'hits', and a low level of 'false alarms' to give a positive numerator in the sensitivity index equation.

	Go Trial	No Go Trial
Response	Correct response (*hit*)	False alarm
No response	Omission (*miss*)	Correct rejection

Table 2.6 – Behaviours used to calculate the sensitivity index

2.4.3 Light-Dark Exploration Box

The light-dark exploration box (LDEB) is one of many approach-avoidance conflict tests designed to evaluate emotionality, based on a conflict between animals' innate curiosity to explore novel environments, and their aversion to brightly lit, open spaces (Crawley 1981). However, here, unlike in the light-dark test pioneered by Crawley, the light zone was dimly lit, to render it 'novel' but not strongly aversive. The LDEB consisted of two zones; a smaller, darker, black Perspex arena (15 cm x 20 cm, 4 Lux), and a larger, brighter, white Perspex arena (30cm x 20cm, 20 Lux), separated by a removable door. A grid of 5cm squares is marked on the floor, and used to score locomotor activity. Manually scoring behaviour allowed this test to be used as a measure locomotor activity and emotionality, although the latter has not been validated using this protocol. We have previously demonstrated that NK1R-/- mice display increased locomotor activity and decreased time in the light zone (Fisher *et al.* 2007), although a wide range of other behaviours, such as grooming or risk assessment behaviour, can be monitored.

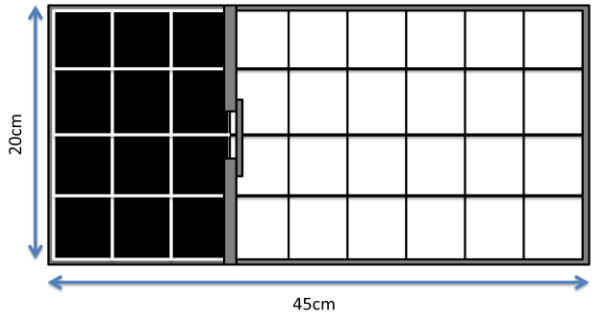

Figure 2.7 – The light-dark exploration box apparatus

Method

Mice were allowed to habituate to the test room between 10.00 – 13.00 h. At either 13.00 h or 15.30 h, mice were placed in the dark zone of the LDEB for 60 min, injected with their allocated treatment or left untreated, and placed back in the dark zone for a further 30 min. Mice were then transferred to the light zone facing away from the door while the door was removed, allowing them to travel between the two zones. One wildtype and one NK1R-/- mouse were

tested alongside each other in adjacent LDEBs with the same treatment, to minimize any random confounding factors. Behaviour was recorded for 30 min by a video camera placed above the LDEB, and was later scored blind (see Table 2.7). Treatments were assigned in a randomized order.

Behaviour	Measurement method	Indications
Locomotor activity (lines per unit time)	[Number of lines crossed per second in the given zone] x 1000	Measure of spontaneous activity, corrected for time spent in the zone
Time in light/dark zones	Time spent with all 4 paws in the zone	Could indicate anxiety-like behavior (unvalidated)
Number of returns to the light zone	Number of times the mouse returns with all 4 paws to the light zone	Could indicate anxiety-like behaviour and/or locomotor activity (unvalidated)
Latency to leave the light zone	Latency to leave the light zone the first time	Could indicate active avoidance (unvalidated)

Table 2.7 – Behavioural outcomes in the LDEB and their indications

2.4.4 Elevated Plus Maze

The elevated plus maze (EPM) is another approach-avoidance conflict test that is used specifically to measure anxiety, giving a behavioural response which can be modulated in a bidirectional manner (Lister 1987). It has the advantage of being an ethological approach, which requires no training or painful stimuli. The EPM, built from Perspex, consists of two 'open' (5 cm x 30 cm, 2 mm wall height) and two 'closed' arms (5 cm x 30 cm, 16 cm wall height), arranged in a 'plus' shape, elevated 30cm above the floor. The animal is placed in the centre, and allowed to explore the maze. A large number of factors can influence behaviour on the plus maze; procedural factors (height above the floor, light intensity, repeated testing) organismic variables (background strain, gender, age) and prior stress (e.g. other anxiety tests) (for review see Rodgers and Dalvi 1997). To that end, the plus maze was performed in low level lighting (10 Lux), at the same time of testing as the LDEB, with mice age-matched as closely as possible.

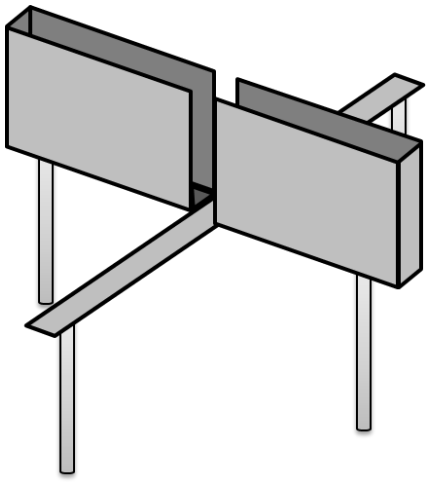

Figure 2.8 – The elevated plus maze apparatus.

Behaviour	Description	Indication
%Time on the open arms	[time on open / (time on open + time on closed)] * 100	Longer time indicates decreased anxiety (Pellow *et al.* 1985)
Number of whole body entries to the open arms	All four paws in the open arms	Increased number indicates decreased anxiety (Pellow *et al.* 1985)
Number of whole body entries to the closed arms	All four paws in the closed arms	Indicator of activity level (Rodgers and Johnson 1995)
Number of head entries to the open arms	Head only enters open arm	Increased number could indicate decreased anxiety (unconfirmed)
%Time in centre square	[time in centre / total time] * 100	Unknown/unconfirmed

Table 2.8 – The behavioural outcomes in the EPM and their possible indications

Method

Mice were allowed to habituate to the test room between 10.00 – 14.00 h, and were tested between 14.00 – 16.00 h on the EPM. Mice received an intraperitoneal injection of either vehicle or drug, 30 min prior to testing, or received no injection. Treatments were assigned in a randomized order. A single mouse was tested at one time: the mouse was placed in the centre

of the 4 arms, facing between an open and closed arm, and allowed to explore the maze for 5 min. The plus maze was cleaned between tests with 70% EtOH. Behaviour (see Table 2.8) was recorded by a video camera above the maze, and was later scored blind.

2.4.5 Novel object recognition/location

The spontaneous object recognition task, or novel object recognition (NOR) test utilizes the characteristic of rodents to explore a novel object in preference to a familiar one. Ennaceur and Delacour first tested this phenomenon in 1988: they found that rats, when exposed to two objects, one of which they had previously seen, could discriminate between the two by preferentially exploring the unfamiliar, or novel, object (Ennaceur and Delacour 1988). The NOR test has a number of advantages over other tests of memory in rodents. Firstly, it utilises spontaneous behaviour, and as such requires no lengthy training protocol. The spontaneity of the behaviour also abolishes the need for food restriction or negative reinforcement, which, in the case of pharmacological and genetic manipulations, can cause a confounding effect on the results. It is also similar to visual recognition tests used in non-human primates and humans, allowing inter-species comparisons. The test can be manipulated to investigate a number of different types of memory, as well as novelty seeking and attention.

Here, we have utilized two versions of the test; 1) the novel object recognition (NOR) paradigm to test for short term, declarative memory and/or novelty seeking, and 2) the novel location test (NOL) to test for spatial memory. In the NOR task, two identical objects are presented to the subject for a given time period. One object is then replaced by a novel object, similar in size but different in colour, texture and shape to the original object, and the animal is allowed to explore again, after a predetermined time frame.

In the NOL test, two identical objects are used, but between the two trials the location of one of the objects is moved. This test requires the presence of cues in the environment, although there is evidence that mice can navigate the NOL test based on the position of the overhead camera, alone (Murai *et al.* 2007). Here we used a piece of laminated black card placed against one of the walls of the arena to allow spatial navigation.

Figure 2.9 – Objects used in the novel object recognition tasks

Objects used in these tests were miniature painted wooden 'penguin' skittles, plastic pencil sharpeners and glass bottles filled with blue dye. Objects used as the novel object, and objects used in the NOL were counterbalanced to account for any inherent aversion or preference for any of the objects.

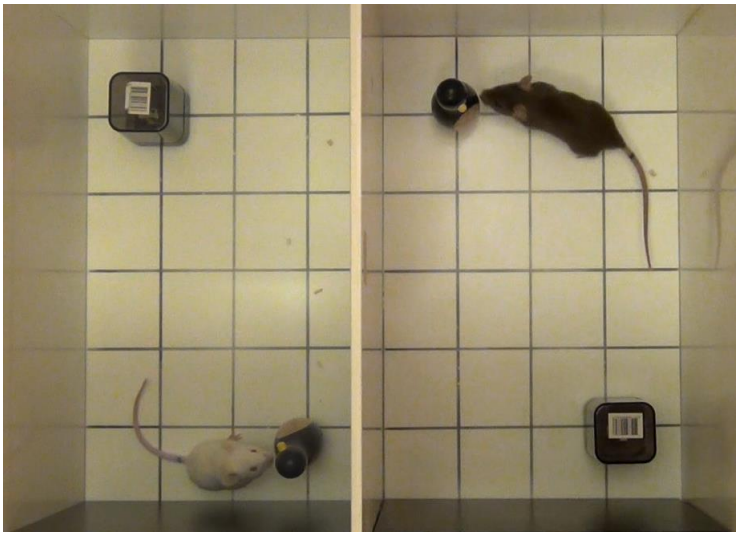

Figure 2.10 – Mice behaving in the novel object recognition task

Method

Mice were brought to the testing room at 10.00 h, and tested between 14.00 – 16.00 h. On day 1, mice were allowed to habituate to an empty arena (the same arena as the light zone of the LDEB) for 30min. On day 2, mice were introduced to the arena with two identical objects, and allowed to explore for 10 min. After an inter-trial interval of 1 h, mice were reintroduced to the arena with one of the objects replaced by a novel object (for the NOR) or one of the objects

moved to a novel location (for the NOL), and allowed to explore for a further 10 min. Mice were injected with vehicle or drug 30 min before the first session on day 2.

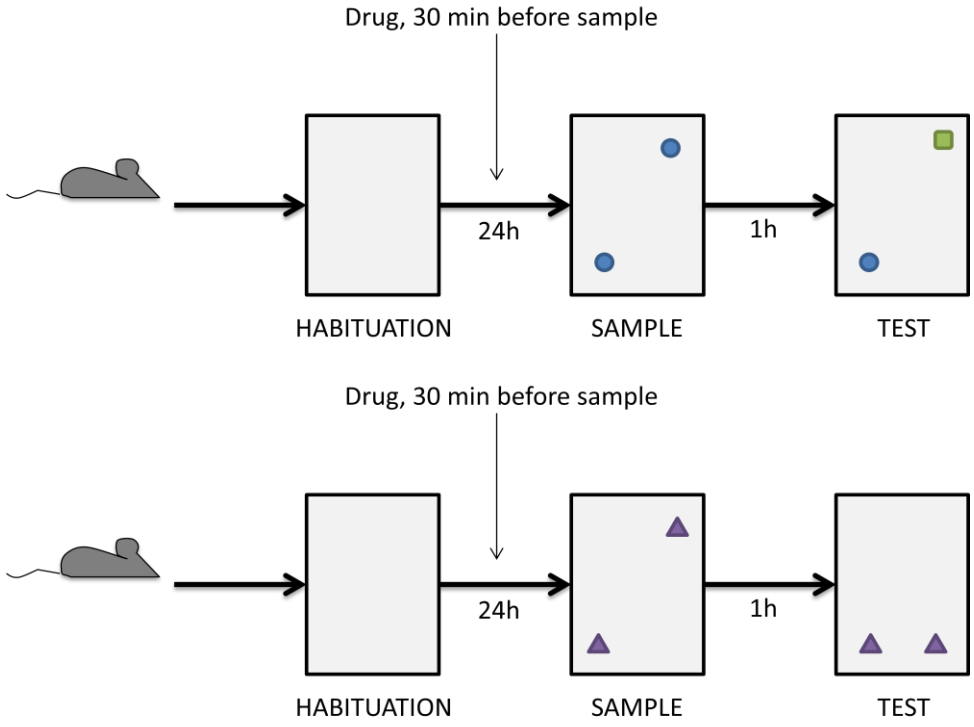

Figure 2.11 – Protocol used in the novel object recognition (top) and location (bottom) tasks.

The following behaviour was recorded in the test sessions by a digital video camera, and scored blind:

- Time exploring the novel object in seconds (N)
- Time exploring the familiar object in seconds (F)
- Number of times the mouse returned to the novel object
- Number of times the mouse returned to the familiar object.

A discrimination index was also calculated according to the following equation:

Discrimination index = (N-F) / (N+F)

The discrimination index gives a value between -1 and +1, with chance level being zero, positive values showing preference for the novel object and negative values showing preference for the

familiar object. In all cases exploration of objects was defined as attention directed at the object, sniffing, touching or nose pointed at, and within 2 cm of the object. Climbing on, walking past and gnawing the object were excluded, in line with previous studies (Leger *et al.* 2013).

2.5 Body Composition Analysis

Body composition analysis was completed on each of two cohorts of animals; lean animals and those fed a high fat diet. Two methods of analysis were used: dual energy X-ray absorptiometry (DEXA) analysis was performed on whole carcasses, and chemical analysis was performed on samples of milled carcass. Chemical analysis on cadavers remains the most accurate and precise method for determination of body composition, and is considered to be the gold standard method.

2.5.1 DEXA

DEXA analysis is a method used clinically to determine bone density and fat mass in humans. It is a non-invasive technique, and multiple measurements can be taken over time. DEXA uses specialized software and X-ray beams at two different energy levels to discriminate between fat, lean tissue and bone. When fired at the subject, the lower energy beam (35kV, 500μA) is absorbed mainly by soft tissue, and the higher energy beam (80kV, 500μA) is absorbed by bone. The X-rays which are not absorbed by the subject are measured by the machine, and the ratio of attenuation of the high to low energy X-rays allows the machine to calculate the body composition of the subject.

DEXA machines have recently been adapted to measure the body composition of rodents and small animals. Animal carcasses or anaesthetized subjects can be used: if it is the latter, multiple measurements can be taken. This is particularly advantageous in drug studies, where the effect of drug can be measured over time. Several studies have investigated the precision and accuracy of these rodent DEXA machines: it seems that results are consistently precise and

reliable, but accuracy can be poor, particularly in terms of body fat (Nagy *et al.* 2001; Brommage 2003; Iida-Klein *et al.* 2003; Johnston *et al.* 2005).

Here, DEXA analysis was performed with the Lunar Piximus II Densitometer (GE Medical Systems, Madison, WI, USA) under supervision of a trained operator. First, a 'phantom' mouse of known composition was used to calibrate the machine. Then, thawed mouse carcasses were scanned individually on the Piximus II machine. Mice were positioned in the detectable area, and the machine performed three scans of each subject. The resulting output to a computer was an average of the three scans.

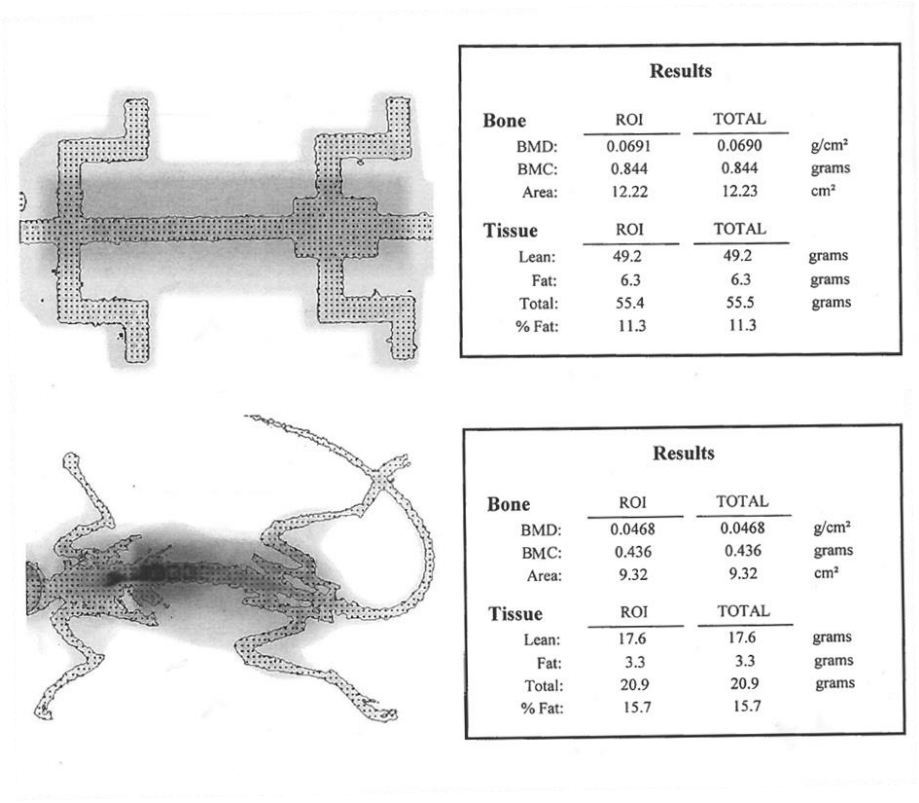

Figure 2.12 – Computer output from a DEXA scan of (A) the "phantom" mouse and (B) an example of a test subject. ROI: region of interest, BMD: bone mineral density, BMC: bone mineral content.

2.5.2 Chemical Analysis

Chemical analysis was completed in four separate parts, to determine water, ash, fat and protein content.

Water

Mice were frozen at -80°C for at least 5 h after the DEXA scanning. Frozen carcases were then kept on a freeze dryer (Heto PL9000) for 2 weeks using a shelf temperature of 25°C. Freeze drying converted ice (solid H_2O) in the frozen carcasses directly to water vapour (gaseous H_2O) by sublimation (see Figure 2.13). This prevented melting and allowed all water to be vaporised and removed.

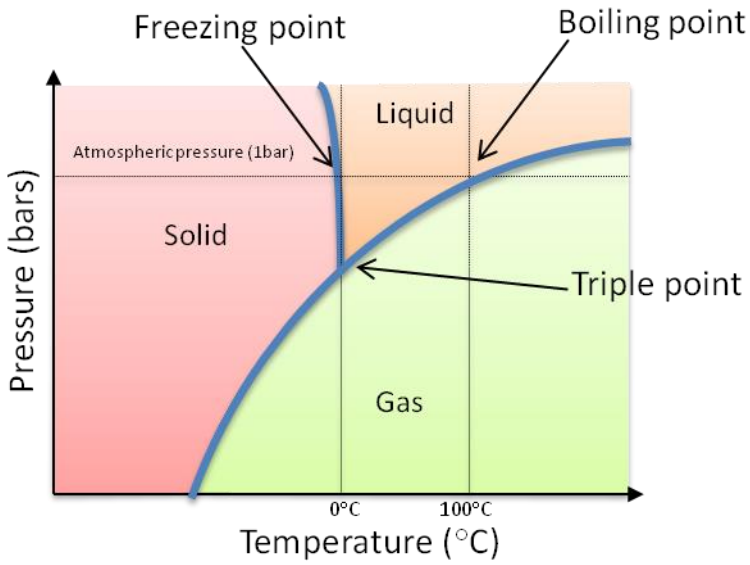

Figure 2.13 – Phase diagram of water.

After freeze drying, mouse carcases were weighed and stored in sealed jars in drying cabinets to prevent water reabsorption prior to subsequent analyses. Water content was defined as:

*%water in mouse = 100 – ((weight of dried carcass / weight of carcass) * 100)*

Dried carcases were milled on a Buchi Mixer B-400 homogeniser, and samples of the ground carcases were used in subsequent analyses.

Ash

Carcass ash was determined on freeze dried samples. Samples weighing ~1g were placed in silica crucibles, and fired at 600°C for 6 hours in a muffle ashing furnace (Carbolite, OAF 11/1).

Crucibles were left to cool in drying cabinets, and then reweighed. Residual ash was used to calculate the ash content of the sample, and of the original carcass using the determined water content:

*%ash in sample = (sample weight after ashing/sample weight before ashing) * 100*

*%ash in mouse = %ash in sample * ((100 – %water content of mouse)/100)*

*ash (g) in mouse = (%ash in mouse * mouse weight)/100*

Fat

Carcass fat content was determined by a modified Soxhlet extraction protocol. Samples of carcass (~1g) were weighed into cellulose extraction thimbles (Whatman 26mm x 60mm: 2800-266), and plugged with approximately 0.5 g of cotton wool. 90ml of petroleum ether (Fisher 40-60°C: P/1760/17) was used to extract the fat from each thimble using a Tecator Soxtec HT2 system (Foss, UK) /Tecator Soxtec 2050 system (Foss UK Ltd, Wheldrake, UK), with a modified manufacturers protocol (35 min extraction, 30 min wash and 10 min dry). Fat extraction occurred as boiling solvent (petroleum ether) dissolved lipids in the sample, which were then collected as the solvent was evaporated (see Figure 2.14). Extracted fat was weighed and calculated as a percentage of the dried sample. Carcass fat in the original carcass was then calculated using the determined water content:

*%fat in sample = (weight of fat in cup/sample weight) * 100*

*%fat in mouse = %fat in sample * ((100 – %water content of mouse)/100)*

*fat (g) in mouse = (%fat in mouse * mouse weight)/100*

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Figure 2.14 – The Soxhlet fat extraction method (taken from www.foss.dk)

Protein

The protein assay used a Kjeldahl method, using an acid titration to determine nitrogen content.

Digestion

Carcass samples (0.3-0.4g) were weighed into Kjeldahl test tubes, and digested for 1 hour at 420°C, using a Tecator 2012 (FOSS, UK) digestion block in the presence of 10 ml of concentrated sulphuric acid (Fisher S/9240/PB17), two Kjeltab CQ catalyst tablets (containing potassium sulphate and copper sulphate) and one Antifoam S tablet (sodium sulphate and silicone antifoam, Thompson & Capper) to prevent acid boiling over. A FOSS 2001 Scrubber Unit with sodium hydroxide solution and water was attached to the top of the test tubes to neutralize and remove any acidic waste gases produced by the reaction.

$$\underset{(s)}{\text{amino acids}} + \underset{(aq)}{H_2SO_4} \rightarrow \underset{(aq)}{(NH_4)_2SO_4} + \underset{(g)}{CO_2} + \underset{(g)}{SO_2} + \underset{(g)}{H_2O}$$

Distillation

Cooled samples were distilled, one at a time by a Tecator 2020 distilling unit /FOSS 2200 Kjeltic Auto Distillation unit (FOSS, UK). 40ml of concentrated (10M) sodium hydroxide (Fisher J/7800/21) and 20ml water were added to the digested sample, and steam was bubbled through. The sample was distilled into 30ml of Kjeldahl receiver solution (4% boric acid with bromocresol green/methyl red indicator, Fisher K/0200/21). The addition of sodium hydroxide neutralized the acid and produced ammonia, which was 'captured' by distillation into the receiver solution:

Liberation of ammonia

$$(NH_4)_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + H_2O + 2NH_3$$
$$(aq) \quad (aq) \quad (aq) \quad (aq) \quad (g)$$

Capture of ammonia

$$H_3BO_3 + NH_3 \rightarrow NH_4^+ + H_2BO_3^-$$
$$(aq) \quad (g) \quad (aq) \quad (aq)$$

Titration

Each sample was titrated with 0.1M volumetric grade hydrochloric acid (Fisher J/4350/17), to a colour change end point:

$$NH_4^+ + H_2BO_3^- + HCl \rightarrow NH_4Cl + H_3BO_3$$

1 mole of HCl used = 1 mole of NH_4 in sample = 1 mole of nitrogen in sample

The volume of acid needed to neutralize the alkaline ammonium solution is proportional to the concentration of ammonium ions in the solution, which is proportional to the concentration of nitrogen in the sample, which is proportional to the sample protein (amino acid) content.

Therefore, the volume of acid used in the titration can be used to calculate the mass of nitrogen, and hence, protein, in the sample:

Moles = concentration (M) x volume (L)

*Moles HCl = 0.1M * volume used in titration (L)*

*mass = moles * RMM*

*mass of nitrogen = moles * 14.0067 (RMM of nitrogen)*

Protein content is given as:

*%protein in sample = %nitrogen = (mass of nitrogen / mass of sample)*100*

*%protein in mouse = %protein in sample * ((100 – %water content of mouse)/100)*

*protein (g) in mouse = (%protein in mouse * mouse weight)/100*

2.6 Statistics

Graphs were generated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using InVivoStat software (Clark *et al.* 2012).

The sample sizes for behavioural experiments were estimated using the Mead's resource equation, and were kept to a minimum in keeping with the aims of the NC3Rs.

2.6.1 Single Measures Analyses

For single measures analyses, two or three-way analyses of variance (ANOVA) were used. Factors are detailed in the relevant Chapters.

2.6.2 Repeated Measures Analyses

For repeated measures (RM) analyses, InVivoStat uses a mixed-model approach. The mixed-model approach was chosen because it allows the user to choose how the within-animal correlations are modelled: this can be in three different ways (see Table 2.9), each with its own assumptions. Moreover, the mixed-model approach, as opposed to the ANOVA-based approach, deals with missing data more effectively: in ANOVA, if an animal misses a response, all the data points from that animal are excluded from the analysis.

Within-animal correlation model	Assumptions
Compound symmetric covariance structure	- **Assumes sphericity, i.e. variances are the same at all time points** - **Suitable for small sample sizes**
Autoregressive structure	- Assumes the variability is the same across all time points, but the strength of the covariance between responses depends on the distance between them, i.e. responses close in time will have a stronger covariance - Suitable for equally spaced time points
Unstructured	- Does not assume sphericity - The strength of the covariance between any pair of repeated measures is the same - Suitable for large sample sizes only

Table 2.9 – Models of within-animal correlations in a mixed-model analysis of repeated measures data

All experiments in this thesis used a compound symmetric covariance structure. This structure assumes sphericity of the variance-covariance matrix. Instead of Mauchley's test of sphericity (which is inaccurate in cases where the sample sizes are small, as is the case in most animal experiments), the 'predicted *vs.* residuals' plot in InVivoStat was used to ascertain the best level of sphericity. This was achieved by transforming (square-root, log10 or arcsine) data as appropriate.

Parametric analyses also assume the data are normally distributed. Where this assumption was violated, a rank transformation was applied; that is, the data were ranked, as for a non-parametric analysis, and the assigned ranks then underwent the parametric test.

2.6.3 Post-hoc analyses

A main effect of one of the variables, or an interaction between them, in the first analysis was used as criteria for progression onto post-hoc analysis. Post-hoc analyses were planned comparisons: comparisons were made using the least square (i.e. predicted means) rather than the observed means (which can lead to false positives when multiple comparisons are being made).

2.6.4 Correlation analyses

In Chapter 7, Pearson's correlation analyses were performed as a measure of the linear correlation between independent measures of body composition. The correlation coefficient (R^2) was used to determine the strength of the relationship in each case.

Chapter 3

The effect of guanfacine on hyperactivity, inattention and impulsivity in NK1R-/- and wildtype mice

Chapter 3. The effect of guanfacine on hyperactivity, inattention and impulsivity in NK1R-/- and wildtype mice

3.1 Introduction

The noradrenergic system could play a critical role in the aetiology of ADHD (see Biederman and Spencer 1999). As discussed in Chapter 1, psychostimulant treatments for ADHD target noradrenergic neurones. Moreover, the only approved, non-stimulant treatments are either a noradrenaline reuptake inhibitor (atomoxetine), or α2-adrenoceptor agonists (guanfacine and clonidine). The first α2-adrenoceptor agonist to be tested as an ADHD treatment was clonidine, after its success in treating Tourette's syndrome (Cohen *et al.* 1979; Hunt *et al.* 1985). Guanfacine was originally used as an anti-hypertensive agent in the late 1970s, but prompted by extensive evidence for the beneficial effects of clonidine in treating ADHD (Dubach *et al.* 1977; Cohn and Caliendo 1997), guanfacine was developed as a treatment for this disorder with the aim of fulfilling a need for a longer-acting and better tolerated compound (Hunt *et al.* 1985; Chappell *et al.* 1995). The FDA (Food and Drug Administration) approved guanfacine for the treatment of ADHD in 2009, after two randomized, double-blind, placebo controlled studies revealed its safety and efficacy in treating the disorder (Biederman *et al.* 2008; Sallee *et al.* 2009).

3.1.1 Noradrenergic neurotransmission

Noradrenaline acts at adrenoceptors (ARs), which were among the first G-protein coupled receptors (GPCRs) to be cloned (Kobilka *et al.* 1987). They are divided into three groups; α1-ARs ($\alpha1_A$, $\alpha1_B$, $\alpha1_D$), α2-ARs ($\alpha2_A$, $\alpha2_B$, $\alpha2_C$) and β-ARs (β1, β2, β3). Originally, the α1 and α2 receptors were classified by their presumed respective post- and presynaptic locations (Langer 1974). However, a more accurate distinction was later made according to their

pharmacological profiles (Bylund *et al.* 1994; Ruffolo and Hieble 1994): i.e. it is now known that α2-ARs are located at both presynaptic and postsynaptic sites.

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Figure 3.1 – Sequence homology of alpha2-adrenoceptors. Taken from Saunders and Limbird 1999.

The amino acid sequence of α2-AR subtypes is homologous throughout the 7 transmembrane regions of the GPCRs, but heterogeneous in the intra- and extracellular loops (see Figure 3.1). The subtypes also differ in their post-translational modifications (e.g. glycosylation; see Figure 3.1), although the significance of this is not yet understood, given that agonist selectivity is determined by the third cytoplasmic loop (Eason and Liggett 1996).

The mRNA of α2A-ARs is present in the midbrain, hypothalamus, amygdala, cerebral cortex, hippocampus, and is particularly prominent in the locus coeruleus and brain stem nuclei controlling blood pressure (Nicholas *et al.* 1993; Wang *et al.* 1996). Similar to α2A-ARs, α2C-ARs are expressed in the basal ganglia, hippocampus and cerebral cortex, whereas α2B-AR expression is limited to the thalamic nuclei (Scheinin *et al.* 1994).

The expression of α2A-ARs in noradrenergic cells of the locus coeruleus suggests that this subtype may primarily function as an autoreceptor. However, α2A-ARs are also present in brain areas innervated by LC neurones, suggesting the receptor also has an important role at postsynaptic sites (Scheinin *et al.* 1994). This is supported by evidence that the sedating properties of clonidine are due to activation of presynaptic α2-ARs, whereas the mydriasis response is mediated by postsynaptic α2-ARs (Heal *et al.* 1989; Heal *et al.* 1995).

The main origin of noradrenergic terminals in the brain is the locus coeruleus (LC), which projects to almost all brain areas, including the prefrontal cortices (PFC), with the exception of the majority of the basal ganglia (see Figure 3.2). The noradrenergic system is strongly implicated in arousal, attention and vigilance (Harley 1987; McCormick 1989; Sara and Segal 1991): the PFC plays a critical role in high-level cognitive functions that are often impaired in ADHD, and so it is the neocortical projection which is thought to be dysfunctional in this disorder. One hypothesis is that a moderate level of noradrenergic transmission is concordant with optimal cognition, whereas peaks in noradrenaline release due to high stress situations can lead to worsened cognition (Robbins and Arnsten 2009; Arnsten 2011; Del Campo *et al.* 2011).

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Figure 3.2 – Schematic diagram of a sagittal section through the rat brain, showing noradrenergic projections from the locus coeruleus (LC). Anterior olfactory nucleus (AON), ansa peduncularis–ventral amygdaloid bundle system (AP-VAB), brainstem nuclei (BS), cingulum (C), corpus callosum (CC), cerebellum (CER), central tegmental tract (CTT), cortex (CTX), dorsal bundle (DB), dorsal periventricular system (DPS), entorhinal cortex (EC), fornix (F), fasiculus retroflexus (FR), hypothalamus (H), hippocampal formation (HF), medial lemiscus (ML), mamillothalamic tract (MT), olfactory bulb (OB), pretectal area (PT), reticular formation (RF), septum (S), spinal cord (SC), stria terminalis (ST), tectum (T), thalamus (TH). Modified from Moore and Bloom 1979; Sara 2009.

3.1.2 Guanfacine and clonidine

Treatment with an α2-AR agonist could help normalize arousal and attention in ADHD by modifying the noradrenergic system. Guanfacine and clonidine both have some efficacy in alleviating the signs of ADHD, although the two drugs have slightly different behavioural/physiological profiles: guanfacine is a weaker hypotensive and sedative, but more potently enhances cognition (Arnsten *et al.* 1988). This profile better lends itself to ADHD, and as such, guanfacine is now the α2-AR agonist of choice in treating this disorder. The difference

between the two drugs could be explained by their slightly different pharmacological profiles: clonidine is only a partial α2-AR agonist, but guanfacine is a full agonist which is approximately 6-fold more selective for α2-ARs than it is for α1-ARs (Jarrott *et al.* 1983). Moreover, guanfacine is selective for the α2A over the α2B/C subtypes (Uhlen and Wikberg 1991). Nevertheless, extended release versions of both guanfacine (Intuniv ®) and clonidine (Kapvay ®) are approved treatment options. Furthermore, both agonists can be used as an adjunctive therapy to psychostimulants, as the therapeutic effects are additive, but side-effects are resolved, e.g. insomnia caused by psychostimulants can be relieved by somnolence caused by guanfacine (Childress 2012).

3.1.3 Aims

The first objective of this study was to investigate whether guanfacine ameliorates the deficits in cognitive performance and response control that are expressed by NK1R-/- mice in the 5-CSRTT, in line with its efficacy in treating ADHD. A second objective was to establish whether or not guanfacine prevents the locomotor hyperactivity of NK1R-/- mice. However, animals' emotional status (anxiety-like behaviour) can confound measures of locomotor activity (see Wilcock and Broadhurst 1967; Stanford 2007; Stanford 2007). This is especially important for studies on the effects of guanfacine on motor behaviour because this drug is used to treat anxiety, which is a common comorbid disorder in ADHD (Sobanski 2006). Therefore, we tested mice in the light-dark exploration box, to measure sponatenous locomotor activity and take note of any changes in emotional status.

3.2 Methods

3.2.1 Light-Dark Exploration Box

The LDEB was used as described in Chapter 2, section 2.4.3. Doses of guanfacine were chosen based on a survey of recent literature (see Appendix 2). Guanfacine (0.1, 0.3 or 1 mg/kg, i.p.), vehicle (saline) or no injection was administered 30 min before mice were transferred to the light zone. Treatments were assigned in a counterbalanced order. Mice were then allowed to

move freely between the two zones while their behaviour was recorded for 30 min with a video camera. Behaviour (listed in Table 2.7 in Chapter 2) was later scored blind. Because the activity of mice in the LDEB declined progressively to reach a 'floor' by approximately 15 min, only the first 10 min of activity after transfer to the light zone were used in the statistical analysis.

3.2.2 5-Choice Serial Reaction Time Task

The 5-CSRTT followed the protocol described in Chapter 2, section 2.4.1. Wildtype and NK1R-/- mice from homozygous breeding colonies (homs) and NK1R-/- and wildtype littermates (hets) were used in this experiment (see Table 2.7). The use of wildtype and NK1R-/- littermates was part of a larger study examining the effects of maternal and early life environment, which is outside the scope of this thesis. All mice were 6-8 weeks old at the start of training.

Genotype	Number used ('n')	Number of breeding pairs derived from	Start weight (g: mean ± SD)
WT *hom*	6	2	32.2 ± 1.9
NK1R-/- *hom*	6	2	29.1 ± 1.7
WT *het*	6	} 4	35.9 ± 4.2
NK1R-/- *het*	6		32.5 ± 1.8

Table 3.1 – Details of mice used in the 5-CSRTT experiments

This 5-CSRTT experiment utilized the VITI (variable inter-trial interval) to challenge the response control and cognitive abilities of the mice. This test prevents the use of interval-timing, and has been utilized to draw out genotype differences, in particular in premature responding.

The mice were first tested in two no injection (NI-1) sessions (one VITI and one long inter-trial interval (LITI)). The findings of these initial tests in naive mice are reported elsewhere (Porter *et al.* 2015). After the initial NI-1 sessions, mice were tested once-weekly, 30 min after treatment with either guanfacine (0.1, 0.3 or 1 mg/kg, i.p.), vehicle (saline) or no injection (NI-2). Each mouse received each treatment once, and the treatments were assigned using a pseudo-Latin square design, to account for any effects of repeated testing and possible long term drug effects. One wildtype and one NK1R-/- mouse failed to graduate through to the testing phase of the experiment, and were dropped from the experiment.

3.2.3 Statistics

Data were analysed as described in Chapter 2, section 2.6. In the LDEB, two-way ANOVAs were performed on raw or, if the sample variances were not uniform, transformed data, using the main factors of *genotype* and *drug treatment*. First, the ANOVA compared the factors across all groups (uninjected, vehicle and drug). A main effect of either factor, or an interaction between them, allowed progression to further analysis. Second ANOVAs compared uninjected controls versus vehicle injected controls (main effect of *'injection'*), and vehicle versus drug doses (main effect of *'drug'*). Where there was a main effect of *genotype*, *injection* or *drug*, or interactions between the factors, post-hoc LSD tests were performed.

In the 5-CSRTT, two- or three-way repeated measures (RM) analyses used '*genotype'* as the between-subjects factor and '*drug'* as the within-subjects factor. The third factor was either '*colony*' or '*time of day'*. Because '*time of day'* (AM session/PM session) can influence behaviour in the 5-CSRTT (Yan *et al.* 2011; Weir *et al.* 2014), this factor was also investigated. Where there were main effects of '*time of day'*, it was used as a blocking factor in the analysis, such that any variability within the data caused by *time of day* was taken into account, but not studied independently. This was possible because there were no *time of day*drug* interactions, i.e. drug treatment had the same effect on behaviour regardless of time of testing. Where there were no main effects of *time of day*, this factor was collapsed. Here, there was no interaction between '*colony'* and '*drug'* in any variable, and so the '*colony'* factor was collapsed. A main effect of '*genotype'* or '*drug'*, or an interaction between them, was used as criterion to post-hoc pairwise comparisons.

Analyses of both experiments were performed on raw or transformed data (arcsine, log10 or square-root), whichever gave the best homogeneity of variance in the 'predicted *vs.* residuals' plot in *InVivoStat*. The 'normal probability plot' in *InVivoStat* was used to test whether the data were normally distributed. If not, a rank transformation was applied, i.e. the data were ranked as for a non-parametric analysis, and were then subject to the parametric test. The transformation applied is noted in square brackets throughout. Statistical significance was set at $P<0.05$.

3.3 Results

3.3.1 Guanfacine reduces activity of mice in the Light-Dark Exploration Box

Compared with untreated wildtypes, NK1R-/- mice were hyperactive in the light zone of the LDEB [[SQRT]geno: $F_{(1,16)}$=4.75, P=0.044; NI, WT vs. KO: P=0.024, Figure 3.3A]. However, vehicle injection alone was sufficient to abolish this hyperactivity [VEH, WT vs. KO; P=0.561]. An apparent hyperactivity in the dark zone just missed the criterion for statistical significance [[SQRT]geno: $F_{(1,16)}$=3.79, P=0.069, Figure 3.3B].

Guanfacine reduced *locomotor activity* in the light zone [[SQRT]drug: $F_{(3,32)}$=15.19, P<0.001]: at the highest dose, activity was decreased in both genotypes [VEH vs. GFC1, WT: P=0.007, NK1R-/-: P<0.001, Figure 3.3A]. The same effect was seen in the dark zone [[SQRT]drug: $F_{(3,30)}$=5.07, P=0.006, Figure 3.3B]. However, an apparent reduction in *locomotor activity* in the dark zone was statistically significant in NK1R-/- mice, only [VEH vs. GFC1, WT: P=0.068, KO: P=0.002].

Guanfacine also reduced the *number of returns* to the light zone in both genotypes [[SQRT]drug: $F_{(3,32)}$=4.99, P=0.006, Figure 3.3C]. This was apparent at 0.1 mg/kg and 1 mg/kg in NK1R-/- mice [VEH vs. GFC0.1: P=0.017, VEH vs. GFC1: P=0.005], but only at 1 mg/kg in wildtype animals [VEH vs. GFC1: P=0.023].

NK1R-/- mice spent less *time in the light* zone than wildtypes [[RANK]geno: $F_{(1,32)}$=4.75, P=0.037] but this was unaffected by guanfacine [[RANK]drug: $F_{(3,32)}$=2.05, P=0.127, Figure 3.3D]. However, the drug increased the *latency* to leave the light zone [[LOG10]drug: $F_{(3,30)}$=4.21, P=0.013 Figure 3.3E], but this was evident only in NK1R-/- mice [VEH vs. GFC1: P=0.039]. However, the effect of guanfacine overall did not depend on genotype in any measure.

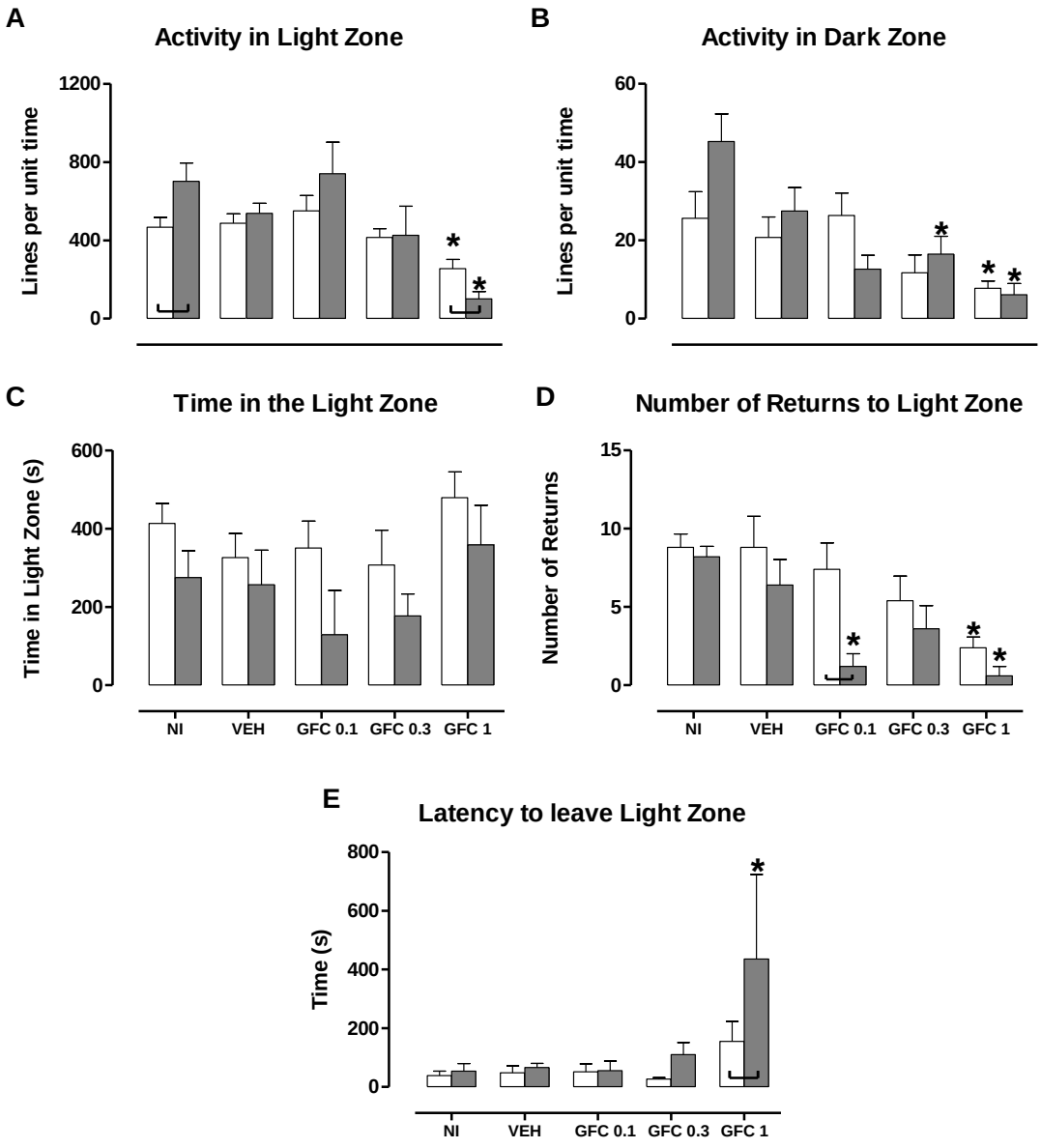

Figure 3.3 - The effects of guanfacine (0.1, 0.3 and 1 mg/kg, i.p.), vehicle (saline) or no injection (NI) on A: activity per unit time in the light zone, B: activity per unit time in the dark zone, C: time in the light zone, D: number of returns to the light zone, E: latency to leave the light zone in wildtype (white bars) and NK1R-/- mice (grey bars) in the light-dark exploration box. Data show mean ± SEM. Lines linking bars indicate statistical significance of P<0.05 and * P<0.05 versus vehicle within genotype. n=5.

3.3.2 Wildtype and NK1R-/- mice learn the 5-CSRTT at the same rate

Both genotypes took the same length of time to learn the 5-CSRTT [[RAW]geno: $F_{(1,20)}$=1.32, P=0.264, Figure 3.4A], and this was unaffected by time of day or colony. Wildtypes and NK1R-/- mice took an average of 46.5 and 38.2 days to reach testing, respectively.

3.3.3 Guanfacine has bi-directional effects on omission errors in NK1R-/- mice

There were no differences in *%omissions* between the genotypes overall [[SQRT]geno: $F_{(1,18)}$=3.40, P=0.082, Figure 3.4B] and, over the whole dose range, both genotypes responded to guanfacine in the same way [[SQRT]drug*geno: $F_{(3,49)}$=1.33, P=0.276]. However, this drug had bidirectional effects on this behaviour [[SQRT]drug: $F_{(3,49)}$=48.00, P<0.001]. The lowest dose (0.1 mg/kg) of guanfacine selectively reduced *%omissions* in NK1R-/- mice in comparison with vehicle-treated NK1R-/- mice [VEH vs. GFC0.1: P=0.004] and with drug-treated wildtypes [GFC0.1, WT vs. KO: P=0.049]. The highest dose of guanfacine (1 mg/kg) increased *%omissions* in both genotypes to a similar extent [VEH vs. GFC1, WT: P<0.001, KO: P<0.001].

Another measure of attention, %accuracy, was also reduced by guanfacine [[ARCSIN]drug: $F_{(3,49)}$=3.57, P=0.020, Figure 3.4C], but this effect did not depend on genotype [[ARCSIN]drug*geno: $F_{(3,49)}$=0.49, P=0.692].

3.3.4 Guanfacine attenuates premature responding in wildtype and NK1R-/- mice

NK1R-/- mice expressed more *%premature responses* than wildtypes overall, as previously reported [[SQRT]geno: $F_{(1,18)}$=8.39, P=0.010, Figure 3.4D]. Guanfacine reduced the frequency of this behaviour, [[SQRT]drug: $F_{(3,49)}$=9.45, P<0.001], and this effect was apparent at the highest dose of drug (1 mg/kg), only [VEH vs. GFC1, WT: P=0.012, KO: P<0.001]. However, the effect of the drug did not depend on genotype [[SQRT]drug*geno: $F_{(3,49)}$=0.39, P=0.759].

3.3.5 Guanfacine does not affect perseveration

Perseveration did not depend on genotype [[SQRT]geno: $F_{(1,19)}$=1.31, P=0.267, Figure 3.4E] and guanfacine had no effect on this behaviour [[SQRT]drug: $F_{(4,66)}$=0.80, P=0.528]. Moreover, the two factors did not interact [[SQRT]geno*drug: $F_{(4,66)}$=0.95, P=0.440].

3.3.6 Guanfacine blunts behaviour in measures of arousal and motivation

The *total number of trials*, *latency to correct response* and *latency to magazine* were blunted by guanfacine in both genotypes (Figure 3.4F, G and H). Guanfacine decreased *total trials* [[ARCSIN]*drug*: $F_{(3,49)}$=3.84, P=0.015], and increased both latencies overall [*Latency to correct*; [LOG10]*drug*: $F_{(3,49)}$=16.47, P<0.001. *Latency to magazine*; [[RANK]*drug*: $F_{(3,49)}$=39.42, P<0.001]. The effect of guanfacine did not depend on genotype in any of these measures.

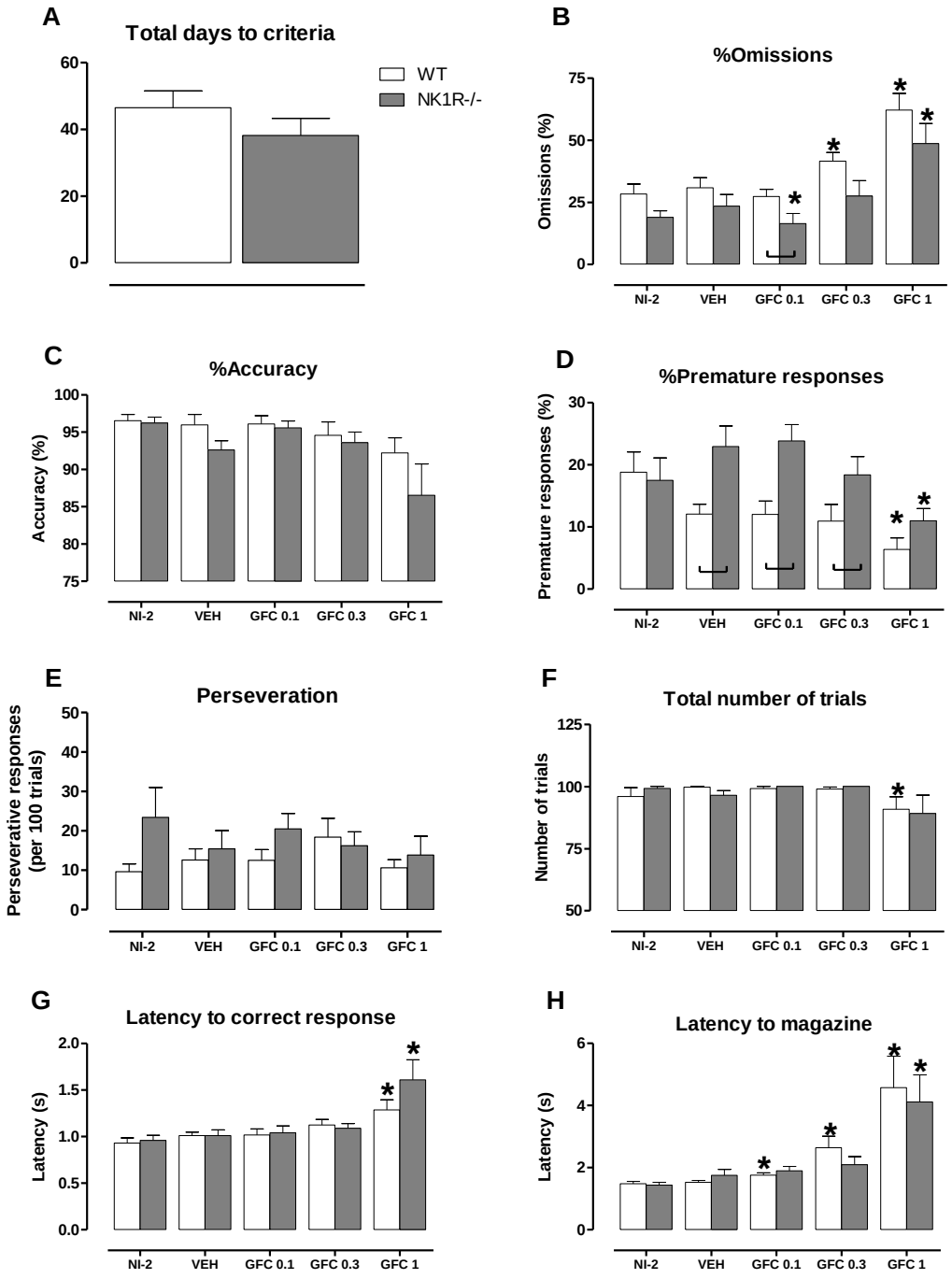

Figure 3.4 – A: The total number of days taken to reach testing criteria in the 5-CSRTT by wildtype and NK1R-/- mice, and the effects of guanfacine (0.1, 0.3 and 1 mg/kg, i.p.) on B: total number of trials, C: %accuracy, D: %omissions, E: premature responses, F: perseveration, G: latency to correct response and H: latency to magazine, compared with vehicle (saline) and no injection (NI-2). Data show mean ± SEM. n=9-10 per group. Lines linking bars indicate statistical significance of P<0.05, * indicates P<0.05 versus vehicle within genotype.

3.4 Discussion

The aim of these experiments was to test the prediction that the effects of guanfacine on ADHD-like behaviours displayed by NK1R-/- mice mirror those seen in ADHD. In line with this prediction the results suggest that guanfacine reduces locomotor activity, improves attention and reduces impulsivity in NK1R-/- mice. However, the cognitive effects depend on dose, and the effects on impulsivity in particular may be secondary to a reduction in arousal.

3.4.1 Effects of guanfacine on hyperactivity

The results of the LDEB are consistent with our previous findings: naïve (uninjected) NK1R-/- mice are hyperactive compared with wildtypes (Herpfer *et al.* 2005). This is consistent with findings that acute administration of NK1R antagonists (L733060 or RP67580) induces locomotor hyperactivity in wildtype mice (Yan *et al.* 2010). Together these results strongly suggest functional NK1R are necessary for normal regulation of motor activity. However, vehicle injection alone was sufficient to abolish this hyperactivity. The interaction between the substance P/NK1R system and the response to stress has been well documented (Ebner and Singewald 2006). For example, acute stress or a novel arena, such as the light zone of the LDEB, increases noradrenaline release (Dalley and Stanford 1995; McQuade *et al.* 1999), but NK1R antagonists blunt this noradrenergic response (Renoldi and Invernizzi 2006; Ebner and Singewald 2007). How this interaction (between NK1R and the stress response) affects locomotor activity remains unclear, but the result presented here confirms that it is important to include an uninjected control when studying the behaviour of NK1R-/- mice.

Guanfacine reduced locomotor activity in both genotypes in the LDEB. This finding was corroborated by the results of the 5-CSRTT: guanfacine reduced measures of arousal (number of trials and both measures of latency) in both genotypes. Together this suggests that functional NK1R are not necessary for this response, at least when guanfacine is administered at a high dose, and the response can be attributed to the sedative effects of the drug (for review see Scheinin *et al.* 1989). Guanfacine is a less potent sedative than its sister drug, clonidine (Arnsten *et al.* 1988), and as such is used in preference to clonidine in treating ADHD.

Nevertheless, in the clinic, somnolence, sedation and fatigue are the most frequent reasons for discontinuation of guanfacine therapy (Faraone *et al.* 2013; Hirota *et al.* 2014).

The lowest dose of guanfacine reduced locomotor activity and number of returns to the light zone in NK1R-/- mice, but not in wildtypes, suggesting that NK1R-/- mice could be more sensitive to the drug at low doses. NK1R-/- mice have disruptions to noradrenergic signalling, including increased extracellular noradrenaline in the prefrontal cortex under anaesthesia and an augmented release of noradrenaline induced by a local pulse infusion of CSF with concentrated (80mM) K^+ in awake animals (Fisher *et al.* 2007; Yan *et al.* 2009; Yan *et al.* 2010), which could explain a difference in sensitivity to guanfacine. In line with the results reported here, guanfacine (1 mg/kg) reduces the activity of spontaneously hypertensive rats (SHRs) without affecting their control (WKY and Wistar rat) strains (Langen and Dost 2011), suggesting that SHRs are also more sensitive to guanfacine. The proposal that ADHD patients, particularly those with *TACR1* polymorphisms, could also be more sensitive to guanfacine than healthy subjects merits further investigation.

Although Crawley originally developed a light-dark test as a screen for anxiolytic drugs (Crawley 1981), in the present experiment, the apparatus was dimly lit (DZ: 4 lux, LZ: 20 lux) so as to render the light zone novel, but not strongly aversive. Animals were also habituated to the dark zone for 90 min, to enable them to recover from the stress of transfer to the apparatus (Dalley and Stanford 1995). Anxiolytics have not been tested with this arrangement or protocol, which would be necessary to validate any conclusions drawn about the effect of drugs on anxiety in this test. However, in this test, guanfacine did reduce the number of crosses between the light and dark zones, which could suggest an anxiogenic effect of the drug. In contrast, guanfacine reduced active avoidance (i.e. increased latency to leave the light zone), suggesting an anxiolytic effect. The likely explanation for both is that these behaviours were secondary to a reduction in arousal: mice simply moved more slowly, and moved between the two zones less frequently. This is corroborated by the finding that guanfacine had no effect on time spent in the light zone. Therefore it cannot be concluded from this experiment alone that guanfacine has an effect on anxiety-like behaviour, and moreover, any effects on locomotor activity are unlikely to be secondary to an effect on emotionality.

3.4.2 Guanfacine improves attention in NK1R-/- mice, only

In mice, sustained attention is best described in the 5-CSRTT by omission errors (Amitai and Markou 2010). Mice appear to have better entrainment to the light cue than rats, that is to say, that after failing to attend to the light cue, mice tend to withhold their response, instead of guessing (thus making an omission instead of an incorrect response). Therefore omission errors can be used as a good measure of attention in mice, whereas in rats, accuracy is a more appropriate measure (Amitai and Markou 2010). This theory is corroborated by the extremely high level of accuracy (~96%) in both genotypes at baseline.

Unlike in our previous studies (Yan *et al.* 2011; Dudley *et al.* 2013), here, NK1R-/- mice did not display an inattentive phenotype at baseline. The explanation for the difference may relate to normal variation within populations of animals, or relate to our previous report that repeated testing improves performance in the 5-CSRTT (Weir *et al.* 2014) (it should be noted that mice had already been tested in NI-1 in this experiment).

Nevertheless, at the lowest dose studied here, guanfacine selectively improved attention, by way of reducing omission errors in NK1R-/- mice, only. This improvement was modest (~7% *c.f.* vehicle), but is unlikely to be explained by a change in animals' state of arousal because, at this dose, guanfacine did not affect the latency to correct response or the total number of trials completed in the 5-CSRTT. Numerous preclinical studies have dissociated the sedative and cognitive effects of α2-AR agonists (Arnsten *et al.* 1988; Franowicz and Arnsten 1998; Jakala *et al.* 1999), for instance, the spatial working memory of rhesus monkeys was improved at a dose of guanfacine that had no sedative or hypotensive effects (Arnsten *et al.* 1988). This finding is also consistent with the improvement in attention when SHRs are tested in an operant conditioning task (Sagvolden 2006). Similarly, in tests of vigilance and working memory, activation of α2A-AR enhances performance of both rats and monkeys in delayed-alternation (Carlson *et al.* 1992) and delayed-response tasks (Arnsten *et al.* 1988), respectively. Low doses of α2A-AR agonists improve cognitive performance, particularly in animals with either artificial depletion of cortical noradrenaline (Milstein *et al.* 2007) or in older animals showing significant natural loss of this neurotransmitter (Arnsten *et al.* 1988).

An improved attention subscale score has been reported in ADHD patients upon GXR (guanfacine extended release) treatment, especially when given chronically (Biederman *et al.* 2008; Sallee *et al.* 2009; Sallee *et al.* 2012; Newcorn *et al.* 2013), or in combination with a psychostimulant (Wilens *et al.* 2012). One study has also reported that when ADHD patients are tested after GXR treatment, they similarly show an improvement on ADHD rating-scales, but no reduction in reaction speed in a Choice Reaction-Time test compared to placebo (Kollins *et al.* 2011).

A similar improvement was not seen in response accuracy. As stated above, accuracy is arguably an alternative index of attention (Robbins 2002), but here the extremely high level of accuracy at baseline may have prevented any improvement being detected. An improvement may be observable if more challenging task parameters are used, for example, shortening the stimulus duration (see Amitai and Markou 2011). There are disparate reports on the effects of guanfacine on this measure. Whereas accuracy was increased in one preclinical study of aged macaques (O'Neill *et al.* 2000), there was no such response in a human study of cognitive performance (Jakala *et al.* 1999). Conversely, depletion of cortical noradrenaline impairs accurate responding in rats performing the 5-CSRTT (Carli *et al.* 1983) and stop signal reaction-time task (Bari *et al.* 2011).

By contrast, the highest dose of guanfacine (1 mg/kg) increased omission errors (i.e. reduced attention) in both genotypes. This impairment is most likely explained by the well-documented sedative effects of this drug (Van der Laan *et al.* 1985; Jakala *et al.* 1999): both response latencies and the total number of total trials were blunted at this dose, in both genotypes. This is mirrored by studies of rats performing the 5-CSRTT, in which guanfacine and medetomidine (another α2-AR agonist) increase omission errors (Sirvio *et al.* 1994; Fernando *et al.* 2012). The neural mechanisms behind this are unclear, but corroborate the theory that noradrenergic signalling strongly influences prefrontal cortical regions mediating attention and working memory (for review see: Arnsten and Li 2005; Robbins and Roberts 2007). Moreover, the classic bell-shaped treatment / response curve applies: too much or too little cortical noradrenaline leads to suboptimal cognition.

The beneficial effects of guanfacine are thought to be mediated primarily by post-synaptic α2A-ARs located in the prefrontal cortex (PFC). Indeed, deficits in working memory in mice with functional ablation of α2A-ARs are not relieved by guanfacine (Franowicz *et al.* 2002). However, guanfacine could also reduce inattentiveness by activating somatodendritic α2A-ARs, in the locus coeruleus (LC). This nucleus, which is the sole source of noradrenaline in the PFC (Loughlin *et al.* 1982), receives inputs from both GABAergic and glutamatergic projection neurones, from the prepositus hypoglossi (PH) and nucleus paragigantocellularis (PGC), respectively (Ennis and Aston-Jones 1989; Aston-Jones *et al.* 1991). Whereas GABAergic neurones tonically inhibit LC neurones, glutamate triggers their burst-spiking in response to sensory stimuli (Foote *et al.* 1980; Ennis and Aston-Jones 1988; Kawahara *et al.* 1999). Although the mechanism which renders NK1R-/- mice more sensitive to guanfacine was not investigated here, it has been reported that antagonism or functional ablation of NK1R blunts this GABAergic inhibition (Maubach *et al.* 2002; Ebner and Singewald 2007), and so such disinhibition could disrupt the noradrenergic response to guanfacine.

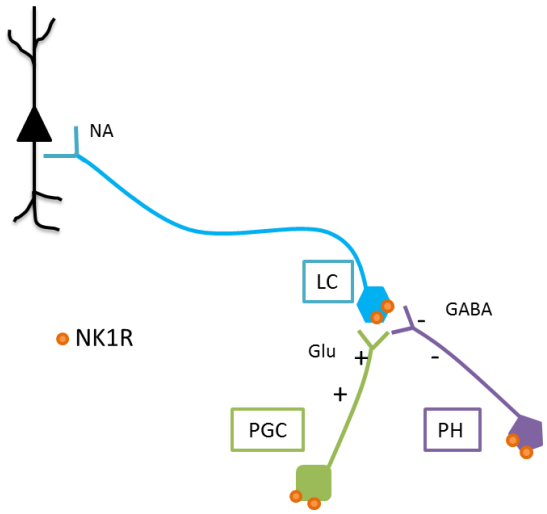

Figure 3.5 – Schematic of GABAergic and glutamatergic inputs to the locus coeruleus (LC), from the prepositus hypoglossi (PH) and nucleus paragigantocellularis (PGC), respectively, showing NK1R localization.

3.4.3 Guanfacine reduces impulsivity

The findings regarding premature responses are concordant with previous reports from this group: NK1R-/- mice were more impulsive than wildtypes, although this was not evident unless

mice had experienced an injection, reiterating the connection between NK1R and stress (see Ebner and Singewald 2006).

Guanfacine reduced impulsivity in both genotypes: at the lower, non-sedative doses (0.1-0.3 mg/kg), guanfacine's effects on impulsivity were not statistically significant, but the high dose (1 mg/kg) decreased impulsivity in wildtype and NK1R-/- mice. Evidently, this improvement does not depend on functional NK1R but could be secondary to a non-specific inhibition of motor behaviour. Since the low doses of guanfacine did affect attention, this also suggests that impulsivity and attention are controlled by different neurocircuitry, although one may influence the other. This corroborates findings by Dudley *et al* (2013), which suggested the same inconsistency between inattentiveness and impulsivity.

Reports have indicated that guanfacine improves impulsivity in rodents. However, these reports are remarkably similar to our own: High Impulsive and Low Impulsive rats displayed decreases in impulsivity in the 5-CSRTT upon guanfacine treatment, but these were similarly accompanied by increases in response latencies (Fernando *et al.* 2012). In contrast, atipamezole (an α2-AR antagonist), increased the number of premature responses made by rats in the 5-CSRTT (Jakala *et al.* 1992). Other paradigms measuring impulsivity, such as the stop signal reaction-time task, have produced similar results: guanfacine reduces impulsivity, but this is in parallel with slowed reaction speeds (Bari *et al.* 2009).

It is important to note that impulsivity is not a unitary construct. If a non-specific blunting of behaviour also explains the efficacy of this drug in the clinic, then it is possible that forms of impulsivity relying on motor activity are more likely to be attenuated than those which are based on cognition. However, it was recently reported that local infusion of guanfacine into the ventral hippocampus of rats causes an improvement in impulsive choice (the considered choosing of small, immediate rewards over larger, delayed rewards) without affecting response latencies, in a delay discounting task (Abela and Chudasama 2014). This improvement was not mirrored by dopaminergic agents, suggesting the effects were due to α2A-AR stimulation, rather than downstream effects on dopamine. Similarly, rhesus monkeys treated with guanfacine chose larger, delayed rewards over smaller, immediate rewards, in an impulsive

choice paradigm (Kim *et al.* 2012), suggesting that 'top-down' effects could certainly underlie the efficacy of this drug in the clinic.

Here, the improvement in impulsivity was likely because this test is one of motoric impulsivity, and guanfacine simply reduced motor behaviour. Therefore it may be of interest to test NK1R-/- mice in an impulsive choice paradigm, such as the delayed discounting test, to determine whether guanfacine could also improve impulsive behaviour which is not determined by motor activity.

3.5 Highlights

- NK1R-/- mice display hyperactivity and impulsivity in the LDEB and 5-CSRTT, respectively.
- NK1R-/- mice show an abnormal stress response, confirming the involvement of substance P and NK1Rs in stress.
- Guanfacine is a potent inhibitor of arousal; high doses lead to non-specific blunting of behaviour in both experiments.
- Guanfacine can improve attention at low doses, but on the whole leads to decreased attention in the 5-CSRTT.
- Guanfacine improves impulsivity, but this may be secondary to decreased arousal.

Chapter 4

The effects of alpha2-adrenoceptor agonists on anxiety and spatial memory

Chapter 4. The effects of alpha2-adrenoceptor agonists on anxiety and spatial memory

4.1 Introduction

The results of Chapter 3 indicated that a low dose (0.1 mg/kg) of guanfacine reduced locomotor activity and improved attention in NK1R-/- mice, only, in the LDEB and 5-CSRTT, respectively. The possibility that these results were underpinned by changes in anxiety-like behaviour and spatial working memory (SWM) is worth investigating, not least because ADHD can be comorbid with anxiety (Sobanski 2006) and deficits in SWM (Vance *et al.* 2013).

4.1.1 Anxiety

Anxiety describes a normal emotion, which can persist chronically or reach pathological levels. Anxiety is common in psychiatric conditions but can also be found independently. The term anxiety broadly refers to two categories; 'state' and 'trait' anxiety. The former refers to acute bouts of anxiety (i.e. situational anxiety), that may be associated with a stressor, and that declines once the stressor is removed. The latter refers to a chronic anxious state which may be heritable, such as in generalized anxiety disorder (GAD). The two categories are separable, in part by their respective pharmacology and therapeutics: for example, MAO inhibitors will prevent panic attacks, but have little effect on baseline anxiety between attacks (Nutt 1990). This Chapter will refer to 'trait' anxiety throughout as it generally discusses the phenotype of NK1R-/- mice.

The pharmacology of anxiety is complex, but interesting given that drugs can actually cause, as well as relieve, anxiety. Benzodiazepines and tricyclic antidepressants were the first classes of drugs to be used to treat GAD, but their use is limited by safety and tolerability issues; selective serotonin reuptake inhibitors and pregabalin are now the first-line treatments for GAD (Both *et al.* 2014).

4.1.2 The noradrenergic system in anxiety

Considerable attention has also been given to α and β adrenoceptor agents, because of well-established links between the noradrenergic system and anxiety. Redmond and Huang were the first to describe that electrical stimulation of the locus coeruleus (LC) evokes behavioural responses in primates which are indicative of increased anxiety or fear (Redmond et al. 1976). Moreover, these responses could be alleviated by clonidine (an α2-adrenoceptor agonist) (Redmond and Huang 1979). In contrast, lesions to LC projections lead to a reduction in fear/anxiety-related behaviour in rats (Verleye and Bernet 1983). In humans, α2-adrenoceptor agonists, which reduce LC activity, also reduce the fear-potentiated startle response (Kumari *et al.* 1996), whereas α2-adrenoceptor antagonists have the opposite effect (Morgan *et al.* 1993).

Gray and McNaughton later proposed that anxiolytic drugs of all types act on a 'behavioural inhibition' system, which mainly comprises the septo-hippocampal system (SHS) (Gray and McNaughton 2003). It was suggested that the rhythmic burst firing of the SHS (known as theta activity) is disrupted by anxiolytics, and lesions to the SHS produce qualitatively similar behaviour as anxiolytic drugs (McNaughton and Gray 2000). It was hypothesized that noradrenergic inputs to the hippocampus (solely from the LC) act as a 'gate' of this theta activity, as lesions to the dorsal ascending noradrenergic bundle (DNAB) also reproduce the effects of anxiolytic drugs (McNaughton and Gray 2000). By this token, it might be expected that stimulation of α2-adrenoceptor (α2-AR) autoreceptors yields an anxiolytic effect, by reducing noradrenergic tone.

However, the evidence for the involvement of α2-ARs in anxiety in a preclinical setting remains mixed. For example, studies have shown that α2-AR antagonists produce anxiogenic effects (Handley and Mithani 1984; Pellow *et al.* 1985; Uzsoki *et al.* 2011) or no effect at all (Durcan *et al.* 1989). Similar discrepancies have been reported for α2-AR agonists: Uzsoski *et al* (2011) found guanfacine to be *anxiogenic* in rats on the elevated plus maze (EPM), whereas in another study, guanfacine was *anxiolytic* in SHRs on the EPM, but without effect in WKY and Wistar rats, even though all strains displayed the same level of anxiety at baseline (Langen and Dost 2011). The latter finding might imply that guanfacine has different effects in normal animals,

and those which express behavioural abnormalities, which is of interest given the findings reported in Chapter 3.

Although reviewing the efficacy of α2-adrenoceptor (α2-AR) agonists for the treatment of anxiety in the clinic is beyond the scope of this thesis, there is some evidence that clonidine and guanfacine are efficacious for this indication: for example, clonidine reduces anxiety in panic disorder (Nutt 1989; Uhde *et al.* 1989) and in withdrawing alcoholics (Glue and Nutt 1987). Guanfacine may be efficacious in treating post-traumatic stress disorder (PTSD) (Connor *et al.* 2013) and ADHD with comorbid PTSD (Connor *et al.* 2013).

4.1.3 ADHD and anxiety are comorbid

The fact guanfacine is used as a treatment for both ADHD and anxiety disorders is interesting, given that the two are often comorbid (Sobanski 2006). This might suggest that the circuitry involved in both is overlapping. Of adults with ADHD, 40-60% are estimated to suffer from an anxiety disorder at some point in their life (Sobanski 2006). Despite this statistic, few studies have examined the impact one disorder has on the other. However, there is some evidence for an interaction between the two: Manchini *et al* (1999) report that patients with anxiety disorders have an earlier age of anxiety onset and more severe anxiety if they had childhood ADHD. There is also evidence that this relationship is bidirectional: ADHD patients with comorbid anxiety had more pronounced attentional deficits than those without comorbid anxiety (Sobanski 2006).

4.1.4 Anxiety is modulated by rodent strain

Any discrepancies between the findings of preclinical research (for review see Haller and Alicki 2012) may arise from a whole host of factors, but a particular factor to consider in rodent tests of anxiety is background strain. Studies have repeatedly shown that strain influences anxiety, sometimes to a greater extent than anxiolytic/anxiogenic drugs (for review see Crawley *et al.* 1997; Sartori *et al.* 2011). The NK1R-/- mouse has been bred on a variety of different background strains since its creation (see Table 4.1). Studies of these other NK1R-/- mice have used different tests in addition to the EPM, such as measuring ultrasonic vocalizations and tail

suspension tests (Rupniak *et al.* 2001; Santarelli *et al.* 2001), but these tests have not been validated as a screen for anxiolytics. The results of Chapter 3, and those reported in Fisher *et al.* (2007), suggest that NK1R-/- mice display a slightly anxiogenic phenotype, since they spend less time in the light zone of the LDEB than wildtypes. However, the same limitation applies: the LDEB has not been validated as a screen of anxiolytic drugs with the current protocol. To that end, it is of interest to determine the anxiety-like phenotype of NK1R-/- mice on the current, mixed background strain, in the EPM.

Background strain	Findings	Reference
129/SvEv	↑ time on EPM open arms	(Santarelli *et al.* 2001)
J129/C57	No difference in time on EPM open arms	(Rupniak *et al.* 2001)
129/Sv/C57Bl6 x MF1	No difference in time on EPM open arms	(Murtra *et al.* 2000)
	↓ time on EPM open arms	Unpublished observations
	↓ time in the light zone of the LDEB	(Fisher *et al.* 2007)

Table 4.1 – Summary of findings regarding the anxiety state of NK1R-/- mice on different background strains. ↑ or ↓ indicates increase or decrease, respectively, compared to wildtypes of the same background strain.

4.1.5 Spatial memory

Although in the clinic, guanfacine's efficacy is generally discussed in terms of effects on inattention, the majority of preclinical work on guanfacine has revolved around 'spatial working memory' (SWM). Spatial memory refers to an individual's ability to remember the location and orientation of objects/surroundings in an environment. Working memory is generally synonymous with short term memory, and involves executive (top down) control/processing of temporarily held information in order to complete goal-directed actions. SWM is a combination of the two: an executive function dependent on recall of spatial information.

Arnsten and colleagues report that guanfacine improves SWM in rhesus monkeys (Franowicz and Arnsten 2002; Arnsten and Jin 2012; Kim *et al.* 2012) and mice (Franowicz *et al.* 2002). What is more, guanfacine can alleviate SWM deficits when they are induced by different methods: phencyclidine and hypobaric hypoxia-induced deficits in rats' SWM were both improved by α2-AR agonist (guanfacine and clonidine) treatment in separate studies (Jentsch

and Anzivino 2004; Marrs *et al.* 2005; Kauser *et al.* 2014). These studies strongly suggest that activation of α2-ARs is involved in optimum SWM (reviewed in Arnsten 2011).

This is interesting given that spatial working memory is disrupted in ADHD (Vance *et al.* 2013), and guanfacine alleviates inattentiveness in ADHD (Faraone *et al.* 2013). Impairments in SWM in ADHD may be a result of decreased attention, or indeed vice versa: selective attention may depend on SWM. To that end, it is of interest to examine the spatial memory of NK1R-/- mice. If deficits in attention in the 5-CSRTT are a result of impaired SWM, it might be expected that their spatial memory is also disrupted.

4.1.6 Aims

The first aim of these experiments was to investigate whether NK1R-/- mice (on the current, mixed background strain) display an anxiogenic phenotype, and moreover, whether guanfacine reduces anxiety-like behaviour at a dose (0.1 mg/kg) which reduced locomotor activity of these mice in the LDEB (see Chapter 3).

The second aim was to investigate whether NK1R-/- mice display deficits spatial memory. To determine whether any deficit in spatial memory was a result of a general disruption to memory function, we also tested the recognition memory of these mice. An additional aim was to determine whether the guanfacine-induced improvement in attention displayed by NK1R-/- mice in the 5-CSRTT (reported in Chapter 3) was underpinned by an improvement in spatial memory.

4.2 Methods

4.2.1 Elevated Plus Maze

The elevated plus maze (EPM) was performed as described in Chapter 2, section 2.4.4. Mice were treated with either vehicle (saline), guanfacine (0.1 mg/kg) or no injection, 30 min before testing. Treatments were assigned in a counterbalanced order. The dose of drug was chosen based on the results presented in Chapter 3: specifically, 0.1 mg/kg guanfacine improved

attention in NK1R-/- mice, only, but did not affect locomotor activity of either genotype. Mice were tested one at a time: the mouse was placed in the centre square of the plus maze, always facing between an open and closed arm, and allowed to explore the maze while its behaviour was recorded for 5 min. The maze was cleaned between each test with 70% ethanol. Behaviour (see Table 2.8 in Chapter 2) was later scored blind.

4.2.2 Novel object recognition and location

The novel object recognition (NOR) and location (NOL) protocol followed that described in Chapter 2, section 2.4.5. One wildtype and one NK1R-/- mouse were always tested simultaneously to minimize the effects of any nuisance factors, and all apparatus was cleaned with 70% ethanol between tests. Objects used were counterbalanced to avoid any potential inherent aversion to the objects confounding the results.

Experiment 1: Naïve mice

The first experiment used naive mice, only, in the NOR and NOL. The two object recognition tests were counterbalanced, and each mouse completed both tests over 2 consecutive days (see Figure 4.1).

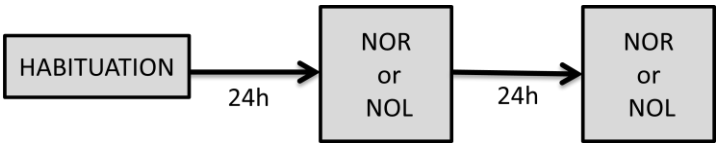

Figure 4.1 – Sequence of events in the novel object tests in naive mice

Experiment 2: The effects of guanfacine

Once baseline object recognition in the two genotypes had been established, the effects of guanfacine on object recognition were studied. In this experiment, mice completed either the NOR, or the NOL (not both), to avoid carryover effects of the drug, and because of a general observation from the first experiment that mice explore objects less with repeated testing. Mice received vehicle (saline) or guanfacine (0.1 mg/kg, i.p.) 30 min before the first trial, and drug treatments were assigned in a counterbalanced fashion. The dose of guanfacine tested

was based on the finding reported in Chapter 3, that 0.1 mg/kg guanfacine improves attention in NK1R-/- mice, only.

Experiment 3: The effects of medetomidine

To determine whether the effects of guanfacine on spatial memory observed in Experiment 2 were due to activation of alpha2A-adrenoceptors, another alpha2-adrenoceptor agonist, medetomidine, was tested. Because guanfacine only affected behaviour in the NOL in Experiment 2, medetomidine was tested in wildtypes and NK1R-/- mice in the NOL test, only. Medetomidine (1, 3 and 10 μg/kg, i.p.) or vehicle (saline) was administered 30 min before the first trial, and drug treatments were counterbalanced. Doses of medetomidine were based on a survey of recent literature (see Appendix 3).

In all experiments, an index of discrimination was used to determine whether mice explored the novel object/location at a greater than chance level, and to take into account any differences in total time exploring the objects.

Discrimination Index = (N – F) / (N + F)

Where 'N' is the time exploring the novel object/location, and 'F' is the time exploring the familiar object/location. This gives a value between -1 and +1, with chance level being zero, positive values showing preference for the novel object and negative values showing preference for the familiar object.

4.2.3 Statistics

Data were analysed as described in Chapter 2, section 2.6. In the EPM, two-way ANOVAs were performed using the main factors of '*genotype*' and '*drug treatment'*. First, the ANOVA compared the factors across all groups (uninjected, vehicle and drug). Where there was a main effect of '*genotype'* or '*drug',* or an interaction between them, post-hoc LSD tests were performed to determine the difference in individual groups.

The NOR and NOL tests were analysed in two different ways, depending on the measurement: repeated measures analyses were performed to investigate the main factors of '*genotype'* and

'*drug'*, using the repeated factor of '*object',* when comparing exploration of the novel and familiar objects. Two way ANOVAs were performed using '*drug'* and '*genotype*' as main factors, when analysing the discrimination index.

Analyses of both experiments were performed on raw or transformed data (arcsine, log10 or square-root), whichever gave the best homogeneity of variance in the 'predicted *vs.* residuals' plot in *InVivoStat*. The 'normal probability plot' in *InVivoStat* was used to test whether the data were normally distributed. If not, a rank transformation was applied, i.e. the data were ranked as for a non-parametric analysis, and were then subject to the parametric test. The transformation applied is noted in square brackets throughout. Statistical significance was set at $P<0.05$.

4.3 Results

4.3.1 NK1R-/- mice display an anxiogenic phenotype in the EPM

In the EPM there was a clear difference between the genotypes in the baseline level of *anxiety* (Figure 4.2). NK1R-/- mice displayed more anxiety-like behaviour than wildtypes in all measures (Table 4.2). There were no differences, however, in the baseline level of *activity* between the genotypes (as measured by entries to the closed arms), or in the time spent in the centre square.

4.3.2 Guanfacine does not affect anxiety-like behaviour in wildtype and NK1R-/- mice

Guanfacine had no effect on the measures of anxiety-like behaviour in the EPM, or on activity, and did not interact with genotype (Table 4.2).

	Main effect			*Post-hoc: WT vs. NK1R-/-*		
Behaviour	***Genotype***	***Drug***	***Drug* Genotype***	**NI**	**Veh**	**GFC**
%Time on open arms	$F_{(1,24)}$= 23.38 **P<0.001**	$F_{(1,16)}$=0.32 P=0.578	$F_{(1,16)}$=0.87 P=0.365	**P=0.009**	**P=0.002**	**P=0.044**
Number of entries to open arms	$F_{(1,24)}$= 42.32 **P<0.001**	$F_{(1,16)}$=2.32 P=0.147	$F_{(2,24)}$=0.37 P=0.551	**P=0.001**	**P=0.002**	**P=0.001**
Number of head entries to open arms	$F_{(1,24)}$=14.94 **P<0.001**	$F_{(1,16)}$=0.01 P=0.938	$F_{(1,16)}$=1.59 P=0.226	**P=0.048**	P=0.179	**P=0.004**
Number of entries to closed arms	$F_{(1,24)}$=2.64 P=0.117	$F_{(1,16)}$=1.36 P=0.260	$F_{(1,16)}$= 0.39 P=0.543	---	---	---
%Time in centre square	$F_{(1,24)}$=2.08 P=0.162	$F_{(1,16)}$=0.06 P=0.815	$F_{(1,16)}$=1.01 P=0.330	---	---	---

Table 4.2 – The results of statistical analyses on measures of anxiety in the EPM, showing main effects of genotype and drug, and post-hoc comparisons showing genotype differences, only. P values in bold show P<0.05. ---: not applicable.

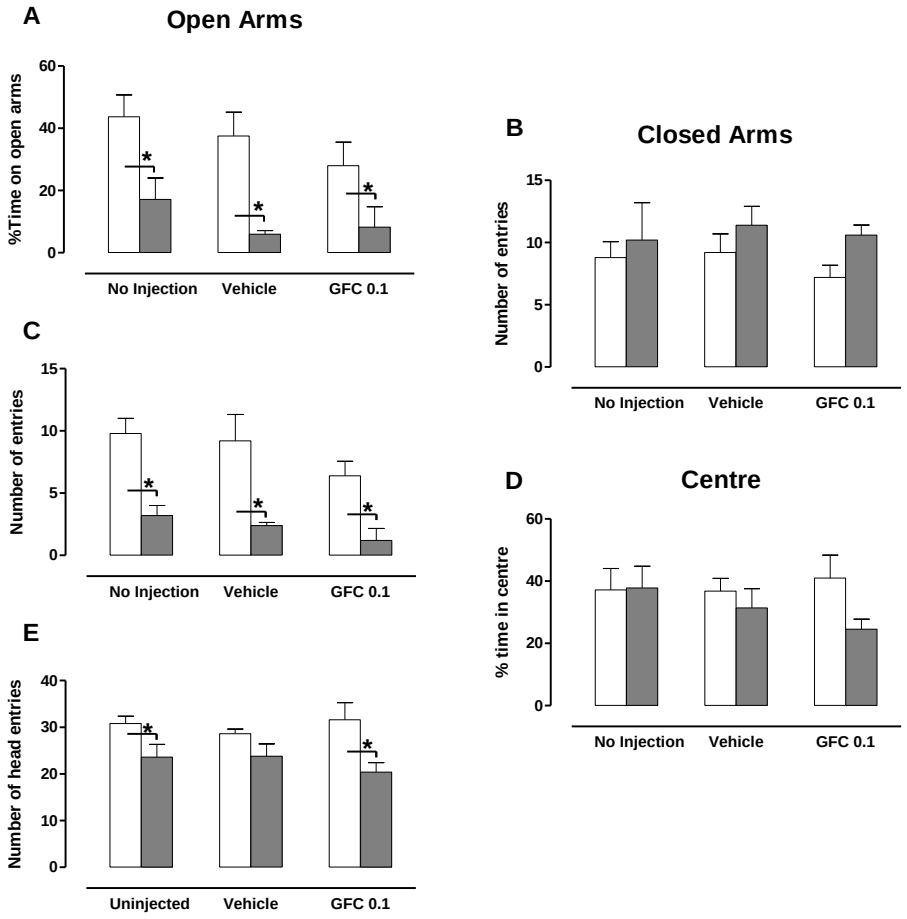

Figure 4.2 – The effects of guanfacine and vehicle injection in NK1R-/- (grey bars) and wildtype (white bars) mice on A: %time spent in the open arms, B: number of entries to the closed arms, C: number of entries to the open arms, D: %time spent in the centre section of the elevated plus maze and E: number of head entries to the open arms. Data show mean ± SEM. * P<0.05, n=5.

4.3.3 Wildtype and NK1R-/- mice have intact recognition and spatial memory

The NOR and NOL tests in naïve mice revealed that both genotypes had intact recognition and spatial memory. In the NOR, both genotypes spent more *time exploring* the novel object [[SQRT]object: $F_{(1,14)}$=41.24, P<0.001, Figure 4.3], and made more *returns* to the novel object [[SQRT]object: $F_{(1,14)}$=27.95, P<0.001]. The same result was seen in the NOL in terms of *time exploring* [[SQRT]object: $F_{(1,10)}$= 12.21, P=0.006] but not *number of returns* [[SQRT]object: $F_{(1,10)}$=2.55, P=0.141]. On the whole there were no differences between the genotypes, but there was a genotype difference in *number of returns* to the novel object in the NOR [[SQRT]geno: $F_{(1,14)}$= 5.28, P=0.038], such that NK1R-/- mice made more returns to both objects (i.e. were more active overall).

Because mice completed both the NOR and NOL in a counterbalanced fashion, direct comparisons between the two tests were valid. Comparison of the NOR and NOL showed that the *discrimination index* was overall higher in the NOR than the NOL [[RAW]type of test: $F_{(1,24)}$= 15.00, P<0.001, Figure 4.4], but this was independent of genotype [[RAW]geno*type of test: $F_{(1,24)}$=0.06, P=0.804], such that both genotypes performed better in the NOR than the NOL.

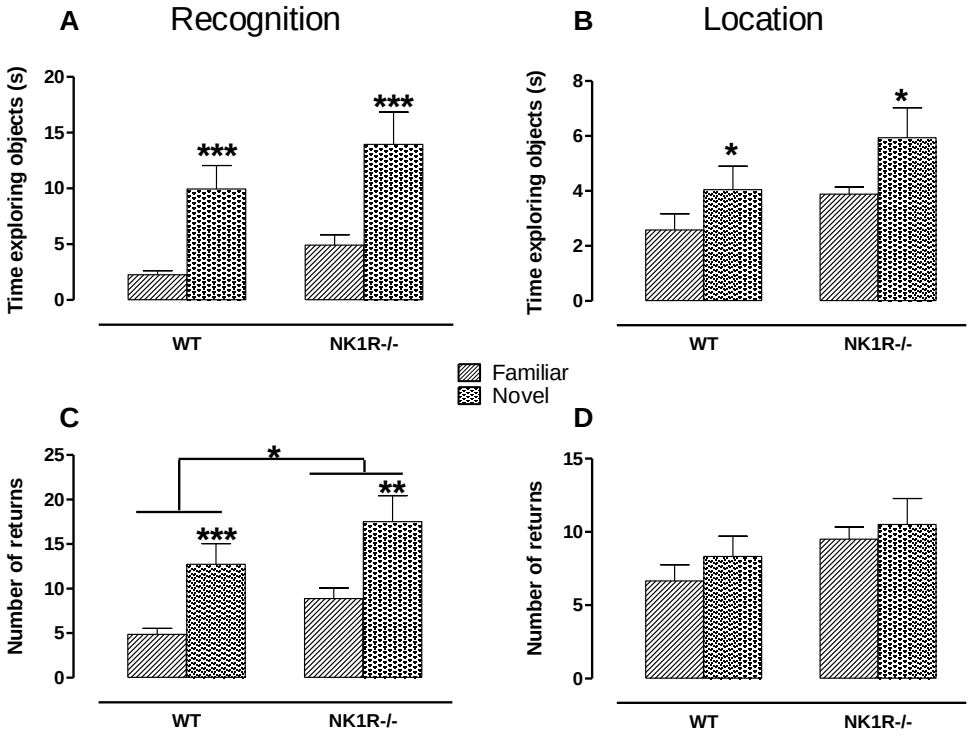

Figure 4.3 – Time exploring objects (A: NOR and B: NOL) and number of returns to objects (C: NOR and D: NOL) made by NK1R-/- and wildtype mice in the novel object recognition and novel object location task. Data show mean ± SEM. * P<0.05, n=6-8.

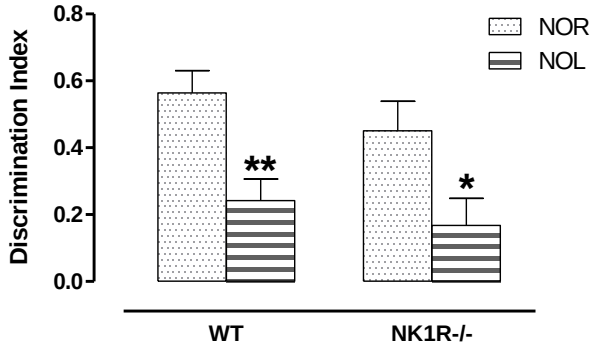

Figure 4.4 – Recognition (NOR) and spatial memory (NOL) memory of NK1R-/- and wildtype mice as determined by an index of discrimination. Data show mean ± SEM. * P<0.05, n=6-8.

4.3.4 Guanfacine has no effect on recognition memory, but improves spatial memory in NK1R-/- mice

When guanfacine was tested in mice in the NOR, all mice spent more *time exploring* the novel object than the familiar one, and made more *returns* to the novel object (Table 4.3, Figure 4.5). This was independent of genotype and drug treatment, and these factors did not interact. Guanfacine also had no effect on the *index of discrimination* in either genotype.

In the NOL, all mice explored the novel object more than the familiar one (see Table 4.3). However, the effect of drug on the *discrimination index* just missed statistical significance [[RANK]drug: $F_{(1,12)}$=4.43, P=0.057]. Nevertheless, the effect of guanfacine did depend on genotype [[RANK]drug*geno: $F_{(1,12)}$=5.00, P=0.045]. NK1R-/- mice treated with guanfacine displayed enhanced spatial memory compared to vehicle treated NK1R-/- mice (NK1R-/-, VEH vs. GFC: P=0.010) and compared to guanfacine-treated wildtypes (GFC, WT vs. KO: P=0.023).

NOR *Main effect*	***Genotype***	***Object***	***Drug***
Time spent exploring objects	$F_{(1,12)}$=0.37 P=0.552	$F_{(1,12)}$=73.84 **P< 0.001**	$F_{(1,12)}$=0.11 P=0.747
Number of returns to objects	$F_{(1,12)}$=0.12 P=0.740	$F_{(1,12)}$=32.77 **P<0.001**	$F_{(1,12)}$=0.12 P=0.7401
Discrimination Index	$F_{(1,12)}$=0.71 P=0.415	N/A	$F_{(1,12)}$=0.03 P=0.859
NOL			
Time spent exploring objects	$F_{(1,12)}$=1.61 P=0.229	$F_{(1,12)}$=114.59 **P<0.001**	$F_{(1,12)}$=0.02 P=0.893
Number of returns to objects	$F_{(1,12)}$=4.23 P=0.062	$F_{(1,12)}$= 52.83 **P<0.001**	$F_{(1,12)}$=0.61 P=0.452
Discrimination Index	$F_{(1,12)}$= 2.09 P=0.1737	N/A	$F_{(1,12)}$=4.43 **P=0.057**

Table 4.3 –The results of statistical analyses on behaviour in the NOR and NOL tests, showing main effects of genotype, object and drug. P values in bold show P<0.05.

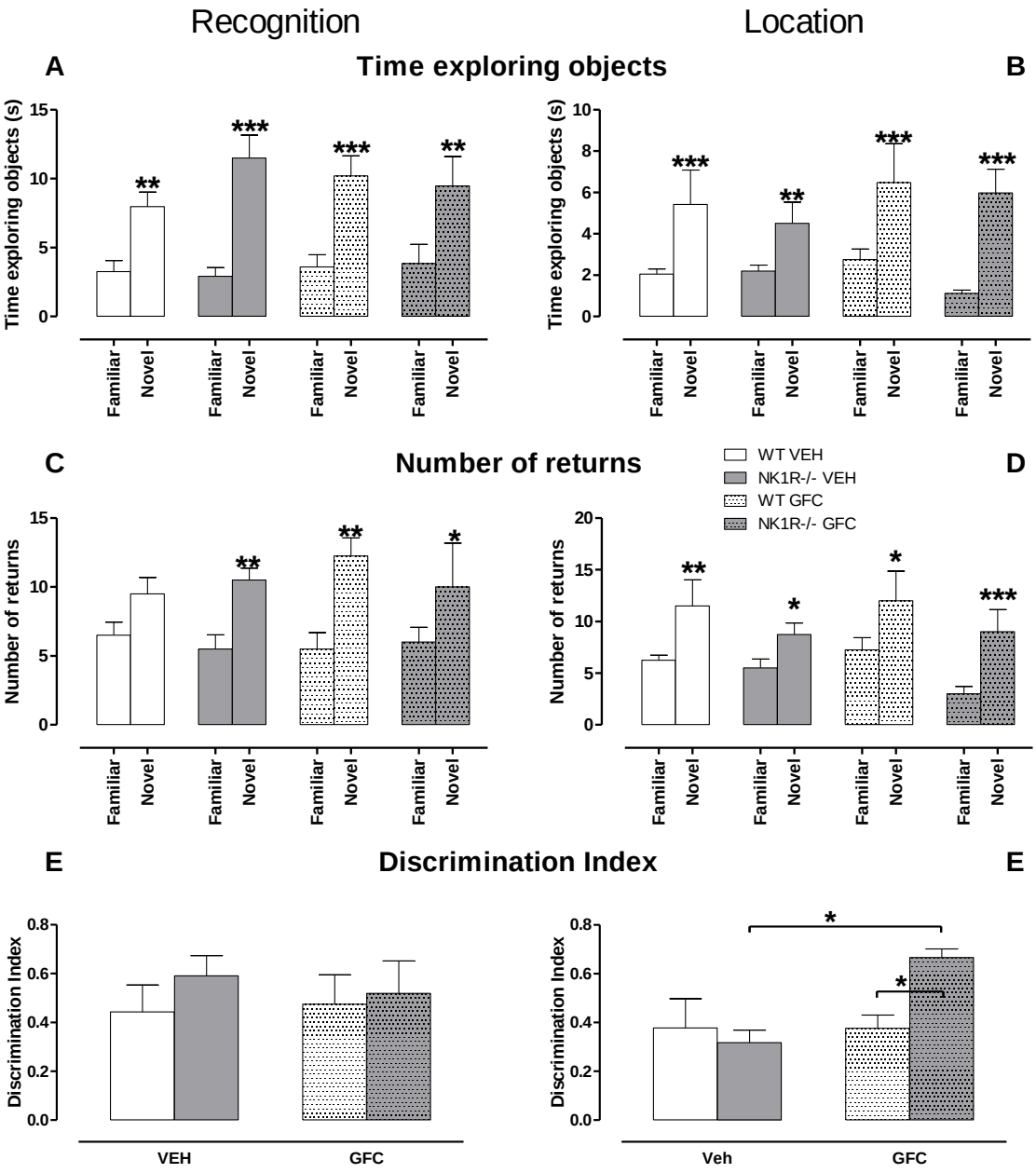

Figure 4.5 – The effects of guanfacine (0.1 mg/kg) on time spent exploring objects (A: NOR and B: NOL), the number of returns to objects (C: NOR and D: NOL), and an index of discrimination (E: NOR and F: NOL) in NK1R-/- and wildtype mice. Data show mean ± SEM. * P<0.05, n=4.

4.3.5 Medetomidine improves spatial memory in wildtype mice

When medetomidine was tested in mice in the NOL test, all mice spent more *time exploring* the novel object [[SQRT]object: $F_{(1,24)}$= 231.63, P<0.001, Figure 4.6], and made more *returns* to the novel object [[RAW]object: $F_{(1,24)}$= 84.48, P<0.001]. There were no effects of drug or genotype on the *time spent exploring*, or the *number of returns*. However, in the index of discrimination, the effect of medetomidine was bidirectional, and depended on genotype [[RAW]drug*geno: $F_{(3,24)}$= 2.91, P=0.05]: a low dose (1 μg/kg) of medetomidine facilitated spatial memory in wildtypes compared with vehicle, although this just missed significance (WT, VEH vs. MED1: P=0.052). However, medetomidine impaired memory at a high dose (10 μg/kg) in wildtypes, inducing a genotype difference, such that NK1R-/- mice had better spatial memory than wildtypes (MED10, WT vs. KO: P=0.037).

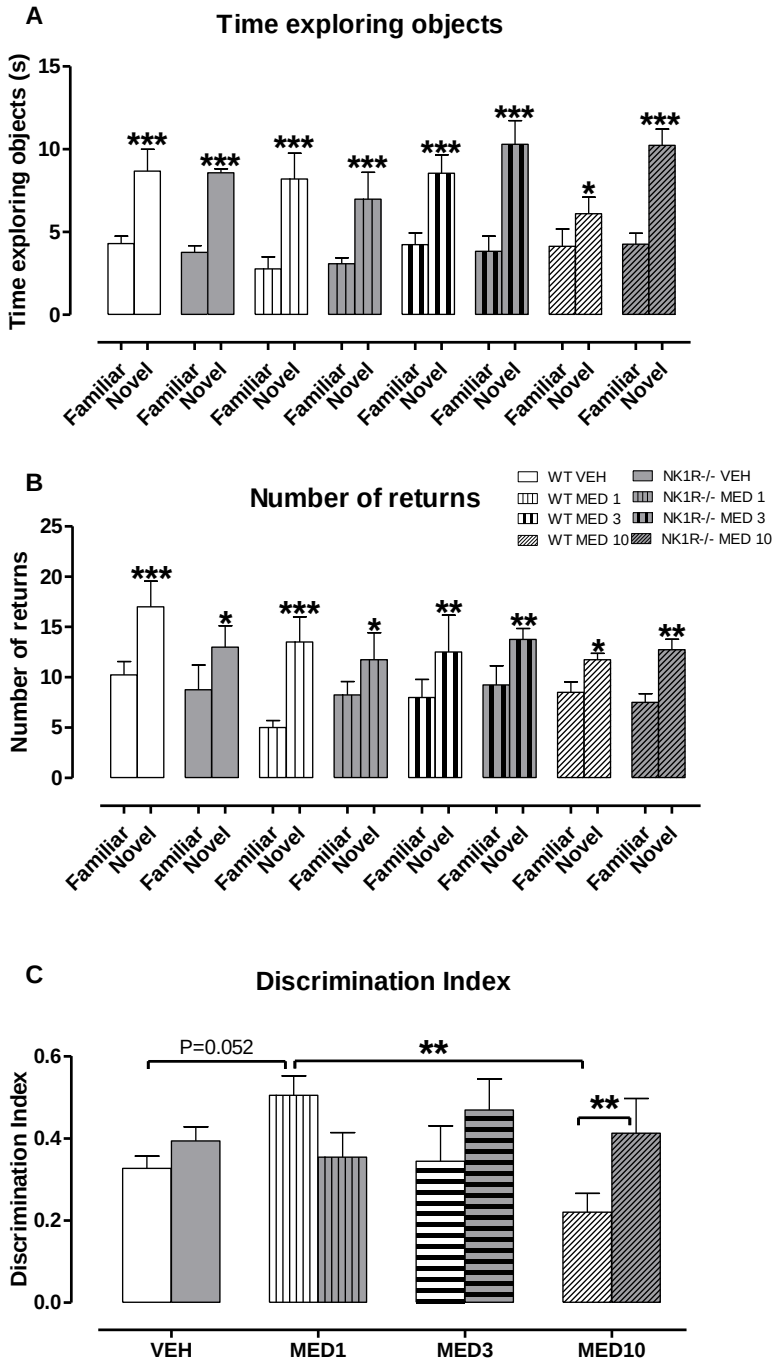

Figure 4.6 – The effects of medetomidine (1, 3 and 10 μg/kg) on A: time exploring objects, B: number of returns to objects, and C: an index of discrimination in wildtype and NK1R-/- mice in the novel object location test. Data show mean ± SEM. * P<0.05, n=4.

4.4 Discussion

The aims of these experiments were two-fold. The first aim was to determine whether NK1R-/- mice display an anxiogenic phenotype which could be alleviated by guanfacine. This hypothesis was borne out in part, as NK1R-/- mice displayed more anxiety-like behaviour than wildtypes. However, guanfacine had no appreciable effect on any measure of anxiety-like behaviour in the EPM, mirroring the results of the LDEB in Chapter 3.

The second aim was to determine whether NK1R-/- mice display deficits in recognition and spatial memory, in the NOR and NOL tests, respectively, which could be relieved by α2-AR agonists. This hypothesis was also partly supported by the results: NK1R-/- mice did not display any memory deficits, but α2-AR agonist treatment facilitated spatial memory, particularly in NK1R-/- mice.

4.4.1 NK1R-/- mice display an anxious phenotype

In the elevated plus maze, NK1R-/- mice displayed an anxiogenic phenotype compared with wildtypes. NK1R-/- mice spent less time (~17% vs. 44% in wildtypes) on the open arms, and made fewer entries to the open arms, than wildtypes. This mirrors the results from Chapter 3, in which NK1R-/- mice spent less time in the light zone of the LDEB than wildtypes. It is also consistent with previous reports that NK1R-/- mice express greater active and passive avoidance of the light zone of the LDEB (Herpfer *et al.*, 2005; Fisher *et al.*, 2007). Perhaps surprisingly, there was no difference between the time NK1R-/- and wildtype mice spent in the central square: an area which can be thought of as "protected", and thus it might be expected anxious NK1R-/- mice would spend more time in the central square than wildtypes (Rodgers and Dalvi 1997). However, NK1R-/- mice made fewer head entries to the open arms than wildtypes when situated in the central square, suggesting these mice were more cautious overall.

The results of this study do contrast with other studies of NK1R-/- mice (Santarelli *et al.* 2001). Although many factors, such as age, gender, maze height, prior exposure to novelty and light intensity affect baseline anxiety (Rodgers and Cole 1993) and will differ between studies, the

effect of functional ablation of the NK1R gene on anxiety-like behaviour seems to depend largely on background strain. Mice lacking NK1Rs on a 129/SvEv background spend a greater proportion of time on the open arms of an EPM than wildtypes (Santarelli *et al.* 2001). By contrast, NK1R-/- mice on a J129/C57 or a 129/Sv x C57BL/6 x MF1 hybrid background do not (Murtra *et al.* 2000; Rupniak *et al.* 2001). The effect of background strain on the temperament of rodents is well documented. For example, a comparison of 15 different mouse strains using the EPM found that anxiety levels differed greatly across the strains: BALB/cByJ mice being the least, and AKR/J mice being the most anxious (O'Leary *et al.* 2013). Here, it seems that behaviour of mice depends on an interaction between NK1R and the genetic background of the animal.

Guanfacine did not affect anxiety-like behaviour of either genotype in EPM. This also corroborates the finding from Chapter 3 that guanfacine did not affect the time spent in the light zone by either genotype in the LDEB. Moreover, changes in the behaviour of NK1R-/- mice in the LDEB, 5-CSRTT and novel object paradigms upon guanfacine treatment are unlikely to be secondary consequences of a change in anxiety-like behaviour. However, this finding does contrast with a report that this drug reduces anxiety-like behaviour of SHR, but not Wistar or WKY control rats, in the EPM (Langen and Dost 2011). This difference could relate to the fact that guanfacine has been developed as an anti-hypertensive (Jerie 1980), and by definition, SHRs are hypertensive animals, which could render them more susceptible to the effects of guanfacine.

4.4.2 NK1R-/- mice show intact recognition and spatial memory

Both wildtype and NK1R-/- mice displayed intact recognition and spatial memory when tested in the novel object recognition and location tasks, respectively. This is unsurprising, given that studies have consistently demonstrated NK1R-/- mice learn complex tasks (e.g. the 5-CSRTT and Morris water maze) with the same ability as wildtypes (Morcuende *et al.* 2003; Yan *et al.* 2011). For the most part, there were no genotype differences in the length of exploration of the objects, but when there were, NK1R-/- mice explored objects for longer, or made more

returns to objects, than wildtypes. This may be a reflection of the hyperactivity of NK1R-/- mice, or an increase in exploratory behaviour, i.e. novelty seeking.

One limitation of using approach-avoidance conflict tests (e.g. the light-dark box and elevated plus maze) is that it is impossible to tell whether a mouse is spending more time in the novel arena because it is less anxious, or because it has an enhanced curiosity for novel environments. These results, together with those from the EPM and LDEB, suggest that NK1R-/- mice are more anxious *and* have a slightly higher tendency to 'novelty seek', i.e. they spend less time in aversive areas than wildtypes, but actually spend slightly more time exploring new objects which are not aversive.

What was also clear from the experiments with naive animals, was that mice generally had better recognition than spatial memory: that is, they discriminated between objects better in the NOR than the NOL. The reason for this difference remains to be seen. It may be that the environmental cues were not clear enough to allow spatial navigation, although this seems unlikely because it has been reported that mice can navigate using an over-head camera, alone (Murai *et al.* 2007). It could also be that mice were using olfactory cues to distinguish between objects in the NOR, although this also seems unlikely as objects were carefully cleaned between tests. Another possibility is that spatial memory and recognition memory are depended on processes in distinct brain areas, such as the hippocampus (Broadbent *et al.* 2004) and PFC (Cassaday *et al.* 2014), respectively, such that the protocol (10 min test/ 1 h interval) allowed better recognition memory consolidation than it did spatial memory consolidation. Whatever the explanation, the difference between the tests could be further explored by changing variables in the two tests, such as the type of visual cues and length of time between sessions.

4.4.3 Alpha2-AR agonists improve spatial memory

The most striking finding from the object recognition experiments was that guanfacine selectively improved spatial memory in NK1R-/- mice, whereas recognition memory in both genotypes, and spatial memory in wildtypes, remained unaffected. In contrast, medetomidine improved spatial memory in wildtypes only: a response that was only evident at the lowest

dose (1 μg/kg). This confirms that both α2-AR agonists improve spatial memory, albeit at specific doses and in a genotype-dependent manner. Even though the test used here is, strictly speaking, one of *short-term* spatial memory rather than spatial *working* memory per se, the two may be close enough in nature to show comparable changes. α2-AR agonists do improve spatial working memory (SWM): for example, in tests of vigilance and working memory, activation of α2A-ARs enhances performance of both rats and monkeys in delayed-alternation (Carlson *et al.* 1992) and delayed-response tasks (Arnsten *et al.* 1988). However, this is not always replicable: guanfacine failed to improve SWM in one study of aged non-human primates (Decamp *et al.* 2011). These effects are likely to be mediated by α2-ARs, rather than α1-ARs, because α1-AR agonists, administered systemically or by local infusion into dlPFC, impair SWM in monkeys (Arnsten and Jentsch 1997; Mao *et al.* 1999).

The differences between guanfacine and medetomidine reported here could be due to their subtype-selectivity: guanfacine is selective for the α2A-AR subtype (K_d values of 19.9 and 344nM for α2A- and α2C-AR subtypes, respectively) (Uhlen and Wikberg 1991; Uhlen *et al.* 1992), whereas medetomidine is non-subtype selective. It seems the effects of α2-AR agonists on SWM are due to actions at the α2A-AR subtype: improvements in spatial working memory in rhesus monkeys are reversed by the α2A-AR antagonist, idazoxan (Franowicz and Arnsten 2002; Arnsten and Jin 2012; Kim *et al.* 2012), and mice with functional ablation of α2A-ARs display deficits in working memory, which are not relieved by guanfacine (Franowicz *et al.* 2002). However, in contrast, α2C-AR -knockout mice also display deficits in spatial memory (Bjorklund *et al.* 2001), but this *is* improved by dexmedetomidine (Tanila *et al.* 1999; Bjorklund *et al.* 2001). Similarly, α2C-AR overexpression impairs water maze learning in mice, and this is alleviated by non-subtype selective α2-AR antagonists (Bjorklund *et al.* 1998; Bjorklund *et al.* 2000). Together this evidence suggests that the α2C-AR subtype can modulate SWM, but this subtype is not necessary for the SWM-enhancing effects of adrenoceptor agents.

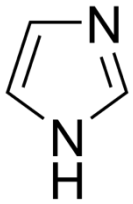

Figure 4.7 – Chemical structure of the imidazole ring

Another explanation for the difference between the two agonists could lie in their affinities for imidazoline receptors (I_1 and I_2). The

structure of medetomidine contains the imidazole ring (see Figure 4.7), whereas guanfacine does not. Moreover, imidazoline I_2 receptors (I_2Rs) are involved in medetomidine-induced impairment of long term potentiation (LTP: a mechanism underpinning learning and memory) in the hippocampus (Takamatsu *et al.* 2008). This could explain why, at a high dose (10 mg/kg), medetomidine actually impaired memory in wildtypes. The hypothesis that imidazoline receptors play a role in spatial memory merits further investigation.

Although the NK1R-/- mice tested here did not display any deficits in spatial working memory, the genotype difference in response to α2-AR agonists suggests that there is some interaction between NK1R and α2-ARs, which affects spatial memory. As discussed in Chapter 3, antagonism or functional ablation of NK1R blunts GABAergic inhibition of locus coeruleus neuronal firing (Maubach *et al.* 2002; Ebner and Singewald 2007). Moreover, NK1Rs are located on noradrenergic neurones of the LC itself (Chen *et al.* 2000; Santarelli *et al.* 2001). This could explain why systemic injection of the NK1R antagonists, WIN 51708 and CP 96345, prevents a clonidine-induced reduction in spiking of LC neurones in rats (Blier *et al.* 2004). However, if NK1R antagonism reduces the response to α2-AR agonists, it might be expected that mice with functional ablation of NK1Rs are less sensitive to α2-AR agonists. This is certainly the case for medetomidine: this drug had no effect on the spatial memory of NK1R-/- mice, but had clear effects in wildtypes. However, this was not the case for guanfacine. This leads to the inference that there is something exceptional about guanfacine, and perhaps by extension, the α2A-AR subtype, in NK1R-/- mice. Indeed, Fisher *et al* reported a 70% reduction in [35S]GTPgammaS binding to α2A-ARs in the locus coeruleus of NK1R-/- mice (Fisher *et al.* 2007). Although the mechanism by which guanfacine improved spatial memory in NK1R-/- was not investigated here, this genotype difference in α2A-ARs could merit further investigation.

Studies of working memory in the most well established model of ADHD, the Spontaneously Hypertensive Rat (SHR), have given inconsistent results (Sontag *et al.* 2013). Mook *et al* found that SHRs had *better* working memory in a radial arm maze, whereas Wyss *et al* demonstrated 12 month old (i.e. aged) SHRs had *impaired* memory in the same test compared to control strains (Wyss *et al.* 1992; Mook *et al.* 1993).

The results presented here are particularly interesting given reports showing that children and adults with ADHD have deficits in verbal and spatial working memory (Hervey *et al.* 2004; Martinussen *et al.* 2005; Vance *et al.* 2013). This executive dysfunction may form the basis of what is described in diagnosis as 'inattention', or indeed vice versa: spatial working memory could depend on selective attention. One functional MRI study suggested the latter could be true: Postle *et al* (2006) argue that spatial working memory is dependent on selective spatial attention, rather than recall of information.

The results presented in this Chapter suggest that guanfacine could be a better treatment option than non-subtype selective α2-AR agonists, for improving spatial memory/attention, particularly in a subset of ADHD patients carrying the *TACR1* gene polymorphism(s).

4.5 Highlights

- NK1R-/- mice display an anxiogenic phenotype compared to wildtypes on a mixed 129/Sv/C57Bl6 x MF1 background strain. This could be because of an interaction between background strain and loss of functional NK1R.
- NK1R-/- mice do not show recognition or spatial memory deficits in tests of short-term memory.
- Guanfacine, at a dose to which NK1R-/- mice are more sensitive (reported in Chapter 3), has no effect on anxiety-like behaviour of NK1R-/- or wildtype mice.
- The same dose of guanfacine improved short-term spatial memory in NK1R-/- mice, only, in an object recognition task. This could help explain guanfacine's efficacy in treating cognitive abnormalities (particularly inattention) in ADHD.
- Medetomidine also induced a genotype difference in spatial memory, but had no effect on NK1R-/- mice. The difference between the two α2-AR agonists is likely due to subtype selectivity, or differential actions at imidazoline receptors.

Chapter 5

The effects of atomoxetine on hyperactivity, inattention and impulsivity in NK1R-/- and wildtype mice

Chapter 5. The effects of atomoxetine on hyperactivity, inattention and impulsivity in NK1R-/- and wildtype mice

5.1 Introduction

5.1.1 Atomoxetine

Atomoxetine is a preferential noradrenaline reuptake inhibitor, which was originally trialled and marketed as 'tomoxetine' for the treatment of depression (Zerbe *et al.* 1985). Development was discontinued after a large scale trial in which the effects of atomoxetine could not be separated from those of placebo (Blier 2006). Nevertheless, atomoxetine (Straterra ®) was re-presented as a treatment for ADHD in 1996, and FDA approved in 2002 (Preti 2002). Atomoxetine is efficacious in reducing all three signs of ADHD in children/adolescents (Wilens *et al.* 2006; Hazell *et al.* 2011) and adults (Faraone and Glatt 2010). However, this drug does fall slightly short of the psychostimulants in terms of patient response rates: 70-80% of patients respond to psychostimulants, compared with approximately 50-60% for atomoxetine (see Heal *et al.* 2009).

Atomoxetine is marketed as a non-stimulant alternative to methylphenidate, despite both drugs being reuptake inhibitors. Atomoxetine inhibits the human noradrenaline (NAT), serotonin (SERT) and dopamine (DAT) transporters with K_i values of 5, 77 and 1451 nM, respectively, whereas methylphenidate preferentially inhibits the DAT (K_i values: 34 nM at DAT, 339 nM at NAT and >10,000 nM at SERT) (Bymaster *et al.* 2002). Both drugs increase extracellular catecholamines in the prefrontal cortex (PFC), but unlike the psychostimulants, atomoxetine causes no increase in extracellular dopamine in mesolimbic areas (Bymaster *et al.* 2002; Carboni *et al.* 2003). This minimizes the abuse potential of the drug in comparison with stimulants. Indeed, atomoxetine is not reinforcing in drug discrimination studies on rats (Swanson *et al.* 2006) or monkeys (Sacchetti *et al.* 1999), and does not facilitate self-

administration in non-human primates (Bymaster *et al.* 2002; Gasior *et al.* 2005) (for full review see Hudson *et al.* 1999). Moreover, there have been no reports of atomoxetine misuse in humans.

5.1.2 Atomoxetine in rodent models of ADHD

The effects of atomoxetine on the cognitive performance and response control of outbred rodents in the 5-CSRTT are remarkably consistent. In Long Evans, Lister-hooded and Sprague-Dawley rats, the drug reduces premature responses (impulsivity) (Blondeau and Dellu-Hagedorn 2007; Robinson *et al.* 2008; Paterson *et al.* 2011; Fernando *et al.* 2012; Robinson 2012), but has negligible effects on omissions (attention). Where omissions are increased, they are generally accompanied by increased response latencies, indicating a drug effect on arousal or motivation for the task (Baarendse and Vanderschuren 2012; Sun *et al.* 2012). The same pattern has even been reported in zebrafish performing a modified version of the 5-CSRTT: atomoxetine attenuated premature responses while response latencies and omissions were unaffected (Parker *et al.* 2014).

Similarly, the locomotor activity of hyperactive rodents is reduced by atomoxetine (Moran-Gates *et al.* 2005; Tamburella *et al.* 2012). However, unlike psychostimulants, the drug does not increase the locomotor activity of normal subjects.

5.1.3 Aims

With the continued aim of testing the predictive validity of the NK1R-/- mouse, the aim of these experiments was to determine whether the ADHD treatment, atomoxetine, alleviates the hyperactivity and impulsivity/inattentiveness displayed by NK1R-/- mice, in the LDEB and 5-CSRTT, respectively.

5.2 Methods

5.2.1 Light-Dark Exploration Box

The LDEB was used as described in Chapter 2, section 2.4.3. Doses of atomoxetine chosen were based on a survey of recent literature (see Appendix 4). Atomoxetine (1, 3 or 10 mg/kg, i.p.), vehicle (saline) or no injection was administered 30 min before mice were transferred to the light zone. Behaviour in the two zones was recorded for 30 min with a video camera, and scored blind.

5.2.2 5-Choice Serial Reaction-Time Task

The 5-CSRTT followed the same protocol as that described in section 2.4.1. The training and drug-free testing (NI-1) part of the protocol was a repeat of the experiment described in Chapter 3, which was designed to test the effects of breeding strategy on behaviour. Mice from both homozygous breeding colonies (homs) and NK1R-/- and wildtype littermates (hets) were used in this experiment (see Table 5.1). Mice started training at 6-8 weeks old. As in Chapter 3, the mice were first tested using two NI-1 sessions (one VITI and one long inter-trial interval (LITI)). These tests in naive mice are reported elsewhere (Porter *et al.* 2015).

Genotype	Number used ('n')	Number of breeding pairs derived from	Start weight (g: mean ± SD)
WT *hom*	6	2	31.3 ± 2.6
NK1R-/- *hom*	6	2	30.3 ± 1.9
WT *het*	6	4	33.2 ± 2.6
NK1R-/- *het*	6		32.0 ± 2.4

Table 5.1 – Details of mice used in the 5-CSRTT experiment

After the initial NI-1 sessions, mice were tested once-weekly, 30 min after treatment with either atomoxetine (0.3, 3 or 10 mg/kg), vehicle (VEH) or no injection (NI-2). The doses of atomoxetine used in this experiment were informed by the results of the LDEB: specifically, a lower dose (0.3 mg/kg) was chosen instead of the 1 mg/kg dose, in order to test whether any cognitive effects of the drug could be dissociated from a reduction in locomotor activity. Each mouse received each treatment once, and the treatments were assigned using a pseudo-Latin

square design, to account for any effects of repeated testing and possible long term drug effects. Two wildtypes (one *hom* and one *het*) and one NK1R-/- (*hom*) mouse failed to graduate through training in the 5-CSRTT.

5.2.3 Statistics

Raw or transformed data from the LDEB and 5-CSRTT were analysed in the same way as described in Chapter 3, section 3.2.3. Statistical significance was set as $P<0.05$. In the LDEB one NK1R-/- mouse from the vehicle group was excluded from the analysis, because it was an outlier (i.e. greater than 3 standard deviations away from the mean) in nearly every behavioural measure. Activity of mice decreased to a 'floor' level by about 15 min, and so, as in Chapter 3, only the first 10 min of recorded activity were used in the analysis.

In the 5-CSRTT there was no interaction between *colony* and *drug* in any variable, so the '*colony*' factor was collapsed. Where there were main effects of *time of day*, time of day was used as a blocking factor in the analysis, such that any variability within the data caused by time of day was taken into account, but not studied independently. This occurred here in %accuracy, %omissions and perseveration. This was valid because there were no *time of day*drug* interactions, i.e. drug treatment had the same effect on behaviour regardless of *time of day*. Where there was no effect of time of day, data were collapsed on this factor.

5.3 Results

5.3.1 Atomoxetine reduces activity in wildtype and NK1R-/- mice (Figure 5.1)

NK1R-/- mice were hyperactive in the light zone [[RAW]geno: $F_{(1,15)}=13.69$, P=0.002, WT vs. KO, NI: P=0.004], and a similar trend was observed in the dark zone [[RAW]geno: $F_{(1,15)}=7.06$, P=0.018, WT vs. KO, NI: P=0.081]. However, the hyperactivity in the light zone was no longer apparent after a vehicle injection [WT vs. KO, VEH: P=0.097].

Atomoxetine reduced the activity both genotypes, overall, in the light zone [[RAW]drug: $F_{(3,31)}$=3.84, P=0.019] and dark zone [[LOG10]drug: $F_{(3,29)}$=4.69, P=0.009]. The high dose (10 mg/kg) reduced locomotor activity of wildtypes and NK1R-/- mice in both the light [LZ, VEH vs. ATX10, WT: P=0.043, KO: P=0.023] and dark zones [DZ, VEH vs. ATX10, WT: P=0.039, KO: P=0.021] compared to vehicle.

Atomoxetine also reduced the number of returns to the light zone [[LOG10]drug: $F_{(3,31)}$=4.13, P=0.014] in both genotypes. However this reached significance in NK1R-/- mice, only, at 10 mg/kg [VEH vs. ATX10, KO: P=0.001] and 3 mg/kg [VEH vs. ATX3, KO: P=0.008]. Atomoxetine had no effect on time spent in the light zone [[RAW]drug: $F_{(3,31)}$=0.09, P=0.964] or on the latency to leave the light zone [[LOG10]drug: $F_{(3,29)}$= 1.78, P=0.1738]. The effect of atomoxetine did not depend on genotype in any measure.

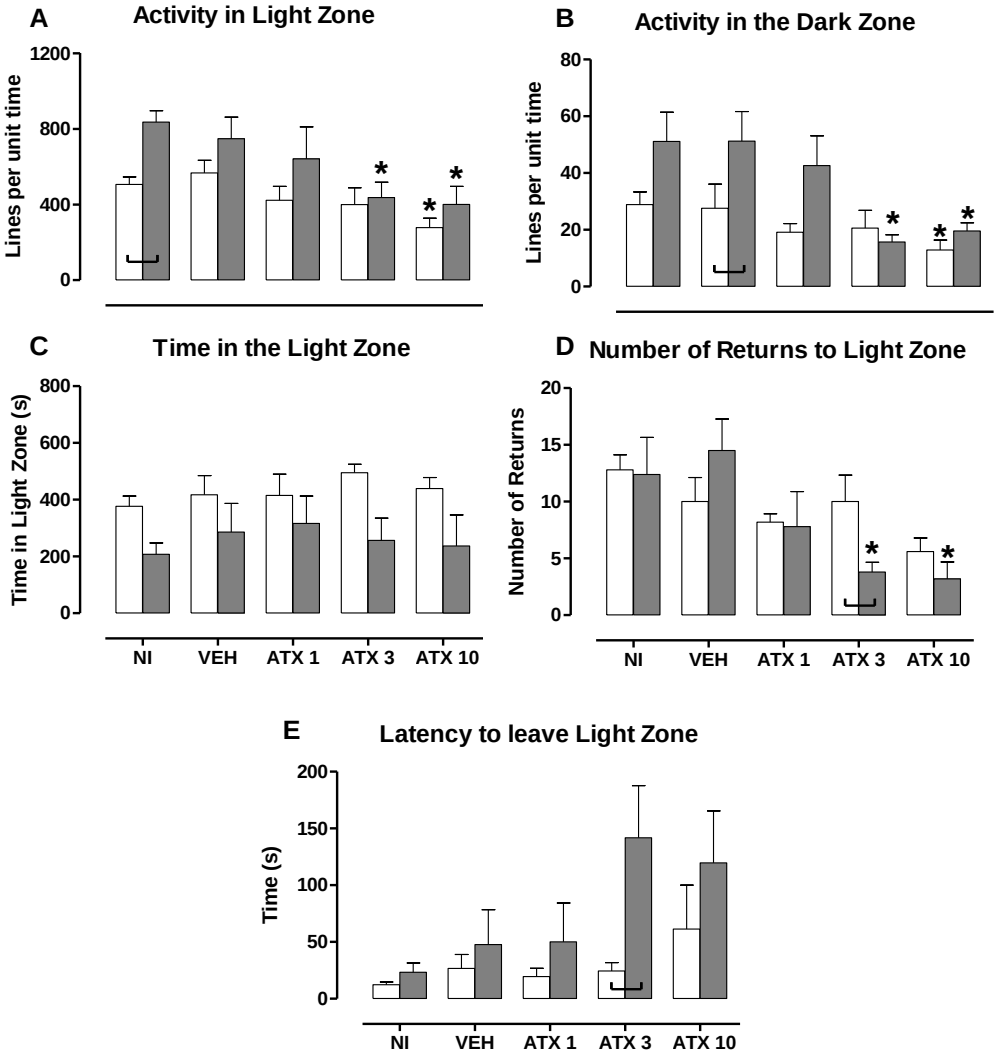

Figure 5.1 The effects of atomoxetine (1, 3 and 10 mg/kg, i.p.), vehicle (saline) or no injection (NI) on A: activity per unit time in the light zone, B: activity per unit time in the dark zone, C: time in the light zone, D: number of returns to the light zone, E: latency to leave the light zone in wildtype (white bars) and NK1R-/- mice (grey bars) in the light-dark exploration box. Data show mean ± SEM. Lines linking bars indicate statistical significance of P<0.05. N=5.

5.3.2 Wildtype and NK1R-/- mice learn to perform the 5-CSRTT at the same rate

Mice learned the task in the same number of days regardless of genotype [[RAW]geno: $F_{(1,19)}$=0.10, P=0.754]. Wildtypes took an average of 35.3 days, and NK1R-/- took an average of 36.8 days (Figure 5.2A). However, mice of both genotypes from the homozygous colony learned the task faster than those from the heterozygous colony [[RAW]colony: $F_{(1,19)}$=4.51, P=0.047]: an average of 31.3 and 40.5 days, respectively. Time of day did not affect the rate of learning.

5.3.3 Atomoxetine attenuates premature responding in NK1R-/- mice but not wildtypes (Figure 5.2E)

NK1R-/- mice did not display an impulsive phenotype in this experiment: there was no difference between the genotypes in the rate of premature responding overall [[SQRT]geno: $F_{(1,13)}$= 0.44, P=0.520]. Atomoxetine reduced %premature responses [[SQRT]drug: $F_{(3,55)}$=2.89, P=0.044], and this main effect did not depend on genotype [[SQRT]drug*geno: $F_{(3,55)}$=0.76, P=0.521]. However, post-hoc analysis revealed that atomoxetine reduced premature responses in NK1R-/- mice, only, at 10 mg/kg [VEH vs. ATX10, KO: P=0.006].

5.3.4 Atomoxetine lengthens the latency to reward, but not to correct response (Figure 5.2G & H)

The latency to correct response [[RANK]geno: $F_{(1,19)}$=0.60, P=0.448] and to magazine [[RANK]geno: $F_{(1,19)}$=0.74, P=0.400] were the same in both genotypes, overall. Atomoxetine increased the latency to magazine [[RANK]drug: $F_{(3,55)}$= 10.59, P<0.001], at the highest two doses in both genotypes [VEH vs. ATX3, WT: P=0.033, KO: P<0.001; VEH vs. ATX10, WT: P<0.001, KO: P<0.001]. However, atomoxetine had no effect on the latency to correct response [[RAW]drug: $F_{(4,74)}$=1.85, P=0.128]. Another measure of motivation, the number of trials completed, was unaffected by genotype [[ARCSINE]geno: $F_{(1,13)}$=0.89, P=0.363] or drug [[ARCSINE]drug: $F_{(4,51)}$=0.89, P=0.479].

5.3.5 Atomoxetine has no effect on accuracy, omission errors or perseveration (Figure 5.2C, D & F)

There was no difference between the two genotypes in %accuracy [[RAW]geno: $F_{(1,18)}$=0.06, P=0.816], %omissions [[SQRT]geno: $F_{(1,18)}$=0.03, P=0.865] or perseveration [[LOG10]geno: $F_{(1,18)}$=0.00, P=0.979]. Moreover, atomoxetine had no effect on these behaviours in either genotype (see Table 5.2).

Main effect	*Drug*	*Geno*Drug*
%Accuracy [RAW]	$F_{(4,74)}$=0.24, P=0.914	$F_{(4,74)}$=0.05, P=0.996
%Omissions [SQRT]	$F_{(4,74)}$=0.27, P=0.897	$F_{(4,74)}$=0.05, P=0.996
Perseveration [LOG10]	$F_{(4,74)}$=0.99, P=0.418	$F_{(4,74)}$=0.18, P=0.949

Table 5.2 – The results of the statistical analyses of behaviour in the 5-CSRTT

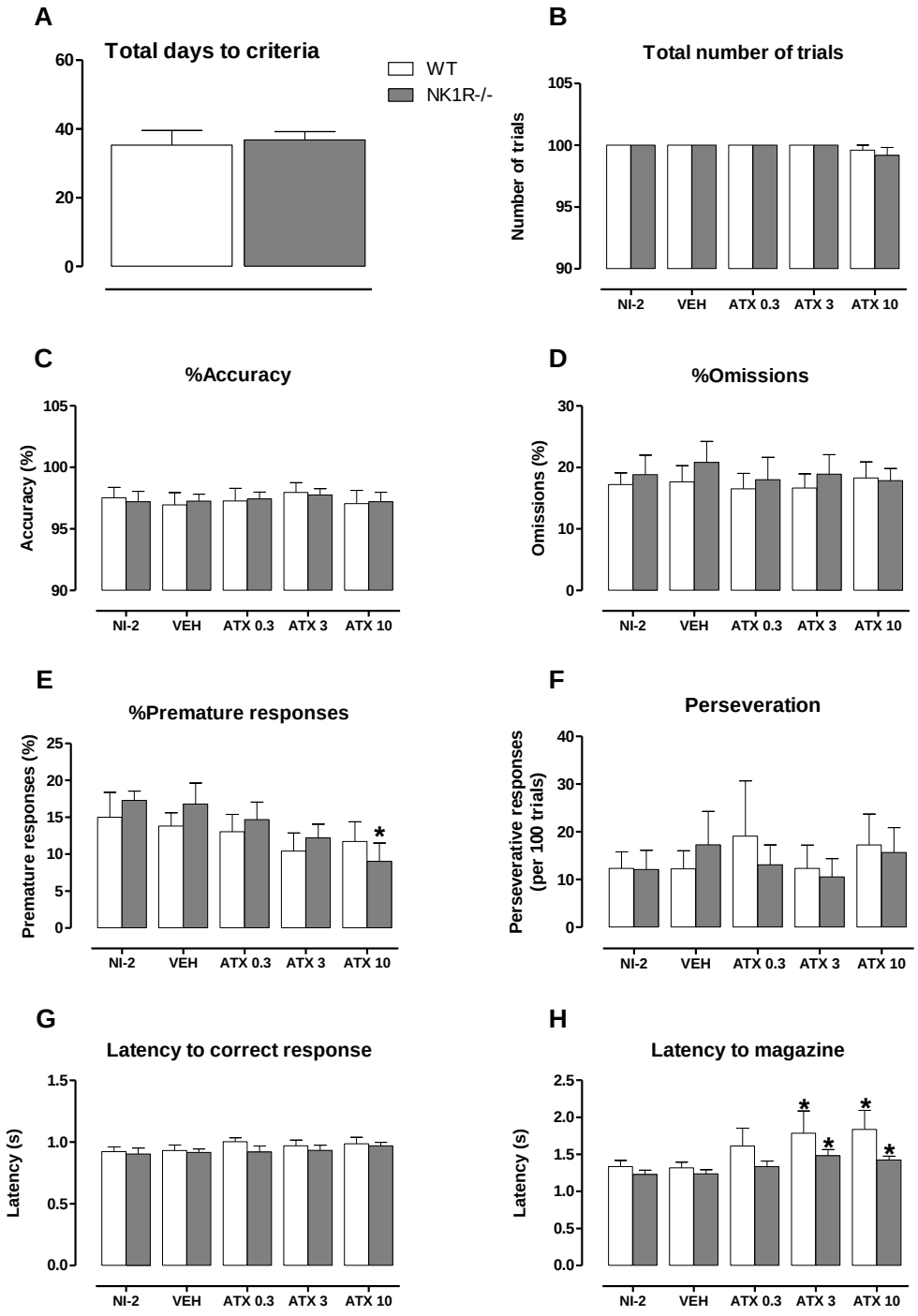

Figure 5.2 – A: The total number of days taken to reach testing criteria in the 5-CSRTT by wildtype and NK1R-/- mice, and the effects of atomoxetine (0.3, 3 and 10 mg/kg, i.p.) on B: total number of trials, C: %accuracy, D: %omissions, E: premature responses, F: perseveration, G: latency to correct response and H: latency to magazine, compared with vehicle (saline) and no injection (NI-2). Data show mean ± SEM. n=9-11 per group. Lines linking bars indicate statistical significance of P<0.05, * indicates P<0.05 versus vehicle within genotype.

5.4 Discussion

The aim of these experiments was to determine whether atomoxetine alleviates the ADHD-like behaviours displayed by NK1R-/- mice. Atomoxetine reduced hyperactive/impulsive behaviours in NK1R-/- mice, at doses which did not affect wildtypes, but had no effect on attention in either genotype. These findings broadly replicate those reported on tests of this drug in outbred rats performing the 5-CSRTT, and further add to the predictive validity of the NK1R-/- mouse.

5.4.1 Atomoxetine prevents hyperactivity

In this LDEB experiment, NK1R-/- mice were hyperactive compared to wildtypes, but this was no longer evident after mice had experienced an injection: strikingly, this exactly replicates the findings reported in Chapter 3, suggesting that this aspect of the behavioural phenotype is a robust one. Atomoxetine reduced the locomotor activity of NK1R-/- mice, and other measures of activity (number of returns to light zone, latency to leave light zone), at a dose (3 mg/kg) which did not affect wildtypes. These behaviours were observed without any corresponding reduction in the time spent in the light zone, which would be expected if there was an appreciable effect on animals' emotionality.

The genotype difference in the locomotor response to atomoxetine is consistent with its effects in other rodent models of ADHD, including spontaneously hypertensive (SHR) rats, trimethyltin chloride-treated (TMT) rats (Tamburella *et al.* 2012) and 6-OHDA-lesioned rats (Moran-Gates *et al.* 2005), but is at odds with a report that the drug did not affect hyperactive DAT-KO mice (Del'Guidice *et al.* 2014). One possible explanation is that the effects of atomoxetine are baseline dependent. Because of a floor effect, greater activity at baseline could be more vulnerable to a reduction than a low baseline.

Another explanation could be that NK1R-/- mice are more sensitive to noradrenergic manipulations: consistent with this is the finding, discussed in Chapter 3, that NK1R-/- mice are more sensitive to the α2-adrenoceptor agonist, guanfacine, in the same behavioural tests. This theory is supported by neurochemical evidence for an underlying difference in noradrenergic

neurotransmission in the two genotypes (Herpfer *et al.* 2005; Fisher *et al.* 2007). The possibility that the noradrenergic response to atomoxetine differs in NK1R-/- and wildtype mice warrants further investigation.

5.4.2 Atomoxetine reduces impulsivity

Unlike in our previous studies (Yan *et al.* 2011; Dudley *et al.* 2013), NK1R-/- mice did not display an impulsive phenotype at baseline (NI-2) in this study. This is likely due to the use of two colonies of mice, as NK1R-/- mice bred from heterozygous parents do not show the same impulsivity as those bred from inbred homozygous parents (see Porter *et al.* 2015). Despite this limitation, atomoxetine (10 mg/kg) reduced premature responding in NK1R-/- mice, but had no effect in wildtypes. This was accompanied by an increased latency to collect the reward in both genotypes, suggesting that the drug may reduce arousal or motivation for the task. However this seems an unlikely explanation, since other indicators of arousal/motivation (latency to correct response and %omissions) were not increased in parallel, and this would occur in NK1R-/- mice, only, if the reduction in premature responses was secondary to reduced arousal in these mice.

These results are supported by consistent reports that atomoxetine reduces impulsivity in outbred rats performing the 5-CSRTT, usually without affecting measures of attention (Robinson *et al.* 2008; Baarendse and Vanderschuren 2012) (see Table 5.3). Studies utilizing different populations of animals report that the drug is particularly effective in reducing premature responding in animals that display high impulsivity at baseline (e.g. high impulsive (HI) rats) (Blondeau and Dellu-Hagedorn 2007; Fernando *et al.* 2012; Tomlinson *et al.* 2014). Similarly, atomoxetine is more effective when impulsivity is increased by manipulating the task parameters, for example, by extending the inter-trial interval (ITI) (Paterson *et al.* 2011; Baarendse and Vanderschuren 2012). A low level of impulsivity at baseline (15 – 20% premature responses) in this study may explain why a relatively high dose of atomoxetine was needed to see an effect, compared with other studies (see Appendix 4); atomoxetine could be more effective when baseline impulsivity is high. However, Robinson (2012) reports that atomoxetine is efficacious when impulsivity is low under baseline conditions and when

impulsivity is increased when the task is made more challenging (e.g. by extending the ITI, reducing the stimulus duration or adding a distracter stimulus).

As the aforementioned studies used outbred rats, it might be expected that atomoxetine would reduce impulsivity in wildtypes, as well as in NK1R-/- mice. However, this was not the case; although atomoxetine reduced impulsivity in a genotype independent manner overall, this reduction was statistically significant in NK1R-/- mice, only. This finding is difficult to reconcile with the literature, as only one study to date has examined the effect of atomoxetine in an animal model of ADHD, the SHR, in the 5-CSRTT, and this study did not include a control strain. This study found no effect of atomoxetine at any dose (0.1 – 5 mg/kg), in any behavioural measure (Dommett 2014). However, this study was limited by a number of factors including a lack of positive drug control and a high animal drop-out rate. Further tests would be needed to confirm whether atomoxetine reduces impulsivity in wildtypes as well as NK1R-/- mice, as the results presented here could be a consequence of testing a limited dose range, or a low level of impulsivity in wildtypes at baseline.

These studies have all used acute doses of the drug. One study examining the effects of chronic atomoxetine treatment in adolescent Long-Evans rats found that chronic dosing had little effect: when tested in the 5-CSRTT, impulsivity was reduced by atomoxetine in both the chronic vehicle-, and chronic drug-treated groups (Sun *et al.* 2012).

To date, atomoxetine is the only noradrenaline reuptake inhibitor (NRI) clinically indicated for ADHD. However, preclinical tests using another preferential NRI, reboxetine, have given similar results to atomoxetine, suggesting a common mechanism. For example, Robinson (2012) found that reboxetine (0.1 and 0.3 mg/kg) reduced premature responding in outbred Lister-hooded rats performing the 5-CSRTT, and improved accuracy in animals which performed poorly at baseline. Similarly, Liu *et al* found that the beneficial effects of reboxetine on impulsivity were prevented by treatment with an alpha2-adrenoceptor antagonist (RX821002) but not an alpha1-adrenoceptor antagonist (prazosin) (Liu *et al.* 2009); a noteworthy finding given the results reported in Chapter 3, that guanfacine, an alpha2-adreoceptor agonist, also reduces impulsivity.

It is interesting to note that reboxetine is clinically indicated for the treatment of major depressive disorder, but reduces impulsivity with a striking resemblance to atomoxetine (Liu *et al.* 2009; Robinson 2012). However, a recent meta-analysis suggested that, much like atomoxetine, this drug may lack efficacy in treating depression (Sepede *et al.* 2012). On the other hand, it is widely accepted that other successful antidepressants such as desipramine and nortriptyline exert their effects by blockade of noradrenaline reuptake (Brunello *et al.* 2002). A key difference between atomoxetine/reboxetine and other noradrenergic antidepressants is that the latter contain the tricyclic moiety (hence the name, tricyclic antidepressants), and exert anticholinergic, adrenergic and histaminergic side effects (such as arterial hypotension and dry mouth) (Dell'Osso *et al.* 2011). Conversely, reboxetine and atomoxetine, are relatively 'pure', and lack any appreciable affinity at other receptor sites, such as 5-HT_{2A}, H_1 receptors, α1-adrenoceptors or muscarinic receptors, which the tricyclics do bind to (Cusack *et al.* 1994; Wong *et al.* 2000). Nevertheless, studies have suggested that both reboxetine and desipramine could be useful in the treatment of ADHD (Biederman *et al.* 1989; Wilens *et al.* 1996; Ghanizadeh 2014).

Reference	Dose	Species/strain	5-CSRTT test	Omissions	Premature responses	Latencies
(Baarendse and Vanderschuren 2012)	0.3, 1 and 3 mg/kg	Rat/ Lister-hooded	Baseline	↑	n.s.	↑
			LITI	↑	↓	↑
(Robinson *et al.* 2008)	0.6, 1 and 3 mg/kg	Rat/ Lister-hooded	Baseline	n.s.	↓	n.s.
(Robinson 2012)	0.3 mg/kg	Rat/ Lister-hooded	Baseline	↑	↓	n.s.
			VITI	n.s.	↓	↑
			LITI	n.s.	↓	↑
(Paterson *et al.* 2011)	0.5, 1 and 2 mg/kg	Rat/ Long Evans	VITI	n.s.	↓	n.s.
(Navarra *et al.* 2008)	0.1, 0.5 and 1 mg/kg	Rat/ Long Evans	VITI	Not reported	↓	↑
(Sun *et al.* 2012)	1 mg/kg/day for 14 days	Rat/ Long Evans	CHRONIC ATX in adolescence before 5-CSRTT training	n.s. effect on baseline	n.s. effect on baseline	n.s. effect on baseline
			ACUTE challenge with ATX (after chronic VEH)	↑	↓	↑
			ACUTE challenge with ATX (after chronic ATX)	↑ (to lesser extent than VEH group)	↓	↑

Table 5.3 – The effects of atomoxetine on behaviour of outbred rats performing the 5-CSRTT. ↑ indicates increase, ↓ indicates decrease and n.s. indicates no significant difference compared to control (vehicle) condition. LITI; long inter-trial interval, VITI; variable inter-trial interval, ATX; atomoxetine, VEH; vehicle.

5.4.3 Atomoxetine has no effect on attention

In measures of attention (%omissions and %accuracy), here, atomoxetine had no effect on either genotype. However, other preclinical studies have reported that atomoxetine improves response accuracy in rats (Baarendse and Vanderschuren 2012; Robinson 2012), but on the whole, this drug tends to increase %omissions (Baarendse and Vanderschuren 2012; Sun *et al.* 2012). This suggests that, in rats at least, attention is best represented in the 5-CSRTT by response accuracy, rather than whether the animal makes a response or not (i.e. %omissions), which may better represent motivation and/or arousal. In mice, the situation is complicated by the extremely high level of accuracy at baseline: a possible explanation of why here, atomoxetine had no detectable effect on this measure. This result highlights a limitation of using mice in the 5-CSRTT, or an aspect of the test which does not translate well between mice and humans, as inattentiveness is consistently reduced by atomoxetine in clinical studies (Wilens *et al.* 2006; Faraone and Glatt 2010; Hazell *et al.* 2011).

5.4.4 Mechanism of action

The mechanism underlying these behavioural responses to atomoxetine is likely to depend on catecholaminergic neurotransmission. Although atomoxetine is ~300-fold more selective for the NAT than the DAT *in vitro*, systemic administration of this drug increases synaptic concentrations of both noradrenaline and dopamine in the prefrontal cortex (PFC) of outbred rats and SHRs (Bymaster *et al.* 2002; Heal *et al.* 2009; Ago *et al.* 2014). This limited, impulse-dependent increase is consistent with its actions as an uptake inhibitor. Local infusion of atomoxetine into the PFC also increases extracellular concentrations of both catecholamines, suggesting the effect, at least in part, could be locally mediated (Bymaster *et al.* 2002). This is corroborated by findings that atomoxetine increases c-Fos expression in the PFC, but not the striatum of mice (Koda *et al.* 2010).

One explanation for the increase in dopamine is that, in the PFC, most of the up-take of dopamine could be through the NAT. This explanation has been proposed because NAT inhibitors reliably increase extracellular dopamine, and 6OHDA lesions of the dorsal noradrenergic bundle (DNAB) prevent this effect (Carboni *et al.* 1990; Di Chiara *et al.* 1992;

Yamamoto and Novotney 1998). This is supported by evidence from genetically modified mice: the efficacy of cocaine to inhibit dopamine uptake into synaptosomes was normal in DAT knockout mice, but reduced by 70% in NAT knockout mice (Moron *et al.* 2002).This explanation seems plausible, because the DAT is sparsely expressed in the PFC (Sesack *et al.* 1998) compared with the NAT (Gehlert *et al.* 1993; Schroeter *et al.* 2000), and interestingly, the NAT has a higher affinity for dopamine than the DAT (Giros *et al.* 1994; Gu *et al.* 1994). Consistent with this hypothesis, local application of alpha2-adrenoceptor agonists and antagonists, reduce and increase, extracellular concentrations of both catecholamines in the PFC, respectively (Gresch *et al.* 1995). Controversially, it has also been suggested that dopamine is co-released with noradrenaline in this brain area, although it is not clear whether this is related to the ectopic uptake of dopamine by the NAT (Devoto *et al.* 2001; Devoto and Flore 2006; Devoto *et al.* 2008).

It has been hypothesized that atomoxetine's blockade of the NAT increases extracellular noradrenaline and dopamine in the PFC, which activates postsynaptic alpha2-adrenoceptors and D_1 receptors, respectively, to enhance executive function (Arnsten and Li 2005; Gamo *et al.* 2010). As discussed in Chapters 3 and 4, optimum activation of prefrontal alpha2-adrenoceptors is particularly important in attention and spatial working memory (Arnsten *et al.* 1988; Franowicz *et al.* 2002). Impulsivity, on the other hand, may be mediated by both prefrontal and striatal brain areas. For example, the nucleus accumbens (NAcc) could be critically involved in response control: this nucleus receives dense dopaminergic inputs from the ventral tegmental area, but only the shell region is innervated by LC noradrenergic neurones (Berridge *et al.* 1997; Delfs *et al.* 1998). It follows that microinfusions of atomoxetine into the shell, but not the core, regions of the NAcc decreased premature responding in rats performing the 5-CSRTT (Economidou *et al.* 2012). Conversely, the beneficial effect of atomoxetine on impulsivity in terms of stopping in the stop signal reaction time task (SSRT) (as opposed to waiting in the 5-CSRTT) could be mediated by the dorsal prelimbic and orbitofrontal cortices (Bari *et al.* 2011). This implies that the effects of systemic atomoxetine on impulsivity may be mediated by multiple neural circuits, and, given that impulsivity is not a unitary construct, could depend on the type of impulsivity exposed by the task.

Other evidence implicates prefrontal serotonin in inhibitory control: systemic administration or local infusion of 5-HT_{2A} receptor antagonists (ketanserin or M100907, respectively), into the mPFC of rats decreases premature responding in the 5-CSRTT (Dalley and Roiser 2012; Passetti *et al.* 2003; Winstanley *et al.* 2003). Although atomoxetine is only approximately 10-fold more selective for the NAT than the SERT, there have been no reports of increased cortical serotonergic efflux with systemic atomoxetine treatment. Thus, the effects of atomoxetine on impulsivity are unlikely to be mediated directly by cortical 5-HT receptors.

However, there is some evidence to suggest that another amine neurotransmitter plays a role in the behavioural effects of atomoxetine. Histamine is well-known to regulate the sleep-wake cycle and arousal, but it may also be involved in cognition. Histamine (H_1 or H_2) receptor-deficient mice show impaired learning and memory in a range of tests (Schneider *et al.* 2014). Atomoxetine increases extracellular histamine in the PFC, in normal rats and in SHRs (Horner *et al.* 2007; Liu *et al.* 2008), and so could facilitate cognitive processes through H_1R or H_2R activation. This possibility warrants further investigation, as histamine receptors could represent a novel therapeutic target.

5.5 Highlights

- Atomoxetine, an established treatment for ADHD, reduced hyperactive and impulsive behaviours in NK1R-/- mice but not wildtypes, and had no effect on attention in either genotype.
- The reduction in impulsivity is unlikely to be explained by a change in animals' motivation for the task, but could relate to parallel changes in motor activity.
- NK1R-/- mice could be more sensitive to noradrenergic manipulations than wildtypes because of underlying differences in their neurochemistry.
- Atomoxetine may exert its effects by increasing extracellular catecholamines or histamine.
- Atomoxetine may be most suitable for the hyperactive-impulsive subtype of ADHD.

Chapter 6

The effects of methylphenidate on inattention and impulsivity in NK1R /- and wildtype mice

Chapter 6. The effects of methylphenidate on inattention and impulsivity in NK1R-/- and wildtype mice

6.1 Introduction

The psychostimulants, amphetamine and methylphenidate, are currently the first-line treatments for ADHD (Leonard *et al.* 2004). Amphetamine was discovered in 1887, and has historically been used to treat a wide variety of medical conditions including nasal congestion, depression, obesity, narcolepsy and ADHD. Methylphenidate was first synthesized in 1944 by Leandro Panizzon. The compound was marketed as a treatment for lethargy and chronic fatigue in 1954 as Ritalin, named after Panizzon's wife, Marguerite or 'Rita' for short (Lange *et al.* 2010). However, it was most efficacious in reducing the signs of ADHD. Since its approval, the prescription of methylphenidate has steadily increased over the years alongside the increasing acceptance of ADHD as a psychiatric disorder (Lange *et al.* 2010). Despite being approved around 60 years ago, when safety regulations were less stringent, methylphenidate maintains a good safety record (Godfrey 2009). Moreover, meta-analysis reveals that psychostimulants are still more efficacious than non-stimulant options: approximately 70% of patients respond to psychostimulant treatment (Faraone *et al.* 2006).

In the clinic, stimulants can be delivered through a variety of formulations, including osmotic release oral systems (OROS), tablets, capsules and transdermal patches. Combinations of active and inactive isomers, as well as immediate and extended release (XR), and pro-drug formulations also increase the number of options available (see Antshel *et al.* 2011). These options allow doctors to better tailor the active window of the drug to patients' needs. Moreover, the XR and pro-drug formulations have further reduced the abuse potential of the stimulants. However, a demand for non-stimulant alternatives remains. Many patients experience intolerable side effects with stimulants, such as loss of appetite, abdominal pain, insomnia and headaches (Spencer *et al.* 2005), or are not eligible because of treatment

contraindications: stimulants are inadvisable in patients with tic disorders, tachycardia, anorexia or bipolar disorder (Kolar *et al.* 2008).

6.1.1 Methylphenidate in rodent models of ADHD

Both psychostimulants robustly increase extracellular catecholamines in a dose-dependent manner in corticostriatal brain areas in rats (Kuczenski and Segal 1997; Bymaster *et al.* 2002) and mice (Koda *et al.* 2010), but the effects of methylphenidate on ADHD-like behaviour of rodents can vary. In the only two studies of methylphenidate in SHRs performing the 5-CSRTT, doses of 0.1, 1 and 10 mg/kg (i.p.) (van den Bergh *et al.* 2006) and 1, 2 and 3 mg/kg (oral) (Dommett 2014) were ineffective in modifying any aspect of behaviour. Methylphenidate was also ineffective in SHRs in a delay discounting task (Wooters and Bardo 2011). However, doses of the drug in the same range improved learning in SHRs in a Morris water maze (10 mg/kg) (Guo *et al.* 2012) and in an attentional set shifting task (2.5 and 5 mg/kg) (Cao *et al.* 2012).

In normal, outbred rodents, methylphenidate induces hyperactivity, whereas in rodent models of ADHD, the drug should reduce baseline hyperactivity. Methylphenidate actually increases the activity of SHRs at doses which increase activity in control strains (Wultz *et al.* 1990; Amini *et al.* 2004), but decreases activity at doses which have no effect in control strains (Umehara *et al.* 2013). A reduction in activity has also been reported in the DAT KO mouse at doses which induce hyperactivity in wildtypes (Gainetdinov *et al.* 1999).

6.1.2 Psychostimulants in the NK1R-/- mouse

We have previously reported that both amphetamine and methylphenidate prevent the hyperactivity of NK1R-/- mice (Yan *et al.* 2010): amphetamine reduces the activity of NK1R-/- mice to that of wildtypes, whereas after methylphenidate treatment, the hyperactivity of NK1R-/-mice is no longer apparent. Amphetamine also increased impulsivity, but reduced perservation, in NK1R-/- mice in the 5-CSRTT (Yan *et al.* 2011). In terms of neurochemistry, NK1R-/- mice display an abnormal response to amphetamine: the drug does not induce an increase in extracellular dopamine in the dorsal striatum of these mice, as it does in wildtypes

(Yan *et al.* 2010). The effects of methylphenidate on cognition and neurochemistry are yet to be tested.

6.1.3 The 5-Choice Continuous Performance Task

In this experiment, methylphenidate was tested in mice performing the 5-Choice Continuous Performance Task (5C-CPT). The 5C-CPT is a refinement of the 5-CSRTT: it utilizes original, 5-CSRTT-type trials where one cue light is illuminated, but the key difference is that it also incorporates 'no-go' trials, where all five cue lights are illuminated simultaneously, signalling that the animal should withhold any response. The 5C-CPT was developed in the mouse as an analogue of human continuous performance tasks (Young *et al.* 2009), and is described as measuring 'vigilance': the ability to correctly discriminate between signal and non-signal stimuli. The no-go trials also provide a measure of impulsivity: on these non-signal trials, mice are required to withhold any response. Inability to withhold a response is termed 'behavioural disinhibition' (Young *et al.* 2011): this is different to the premature responding discussed in Chapters 3 and 5. The two types can be distinguished, because impulsivity is not a unitary construct. Different types of impulsivity have been classified in a number of ways by different researchers (see Evenden 1999), but most seem to be variations on three constructs; timing/motor impulsivity (i.e. premature responding), behavioural disinhibition (i.e. making a response when one should not be made), and delayed reward/impulsive choice (i.e. the choosing of small, immediate rewards over large, delayed rewards). The former two types of impulsivity, together with a measure of vigilance, are tested here to further characterize the NK1R-/- mouse and the effects of methylphenidate on cognition.

6.1.4 Aims

The aim of this experiment was to further characterise the predictive validity of the NK1R-/- mouse by testing whether the first-line ADHD treatment, methylphenidate, reduces two types of impulsivity and improves vigilance in these mice.

6.2 Methods

6.2.1 5 Choice Continuous Performance Task

Only mice from homozygous breeding colonies were used in this experiment. 12 wildtypes and 12 NK1R-/- mice started training at 6-8 weeks of age. Wildtypes were from two, and NK1R-/- mice were from three breeding pairs. Mice were assigned to the four test chambers, counterbalancing for genotype, time of day and cage. The training protocol then followed that described in section 2.4.2. One wildtype failed to graduate through training, and so was excluded from the testing phase.

This 5C-CPT experiment utilized extended VITI (variable inter-trial interval; 7, 8, 9, 10, and 11 s) sessions. The mice were first tested after no injection (NI-1: to be reported elsewhere), and then, at once-weekly intervals, tested 30 min after treatment with either methylphenidate (3, 10 or 30 mg/kg, i.p.), vehicle (VEH) or no injection (NI-2). Each mouse received each treatment once, and the treatments were assigned using a pseudo-Williams' Latin square design, to compensate for any effects of repeated testing and possible long term drug effects. The doses were based on a pilot study of methylphenidate in the 5-CSRTT, in which there was no effect of the drug at doses of 0.5, 2.5 and 5 mg/kg (unpublished observations), and a literature search (see Appendix 5). The behavioural outcomes (see section 2.4.2) were stored online. Sensitivity Index (SI) was also calculated, according to the following formula, to measure 'vigilance', i.e. the ability to correctly distinguish between signal and non-signal stimuli:

$$\mathbf{SI = PH - PFA/(2(PH+PFA))-(PH+PFA)^2}$$

Where PH (%hits) is [(correct responses/ correct responses + omissions) * 100], and PFA (%false alarms) is [(correct rejections/ correct rejections + false alarms) * 100].

6.2.2 Statistics

Raw or transformed data were analysed as described in section 2.6. The number of days taken to progress through training were analysed using a one way ANOVA with '*genotype'* as the factor. For the 5C-CPT test data, two- or three-way repeated measures (RM) analyses used

'*genotype'* and '*time of day'* as between-subjects factors and '*drug'* as the within-subjects factor. Where there was no main effect of *time of day*, this factor was collapsed. Firstly, the analysis compared all 5 groups (NI, VEH, MPH3, MPH10 and MPH30). A main effect of '*genotype'* or '*drug'*, or an interaction between them, was used as the criterion to progression to further analysis. Secondly, the effect of drug (MPH3, 10 and 30) was compared with vehicle: all main effects of *drug* and *drug*genotype* interactions that are reported are based on the results of this second comparison. Post-hoc pairwise comparisons were used to confirm differences between individual groups. Statistical significance was set at $P<0.05$.

6.3 Results

There were no main effects of *time of day*, and no interactions between time of day and any other factor, for any variable. Therefore, the data were collapsed across *time of day*.

6.3.1 NK1R-/- mice learn the 5C-CPT faster than wildtypes

The number of days to reach the criteria for testing was divided into two sections; the number of days taken to learn the 5-CSRTT aspect of the task, and the number of days taken to learn the 'no-go', i.e. 5C-CPT, aspect of the task (Figure 6.1). There was no genotype difference in the time it took mice to learn each of these sections individually [days to pass 5-CSRTT: [SQRT] $F_{(1,21)}=3.04$, $P=0.096$, days to pass 5C-CPT: [RANK] $F_{(1,21)}=2.92$, $P=0.102$], but NK1R-/- mice took fewer days to reach the end of training overall [[SQRT] $F_{(1,21)}=9.03$, $P=0.007$, Figure 6.1C].

6.3.2 Methylphenidate reduces attention in wildtypes

There were no genotype differences in %*accuracy* overall [[RANK]geno: $F_{(1,21)}=2.65$, $P=0.117$, Figure 6.2A], and methylphenidate had no effect on %*accuracy* [[RANK]drug: $F_{(3,60)}=1.88$, $P=0.143$].

There were no differences in *%omissions* between the genotypes overall [[SQRT]geno: $F_{(1,21)}=0.59$, $P=0.452$, Figure 6.2B], or at NI-2 [WT vs. KO: $P=0.356$]. Methylphenidate increased %*omissions* in both genotypes [[SQRT]geno*drug: $F_{(3,63)}=2.09$, $P=0.110$; drug: $F_{(3,63)}=143.19$,

P<0.001]. However, at 10 mg/kg, %*omissions* were increased in wildtypes [VEH vs. MPH10: P=0.026] but not NK1R-/- mice [VEH vs. MPH10: P=0.402]. At the highest dose (30 mg/kg), %*omissions* were increased in both genotypes [VEH vs. MPH30, WT: P<0.001, KO: P<0.001].

6.3.3 Methylphenidate reduces impulsivity in NK1R-/- mice, only

There was no genotype difference in premature responses overall [[SQRT]geno: $F_{(1,21)}$=0.01, P=0.918, Figure 6.2C], or at NI-2 [WT vs. KO: P=0.983]. However, NK1R-/- mice responded with fewer false alarms than wildtypes overall [[RAW]geno: $F_{(1,21)}$=9.15, P=0.006, Figure 6.2D], but this just missed statistical significance at NI-2 [WT vs. KO: P=0.085].

Impulsivity was attenuated by methylphenidate: the drug reduced %*premature responses* [[SQRT]drug: $F_{(3,63)}$=18.95, P<0.001] and this did not depend on genotype overall [[SQRT]geno*drug: $F_{(3,63)}$=1.30, P=0.281]. However, 10 mg/kg MPH reduced premature responses in NK1R-/- mice only [VEH vs. MPH10: P=0.021]. The highest dose of the drug (30 mg/kg) reduced premature responses in both genotypes [VEH vs. MPH30, WT: P<0.001, KO: P<0.001].

Similarly, *%false alarms* were attenuated by methylphenidate [[RAW]drug: $F_{(3,63)}$=41.11, P<0.001], in a genotype independent manner [[RAW]geno*drug: $F_{(3,63)}$=0.36, P=0.779]. However, as with premature responses, 10 mg/kg methylphenidate reduced false alarms in NK1R-/- mice only [VEH vs. MPH10: P=0.005] and 30 mg/kg methylphenidate reduced false alarms in both genotypes [VEH vs. MPH30, WT: P<0.001, KO: P<0.001].

6.3.4 Methylphenidate increases vigilance in NK1R-/- mice, but worsens vigilance in wildtypes

The *sensitivity index* (SI) calculation takes into account omissions, correct responses and false alarms, to give a measure of vigilance (Figure 6.3A). NK1R-/- mice had a higher SI (i.e. were more vigilant) than wildtypes overall [[RAW]geno: $F_{(1,21)}$=4.47, P=0.047], but no genotype difference was evident at NI-2 [WT vs. KO: P=0.519]. Methylphenidate reduced the SI [[RAW]drug: $F_{(3,60)}$=3.57, P=0.019] in a genotype-independent manner [[RAW]geno*drug:

$F_{(3,60)}$=1.960, P=0.130]. However, at 10 mg/kg, a genotype difference (WT<KO) was evident in the SI which was not present at any other dose [WT vs. KO: P=0.003].

6.3.5 Methylphenidate prevents perseveration in NK1R-/- mice

NK1R-/- mice displayed more perseverative responses overall [[LOG10]geno: $F_{(1,21)}$=8.31, P=0.009, Figure 6.3B]. Methylphenidate reduced *perseveration* [[LOG10]drug: $F_{(3,63)}$=11.29, P<0.001], but just missed the criterion for a genotype-dependent effect [[LOG10]geno*drug: $F_{(3,63)}$=2.71, P=0.053]. All doses of methylphenidate reduced perseveration in NK1R-/- mice [VEH vs. MPH3: P=0.017, MPH10: P=0.003, MPH30: P<0.001], but none had any effect in wildtypes.

6.3.6 Methylphenidate has a genotype-dependent effect on motivation for the task

NK1R-/- mice completed more trials overall [[RANK]geno: $F_{(1,21)}$=4.61, P=0.044, Figure 6.3C]. Methylphenidate affected the *total trials* completed in a genotype-dependent manner [[RANK]geno*drug: $F_{(3,63)}$=12.58, P<0.001]: methylphenidate increased the number of trials completed by wildtypes at all doses (bar 30 mg/kg, which just missed significance) [VEH vs. MPH3: P=0.049, MPH10: P<0.001, MPH30: P=0.055], but 30 mg/kg methylphenidate reduced the number of trials completed by NK1R-/- mice [VEH vs. MPH30: P<0.001].

6.3.7 Methylphenidate slows reaction speeds, particularly in wildtypes

Methylphenidate increased the *latency to correct response* in a genotype-dependent manner [[RANK]geno*drug: $F_{(3,60)}$=4.84, P=0.004, Figure 6.3D]. NK1R-/- mice took longer to respond correctly than wildtypes [[RANK]geno: $F_{(1,21)}$=20.29, P<0.001], but this genotype difference was ablated by the high dose of methylphenidate, as the drug lengthened reaction times in wildtypes only [VEH vs. MPH30, WT: P<0.001, KO: P=0.461].

Similarly, NK1R-/- mice had a longer to *latency to false alarms* than wildtypes [[RANK]geno: F(1,21)=11.33, P=0.003, Figure 6.3E], and methylphenidate lengthened this measure [[RANK]drug: $F_{(3,56)}$=2.75, P=0.050]. The *latency to false alarms* was lengthened in wildtypes at

10 mg/kg and 30 mg/kg, only [VEH vs MPH10: P=0.50, MPH30: P=0.048]. However, this response was not genotype-dependent overall [[RANK]geno*drug: $F_{(3,56)}$=0.54, P=0.658].

There was a trend to a genotype difference overall in the *latency to magazine* [[RANK]geno: $F_{(1,21)}$=3.54, P=0.074, Figure 6.3F]. However, in this measure, methylphenidate reduced reaction speeds in both genotypes [[RANK]geno*drug: $F_{(3,60)}$=1.36, P=0.265; drug: $F_{(3,60)}$=7.92, P<0.001]. This was evident in both genotypes at 10 mg/kg [VEH vs. MPH10, WT: P=0.002, KO: P<0.001], but only in NK1R-/- mice at 3 mg/kg and 30 mg/kg [VEH vs MPH3: P=0.040, MPH30: P<0.001].

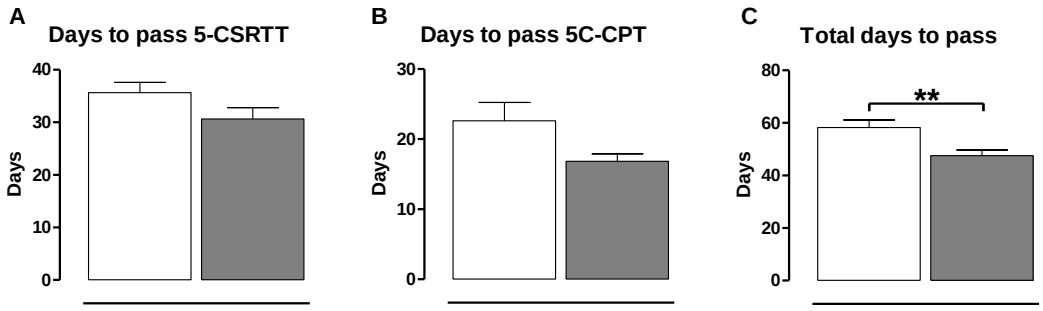

Figure 6.1 – Time taken to graduate through training in the 5-CSRTT (A) and 5C-CPT (B) sections of the 5C-CPT experiment by wildtype (white bars) and NK1R-/- mice (grey bars). C: Days taken to pass training criteria in total (i.e. A + B). ** P<0.01. Data show mean ± SEM. n=11-12

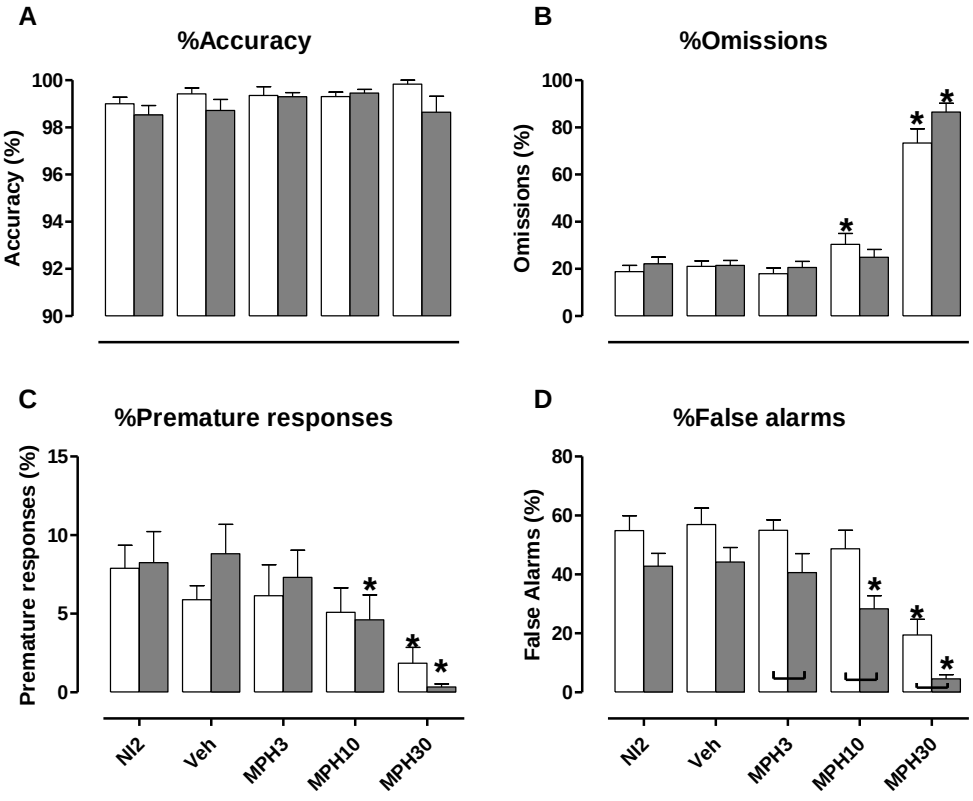

Figure 6.2 – The effects of methylphenidate (3, 10 and 30 mg/kg), vehicle (VEH) and no injection (NI-2) on A: %accuracy, B: %omissions, C: %premature responses and D: %false alarms in the 5C-CPT, in wildtype (white bars) and NK1R-/- mice (grey bars). * P<0.05 vs. vehicle within genotype. Connected bars denote P<0.05. Data show mean ± SEM. n=11-12.

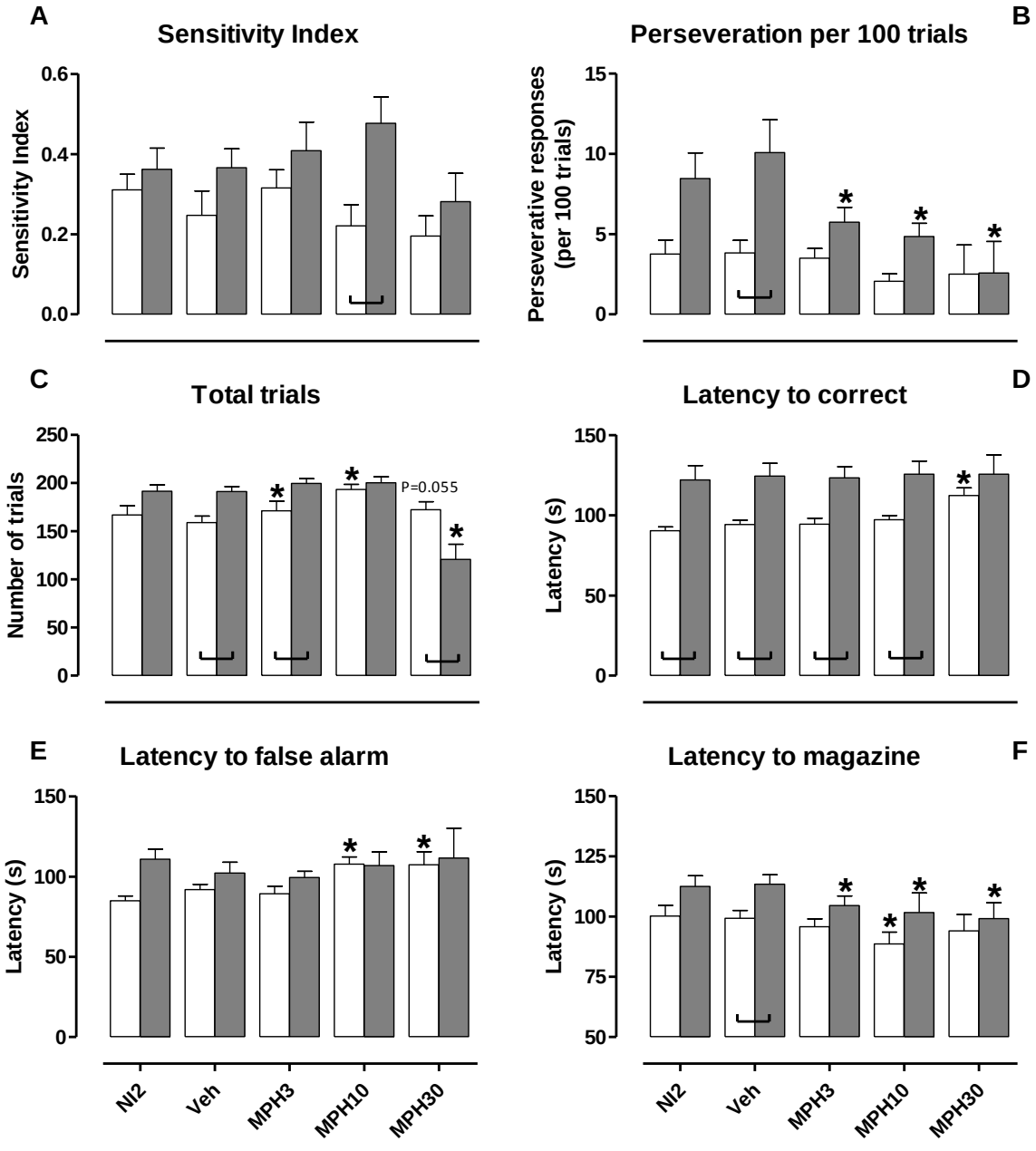

Figure 6.3 – The effects of methylphenidate (3, 10 and 30 mg/kg), vehicle (VEH) and no injection (NI-2) on A: sensitivity index, B: perseveration, C: total number of trials, D: latency to correct response, E: latency to false alarm and F: latency to magazine in the 5C-CPT, in wildtype (white bars) and NK1R-/- mice (grey bars). * P<0.05 vs. vehicle within genotype. Connected bars denote P<0.05. Data show mean ± SEM. n=11-12.

6.4 Discussion

The aim of this experiment was to determine whether the first-line ADHD treatment, methylphenidate, reduces impulsive and inattentive behaviour in NK1R-/- mice performing the 5C-CPT. Overall, the findings suggest that methylphenidate robustly reduced impulsivity in NK1R-/- mice, at a dose which increased inattention in wildtypes. As discussed below, these changes are unlikely to be due to reduced motivation for the task at this dose of drug, but at a higher dose, the drug could have decreased motivation and/or ability to carry out the task, particularly in wildtypes. Interestingly, the most potent effect of methylphenidate was on perseveration: all three doses of the drug prevented perseveration in NK1R-/- mice, but had no effect in wildtypes.

6.4.1 The 5C-CPT

Here, the 5C-CPT was used as an extension of the 5-CSRTT: it utilized novel 'no-go' trials to further challenge the vigilance and response control of mice. In this experiment, NK1R-/- mice took fewer days to reach testing criteria than wildtypes. It could be that NK1R-/- mice coped with the extra step ('no-go' trials) better than wildtypes. Although the reason for this is unclear, it seems a likely explanation, as NK1R-/- mice expressed fewer *false alarms* than wildtypes in the testing phase.

The reason that NK1R-/- mice were not impulsive in this cohort, either in terms of premature responses or false alarms, remains to be explained. This could be because of the training method used: variable inter-trial intervals (VITI) were used in the training phase of the experiment, whereas previously (in Yan *et al.* 2011 and Dudley *et al.* 2013), the first time mice experienced a VITI was during testing. It could be that in this experiment, mice had been trained to a higher degree, i.e. impulsivity had decreased with repeated testing (see Weir *et al.* 2014). This is corroborated by the results for %accuracy, which also improved with repeated testing (Weir *et al.* 2014): here it was around 99%, whereas in the 5-CSRTT, %accuracy is usually around 95% (see Chapter 3 or 5). This may not be a great difference, but could signify the overall level to which mice had learned the task.

6.4.2 Methylphenidate improves impulsivity in NK1R-/- mice

One important point to note is that the results of this experiment corroborate the proposal that this task measures two distinct types of impulsivity; motor impulsivity (*%premature responses*) and behavioural disinhibition *(%false alarms*) (Young *et al.* 2009; Young *et al.* 2011). This is inferred because NK1R-/- mice displayed a similar incidence of, or more, premature responses than wildtypes, but fewer false alarms, suggesting the two are distinct behaviours. A similar finding was reported in a study which used 5,7-dihydroxytryptamine (5,7-DHT)–lesioned rats, suggesting not all measures of impulsivity correlate with each other (Winstanley *et al.* 2004). However, methylphenidate reduced both forms of impulsivity, at 10 mg/kg, in NK1R-/- mice but not wildtypes. At the highest dose, impulsivity was attenuated in both genotypes, but omitted responses were also increased (to around 80%) in both genotypes, indicating that this dose was outside of the beneficial dose-range.

One study comparable to this one, found that methylphenidate reduced impulsivity in rats deemed 'high impulsive' in the 5C-CPT, but *increased* impulsivity in 'low impulsive' rats, i.e. the effects were baseline dependent (Tomlinson *et al.* 2014), as was the case in a stop signal reaction time task (SSRT) (Eagle *et al.* 2007). The results presented here mirror this in part: NK1R-/- mice had a slightly higher baseline of premature responding than wildtypes (although this was not statistically significant), which was attenuated by methylphenidate. At odds with this proposal is the finding that NK1R-/- mice also expressed fewer false alarms than wildtypes, yet methylphenidate further reduced this. The latter result actually strengthens the predictive validity of the NK1R-/- mouse, as the effect of methylphenidate was not baseline dependent, but genotype dependent.

The use of these two different tests of impulsivity together suggests something about the neurobiology of this behavioural trait. As discussed previously, impulsivity is multi-faceted construct: different forms of impulsivity can be measured independently in different tests (Dalley and Roiser 2012), and could be separable by their neurobiological underpinnings. Abnormal serotonergic and/or dopaminergic transmission is believed to be involved in all forms of impulsivity, and the two neurotransmitters may interact (Winstanley *et al.* 2005; Oades 2007). Here, impulsivity is discussed either in terms of "*stopping*" (i.e. on a SSRT, or go/no-go

task), "*waiting*" (i.e. in the 5-CSRTT) or "*choice impulsivity*" (i.e. in a delayed discounting paradigm), although these categories can even be further subdivided.

Rodent studies have consistently suggested that the monoaminergic systems are involved in impulsive behaviour: for example, psychostimulants improve *stopping* in the SSRT (Feola *et al.* 2000; Eagle and Robbins 2003; Eagle *et al.* 2007). In contrast, psychostimulants generally increase impulsivity when *waiting* is involved (Cole and Robbins 1987; van Gaalen *et al.* 2006; Blondeau and Dellu-Hagedorn 2007), as we have previously reported for amphetamine in the 5-CSRTT (Yan *et al.* 2011). This effect is reversed when premature responding is not punished (Bizarro *et al.* 2004). Interestingly, the effect of psychostimulants on *choice* impulsivity may depend on interactions between dopamine and serotonin, because the capacity for amphetamine to reduce impulsivity of rats in a delay-discounting paradigm is attenuated when brain 5-HT is depleted (Winstanley *et al.* 2003; Helms *et al.* 2006).

Dopamine efflux is reduced in the prefrontal cortex (PFC) of NK1R-/- mice (Yan *et al.* 2009), which could be indicative of why the response to methylphenidate differed in these mice. However, because dopamine is taken up by the noradrenaline transporter in the PFC, and methylphenidate increases efflux of both catecholamines (Bymaster *et al.* 2002; Berridge *et al.* 2006), it is difficult to separate the relative contribution of cortical dopamine to the behavioural response to this drug. Nevertheless, the effect of methylphenidate on rats performing a stop-signal reaction time task (SSRT) is not attenuated by the dopamine D_1/D_2 receptor antagonist, α-flupenthixol (Eagle *et al.* 2007), suggesting the involvement of a non-dopaminergic mechanism in impulsivity to some extent.

Serotonin may be critical for some forms of impulsivity, but not others. For example, 5,7-DHT-depletion of 5-HT increases *waiting* impulsivity in rats performing the 5-CSRTT (Harrison *et al.* 1997; Harrison *et al.* 1999), but neither 5-HT depletion nor 5-HT reuptake inhibitors have any effect on *stopping* in a SSRT (Bari *et al.* 2009; Eagle *et al.* 2009). However, it should be noted that serotonin is strongly implicated in *stopping* on a go/no-go task, suggesting that this type of impulsivity can be further subdivided into 'action restraint' (on the go/no-go task) and 'action cancellation' (on the SSRT) (Eagle *et al.* 2008). Furthermore, 5-HT depletion has no effect on impulsive *choice* in a delayed discounting paradigm (Winstanley *et al.* 2004). In either case,

methylphenidate has a negligible affinity for the serotonin transporter and does not increase extracellular serotonin concentrations in the PFC of rats (Bymaster *et al.* 2002). Although downstream effects cannot be ruled out, the main mechanism of action of the drug is unlikely to be serotonergic.

	"Stopping" SSRT/ Go/No-Go	**"Waiting" 5-CSRTT**	**"Choice" Delay Discounting**
Dopamine augmentation (e.g. amphetamine)	↓	↑	↓
Serotonin depletion (e.g. 5,7-DHT-lesions)	No effect or ↑	↑	---

Table 6.1 – A summary of the effects of increasing extracellular dopamine concentrations and decreasing extracellular serotonin concentrations on the three types of impulsivity. ↓ impulsivity is improved, ↑impulsivity is increased, --- inconclusive.

Another property of methylphenidate to consider is the drug's ability to increase histamine release (Horner *et al.* 2007). As discussed in Chapter 5, histamine could contribute to the regulation of cognitive processes (Schneider *et al.* 2014). Studies examining the effects of histamine in the 5-CSRTT corroborate the theory that histamine is involved in impulsivity: the histamine H_3 receptor antagonist, ciproxifan (which increases histamine release (Esbenshade *et al.* 2008)), reduced premature responding, and increased %accuracy in Lister-hooded and Long Evans rats performing this task (Ligneau *et al.* 1998; Day *et al.* 2007). Although histamine is renowned for its regulation of sleep, these effects were apparent without any effects on response latencies, i.e. there was no evidence of sedation.

6.4.3 Methylphenidate attenuates perseveration in NK1R-/- mice

Methylphenidate was particularly efficacious in attenuating the perseveration displayed by NK1R-/- mice: all three doses reduced perseveration in these mice, but not wildtypes. We have also previously reported that amphetamine reduced perseveration in NK1R-/- mice in the 5-CSRTT (Yan *et al.* 2011).

Although the distinct behaviour that 'perseveration' describes is not yet fully understood, it has been suggested that it could be a form of 'checking', which is common in patients with

obsessive compulsive disorder (OCD). This is noteworthy given the overlap between ADHD and OCD (Geller 2006). Another suggestion is that this behaviour could be a type of 'tic', reminiscent of those seen in Tourette's syndrome: a disorder which can also be comorbid with ADHD (Ludolph *et al.* 2012). However, the pharmacology of perseveration does not support this theory: methylphenidate (reported here) and amphetamine (Yan *et al.* 2011) improve this behaviour in NK1R-/- mice, but these drugs are reported to worsen tics, or even induce the onset of tics, in the clinic (Erenberg 2005). Similarly, guanfacine is effective in reducing ADHD with comorbid tics (Bloch *et al.* 2009), but guanfacine had no effect on perseveration in NK1R-/- mice (Chapter 3).

In any case, it is remarkable that the lowest dose of methylphenidate (3 mg/kg) did not affect measures of attention or impulsivity, but did reduce perseveration. At the very least, this suggests that perseveration is controlled by different neurocircuitry/neurochemistry than the key ADHD behaviours. If perseveration does represent a 'checking' behaviour, the results presented here suggest that methylphenidate could be a useful treatment option for patients expressing this type of obsessive/compulsive checking. Moreover, methylphenidate could be particularly effective in alleviating this behaviour in a subset of ADHD patients with *TACR1* polymorphisms.

6.4.4 Methylphenidate reduces attention in wildtype mice

At the same dose (10 mg/kg) which reduced impulsivity in NK1R-/- mice, attention (%omissions) was reduced in wildtypes, but not NK1R-/- mice. This effect on %omissions, combined with the result for %false alarms, equated to a genotype difference in vigilance when measured by the sensitivity index (taking %omissions and %false alarms into account), such that NK1R-/- mice were more vigilant than wildtypes. Another measure of attention, response accuracy, remained unaffected by methylphenidate at any dose. As discussed in Chapters 3 and 5, this is likely due to a) the high level of %accuracy at baseline, and b) attention being better represented by %omissions in mice. The increase in %omissions at 10 mg/kg in wildtypes is unlikely to be explained by reduced motivation for the task, as other measures of motivation (total number of trials, latency to correct response/magazine) either did not change, or

suggested *increased* motivation for the task at this dose. Others have reported that methylphenidate has no effect on attention (%accuracy) (Paterson *et al.* 2011) or increases this measure in rats (Paine *et al.* 2007). The difference between these studies and the results reported here could suggest that methylphenidate has distinct effects on rats and mice, or that the 5-CSRTT does not provide a sensitive measure of the effects of methylphenidate on attention in mice.

At the highest dose (30 mg/kg), methylphenidate increased omissions in both genotypes. At this dose, motivation could be reduced, as the latency to correct response was *increased* in wildtypes. Conversely, the latency to reward was *reduced* in NK1R-/- mice, but this is likely to be explained by the reduction in perseveration, as mice did not spend time 'perseverating' before collecting the reward. Stimulants have been used as appetite suppressants since the 1950s (Fernstrom and Choi 2008). Moreover, a common side effect of methylphenidate in humans is reduced appetite, and so reduced appetitive motivation for the task could certainly explain the behaviour observed when mice were tested with the highest dose of drug.

6.5 Highlights

- NK1R-/- mice did not display impulsivity in terms of 'stopping' (%false alarms) in the 5C-CPT.
- Methylphenidate reduced impulsivity in terms of waiting (%premature responses) and stopping (%false alarms) in NK1R-/- mice at a dose which had no effect on these measures in wildtypes.
- The catecholaminergic response to methylphenidate could underpin the reduction in impulsivity observed here, but the effects of this drug on extracellular histamine concentration could also contribute to the behavioural profile of this drug.
- At the highest dose tested, methylphenidate blunted all forms of behaviour in both genotypes, and could have reduced motivation for the task.
- Methylphenidate was most effective in reducing perseveration in NK1R-/- mice, suggesting this drug could be most useful for alleviating this trait in ADHD patients with *TACR1* polymorphisms.

Chapter 7

Investigating the body composition of wildtype and NK1R-/- mice

Chapter 7. Investigating the body composition of wildtype and NK1R-/- mice

7.1 Introduction

In Chapters 3, 5 and 6, the 5-CSRTT and 5C-CPT were used to examine the behavioural abnormalities of NK1R-/- mice. The results were mostly discussed in terms of neurochemistry. However, other important factors to consider are the weight and food intake of these mice compared with wildtypes, especially given that the 5-CSRTT and 5C-CPT are operant tasks in which mice respond for an appetitive reward. The NK1R-/- mice used in the experiments detailed in Chapters 3, 5 and 6 were smaller than their wildtypes counterparts, as were those in the experiments reported in (Dudley *et al.* 2013), yet it has been suggested that ADHD can be comorbid with obesity (Cortese *et al.* 2008).

7.1.1 ADHD and obesity

Comorbidities between ADHD and other psychiatric disorders have been investigated extensively. However, the overlap between ADHD and medical conditions such as obesity is less clear. Obesity, defined as a BMI (body mass index) greater than 30, is a highly prevalent condition in the Western world. Globally, 35% of adults over 20 years old were overweight, and 12% were obese, in 2008 (www.who.int/gho/en). Obesity is considered one of the most common indirect causes of morbidity. It is difficult to define the cross-over between ADHD and obesity because of many confounding factors, for example, psychiatric conditions comorbid with ADHD, e.g. depression, anxiety or drug addiction, may also influence weight. Despite these limitations, evidence does suggest an association between ADHD and obesity in children/adolescents (Waring and Lapane 2008) and adults (Cortese *et al.* 2013).

The incidence of ADHD in obese individuals varies widely from study to study: estimates vary from 13% to 58%, compared with about 5% in the general population (Altfas 2002; Erermis *et al.* 2004; Agranat-Meged *et al.* 2005; Fleming *et al.* 2005). This relationship is also bidirectional.

Studies on the weight status of individuals with ADHD have similarly shown that these patients have higher BMI-SDS (standard deviation scores, or *z*-scores) than controls (Curtin *et al.* 2005; Anderson *et al.* 2006; Hubel *et al.* 2006).

This link between ADHD and obesity is somewhat counterintuitive: it might be expected that a hyperactive individual is more likely to be underweight than overweight. Nevertheless, inattentiveness and impulsivity may influence an individual to gain weight, more so than hyperactivity might induce weight loss. A number of mechanisms of how ADHD and obesity overlap have been proposed (see section 1.9.1), however, no study has, to date, investigated the temporal relationship between ADHD and obesity: such longitudinal studies would be necessary to determine which theory is most likely.

7.1.2 Weight and food intake of NK1R-/- mice

If any of the aforementioned theories of the overlap between ADHD and obesity also apply to NK1R-/- mice, it might be expected that these mice have a disrupted metabolism and/or eating pattern. The food intake of mice was monitored and controlled over the duration of the 5-CSRTT/5C-CPT experiments (see Appendix 6): from these data it is clear that NK1R-/- mice need to eat more in relation to their body weight, than wildtypes, to maintain the same weight. NK1R-/- mice could have a faster metabolism, which could affect their appetite when food is restricted. They could also have underlying differences in body composition. However, in the 5-Choice tasks, mouse weight and food intake are controlled. It would therefore be of interest to examine the body composition of wildtype and NK1R-/- mice under normal feeding conditions.

We might expect a hyperactive animal to be leaner, despite consuming more food. However, if NK1R-/- mice follow the same paradox that is seen in ADHD, they may have increased percentage body fat compared with wildtypes. By the same token, a high fat ("Western") diet could induce a greater weight and body fat gain in NK1R-/- mice than wildtypes, as a diet rich in fat is particularly obesogenic in humans (Mozaffarian *et al.* 2011).

7.1.3 Gender differences in ADHD with comorbid obesity

In behavioural studies female mice were avoided, because changes in behaviour due to hormonal fluctuations necessitate the use of more animals to reduce variation in the results. Moreover, the process used to measure the stage of oestrous cycle is highly stressful. However, there is limited evidence to suggest that the association between ADHD and obesity is gender-dependent. One study found that an association between obesity and ADHD behaviours was only present in adolescent females (van Egmond-Frohlich *et al.* 2012), and others have reported stronger risk of comorbidity in females than males (Kim *et al.* 2011; Byrd *et al.* 2013). For this reason, it is also of interest to compare the body composition of both male and female mice.

7.1.4 Body composition analyses

In this Chapter, the body composition of wildtype and NK1R-/- mice of both genders, fed a normal or a high fat diet, was examined. This analysis was carried out by two different, but comparable methods. The 'gold standard' remains chemical composition analysis: each component of the animal's carcass is analysed separately using analytical chemistry. Dual energy X-ray absorptiometry (DEXA) analysis is commonly used in a clinical setting to diagnose osteoporosis (see Chapter 2, section 2.5.1), but has recently been back-translated for use in rodents. Here, both of these techniques are used in parallel to allow comparisons to be made between the two. Moreover, chemical analysis is used with the aim of further validating DEXA as an accurate and precise method of measuring body composition in rodents.

7.1.5 Aims

The first aim of these experiments was to test the prediction that NK1R-/- mice might have a higher body fat percentage than wildtypes, under normal conditions and after exposure to a high fat "Western" diet. In doing this, these experiments also aimed to determine whether females are more susceptible to changes in weight and body composition than males, as may be the case in ADHD. The second aim of these experiments was to further test the validity of

the use of DEXA in a preclinical setting, by comparing the results of DEXA analysis with chemical composition analysis.

7.2 Methods

7.2.1 Normal diet

Mice were weaned on to a normal diet (2018 global Rodent Diet, Harlan; see Appendix 7) at 3 weeks old. At 6 weeks (±1 day) of age, nose to tail lengths were measured before the mice were culled by a Schedule 1 method. Carcasses were frozen at -20°C until the analysis.

7.2.2 High fat diet

A separate batch of mice was weaned at 3 weeks of age, onto a diet in which 45% of the calories derived from fat (Research Diets, NJ, USA). The constituents of the diet and their calorific values are given in Appendix 8. Animals were maintained on this diet for 28 days and were weighed daily. The food hopper was also weighed daily to determine food consumption. The amount of food consumed by each cage of mice was divided by the number of mice in that cage, to give the weight of food consumed per mouse. This was used to calculate a ratio of food eaten to body weight. At the end of the 28 days (at 7 weeks of age) mice were culled and nose to tail lengths measured as above. Carcasses were frozen at -20°C until needed.

In both cases mice were group housed, and had *ad libitum* access to water and the respective diet.

7.2.3 Body composition analyses

Body composition analyses were performed as described in Chapter 2, section 2.5. DEXA analysis was performed on whole carcasses of mice, and chemical analysis was performed on samples of milled, freeze-dried carcasses.

DEXA

The DEXA analysis gave data for;

- Total tissue mass (TTM; g)
- Tissue area (TA; cm^2)
- Fat mass (FM; g)
- %Fat
- Bone area (BA; cm^2)
- Bone mineral content (BMC; g)
- Bone mineral density (BMD; g/cm^2)

Chemical analysis

The results of the chemical analysis were used to calculate;

- %Water
- %Fat
- %Ash
- %Protein

7.2.4 Statistics

The weight and food intake of mice over 28 days on a high fat diet were analysed using repeated measures analyses, with between-subjects factors of '*genotype'* and '*gender'*, and within-subjects factor of '*day'*. First, the analysis compared the 4 groups: if there was a main effect of either *genotype* or *gender*, the analysis progressed to a second comparison of each gender separately. Post-hoc LSD tests revealed differences between the genotypes on individual days.

Single measures ANOVA was used the main factors of '*genotype'* and '*gender'* to analyse the results of the body composition studies. A main effect of either of these factors, or interaction between them, was used as the criterion for progression to post-hoc comparisons in the LSD test. The comparisons between DEXA and chemical analysis were performed using Pearson's correlation analysis and linear regression.

7.3 Results

7.3.1 Wildtype and NK1R-/- mice gain weight at the same rate on a high fat diet (Figure 7.1)

Over 28 days of *ad lib* access to high fat diet, the weight gain (Figure 7.1A) of mice depended on gender, such that females weighed less than males [[RAW]gender: $F_{(1,33)}$=103.12, P<0.001], and to some extent, on genotype, though this just missed the criterion for significance [[RAW]geno: $F_{(1,33)}$=3.83, P=0.059]. These two factors did not interact [[RAW]geno*gender: $F_{(1,33)}$=1.15, P=0.292]. However, female wildtype mice weighed less than NK1R-/- females on days 1 - 16 and 24 – 28, but at no point did the weight of the two genotypes differ in males.

The mass of food eaten (Figure 7.1B) reflected the differences in weight: males ate more food than females over the 28 days [[RAW]gender: $F_{(1,7)}$=28.77, P<0.001], but mice ate the same amount regardless of genotype [[RAW]geno: $F_{(1,7)}$=1.39, P=0.278]. However, a difference between wildtype and NK1R-/- mice was evident on a few individual days.

When the ratio of 'food eaten to body weight' was calculated (Figure 7.1C), wildtype and NK1R-/- mice ate the same amount of food in proportion to their body weight [[SQRT]geno: $F_{(1,33)}$=0.1, P=0.749]. The mass of food eaten in proportion to body weight was also independent of gender [[SQRT]gender: $F_{(1,33)}$=0.24, P=0.624], and the two factors did not interact [[SQRT]geno*gender: $F_{(1,33)}$=0.98, P=0.329]. However, mice did eat progressively less food over the 28 days, relative to their weight [[SQRT]day: $F_{(26, 858)}$=53.46, P<0.001].

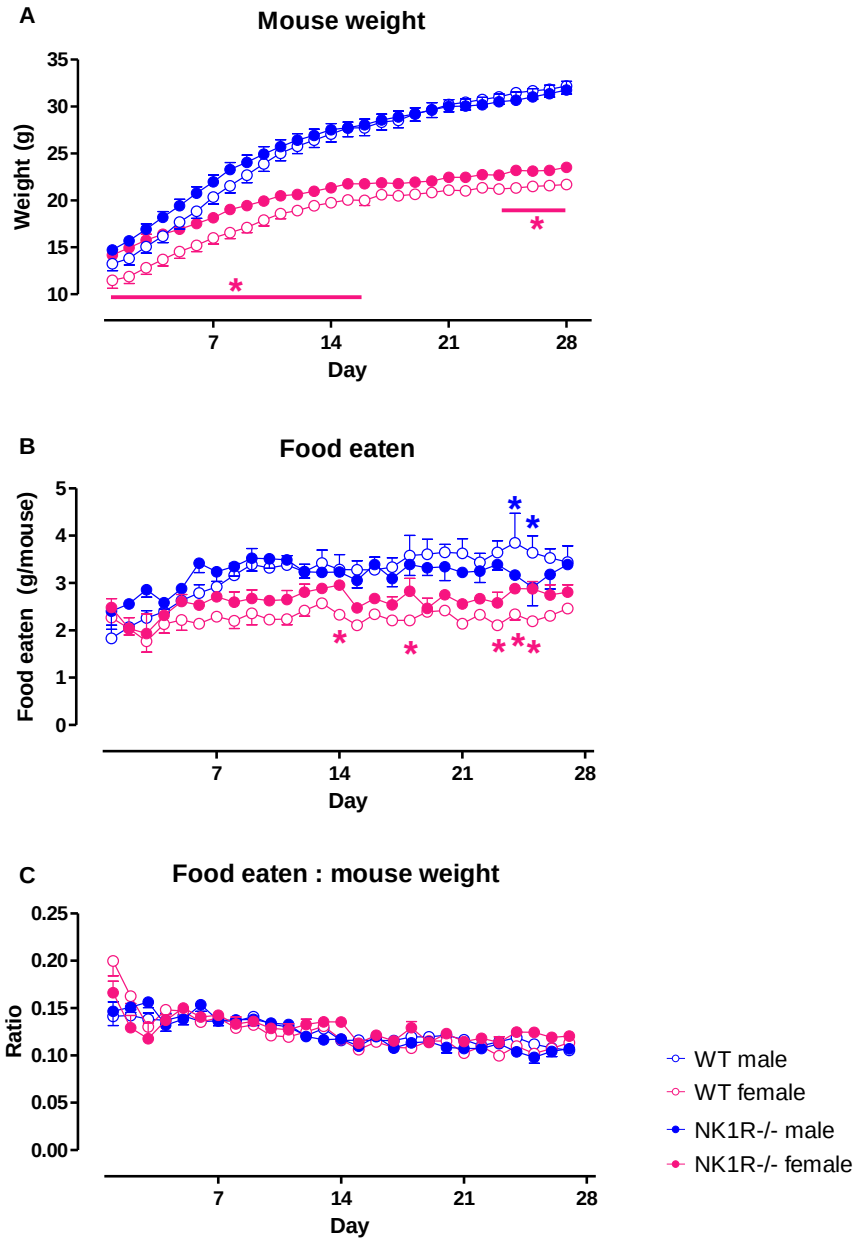

Figure 7.1 – A: Weight gain, B: food eaten, and C: ratio of weight to food eaten by wildtype and NK1R-/-, male and female, mice over 28 days of access to a high fat diet. n=8-10 per group in A and C, n=2-3 cages per group in B. Data shows mean ± SEM. * P<0.05 wildtype versus NK1R-/- within gender.

7.3.2 NK1R-/- mice are smaller than wildtypes (Figure 7.2)

Weight

On a *normal diet*, NK1R-/- mice weighed less than wildtypes [[RANK]geno: $F_{(1,36)}$=5.75, P=0.022]. This did not depend on gender [[RANK]geno*gender: $F_{(1,36)}$=0.79, P=0.380], but did reach statistical significance in males [WT vs. KO: P=0.026], not females [WT vs. KO: P=0.293]. Females also weighed less than males in both genotypes [WT: P<0.001, NK1R-/-: P=0.001].

By contrast, by the end of 28 days on a *high fat diet*, male NK1R-/- mice weighed the same as male wildtypes [[RANK]geno: $F_{(1,33)}$=5.24, P=0.029; male WT vs. KO: P=1.000]. Moreover, female NK1R-/- mice weighed more than female wildtypes [female WT vs. KO: P=0.002], such that overall, weight depended on an interaction between genotype and gender [[RANK]geno*gender: $F_{(1,33)}$=5.59, P=0.024]. Females of both genotypes weighed less than males [[RANK]gender: $F_{(1,33)}$=130.1, P<0.001].

Size

On a *normal diet*, NK1R-/- mice were smaller (nose to tail length) than wildtypes in both genders [[RANK]geno: $F_{(1,36)}$=53.97, P<0.001]. However, males and females were the same size as each other [[RANK]gender: $F_{(1,36)}$=0.25, P=0.623]. This was replicated in the tail length: males and females had the same tail length, but NK1R-/- mice had much smaller tails than wildtypes [[RANK]geno: $F_{(1,36)}$=51.70, P<0.001; gender: $F_{(1,36)}$=0.01, P=0.919]. A similar pattern was observed in mice fed a *high fat diet*: NK1R-/- mice of both genders were smaller than wildtypes, in terms of nose to tail length [[RAW]geno: $F_{(1,33)}$=9.75, P=0.004] and tail length [[RAW]geno: $F_{(1,33)}$=19.87, P<0.001]. However, in this cohort, females were smaller than males in both measures [nose-tail: [RAW]gender: $F_{(1,33)}$=25.59, P=0.001; tail: [RAW]gender: $F_{(1,33)}$=7.01, P=0.012].

Body Mass Index (density)

When BMI was taken as a pseudo measure of 'density' (i.e. grams per cm^2), the same pattern was evident in both groups of mice: NK1R-/- mice had a higher BMI than wildtypes in the

normal diet [[[RANK]geno: $F_{(1,36)}$=14.55, P<0.001] and the *high fat diet* groups [RANK]geno: $F_{(1,33)}$=26.66, P<0.001]. Females also had a lower BMI than males in both groups [normal diet [RANK]gender: $F_{(1,36)}$=160.31, P<0.001]; HFD [RANK]gender: $F_{(1,33)}$=179.06, P<0.001].

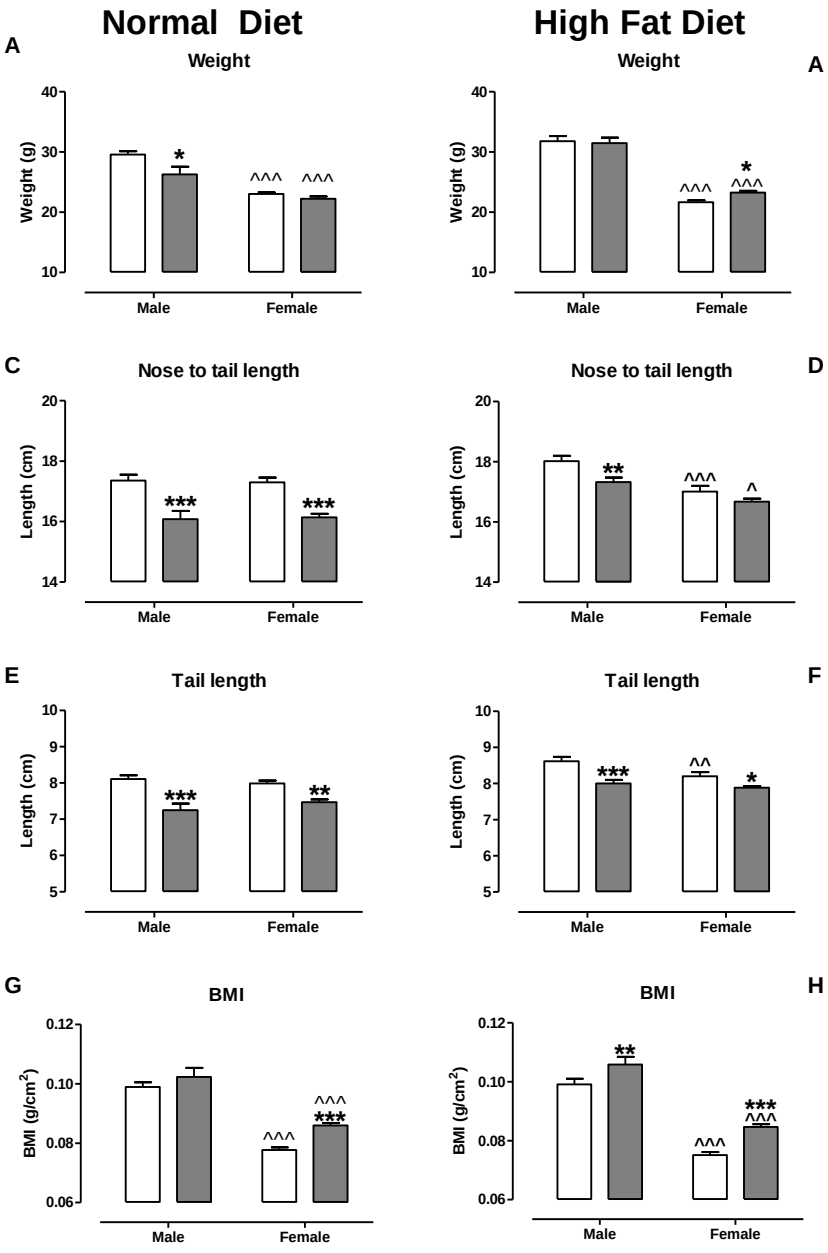

Figure 7.2 – Weight (A & B), length (C & D), tail length (E & F) and body mass index (BMI: G & H) of wildtype (white bars) and NK1R-/- mice (grey bars) after access to a normal or high fat diet. n=8-10, * P<0.05, ** P<0.01, *** P<0.001 NK1R-/- versus wildtype, ^ P<0.05, ^^ P<0.01, ^^^ P<0.001 male versus female. Data show mean ± SEM.

7.3.3 DEXA analysis of body fat (Figure 7.3)

DEXA estimated the total tissue mass, tissue area and fat content of the animals. In mice fed a normal diet, the total tissue mass of mice depended on genotype [[RANK]geno: $F_{(1,36)}$=36.17, P<0.001] and gender [[RANK]gender: $F_{(1,36)}$=105.97, P<0.001]: overall, NK1R-/- mice were smaller than wildtypes, and females were smaller than males. Tissue area also depended on genotype and gender, and these two factors interacted [[RANK]geno*gender: $F_{(1,36)}$=4.68, P=0.037]: NK1R-/- females were smaller than wildtype females [P=0.001], but this was not the case in males [P=0.644]. After exposure to a high fat diet, the genotype difference was lost [[LOG10]geno: $F_{(1,33)}$=0.40, P=0.531], but females still had a lower total tissue mass than males [[LOG10]gender: $F_{(1,33)}$=209.72, P<0.001], independent of genotype [LOG10]geno*gender: $F_{(1,33)}$=3.02, P=0.092]. Tissue area was also smaller in females [[RAW]gender: $F_{(1,33)}$=50.87, P<0.001]. There was a trend towards a genotype difference in tissue area overall [[RAW]geno: $F_{(1,33)}$=3.23, P=0.08]: NK1R-/- females were larger than wildtype females [WT vs. KO: P=0.049], but no difference was observed in males after exposure to a high fat diet.

The mass of fat in mice on a normal diet was the same in both genotypes [[RANK]geno: $F_{(1,36)}$=0.15, P=0.699], but since NK1R-/- mice had a lower total tissue mass, this translated to a higher %fat in NK1R-/- mice [[RANK]geno: $F_{(1,36)}$=12.1, P=0.001], in males [WT vs. KO: P=0.002], but not females [WT vs. KO: P=0.138]. In mice fed a high fat diet, this genotype difference was lost: %fat was the same in males and females [[RAW]gender: $F_{(1,33)}$=1.04, P=0.316], and in wildtypes and NK1R-/- mice [[RAW]geno: $F_{(1,33)}$=0.10, P=0.753]. Fat mass was also independent of genotype [[LOG10]geno: $F_{(1,33)}$=0.00, P=0.951], but was lower in females than males [[LOG10]gender: $F_{(1,33)}$=34.47, P<0.001].

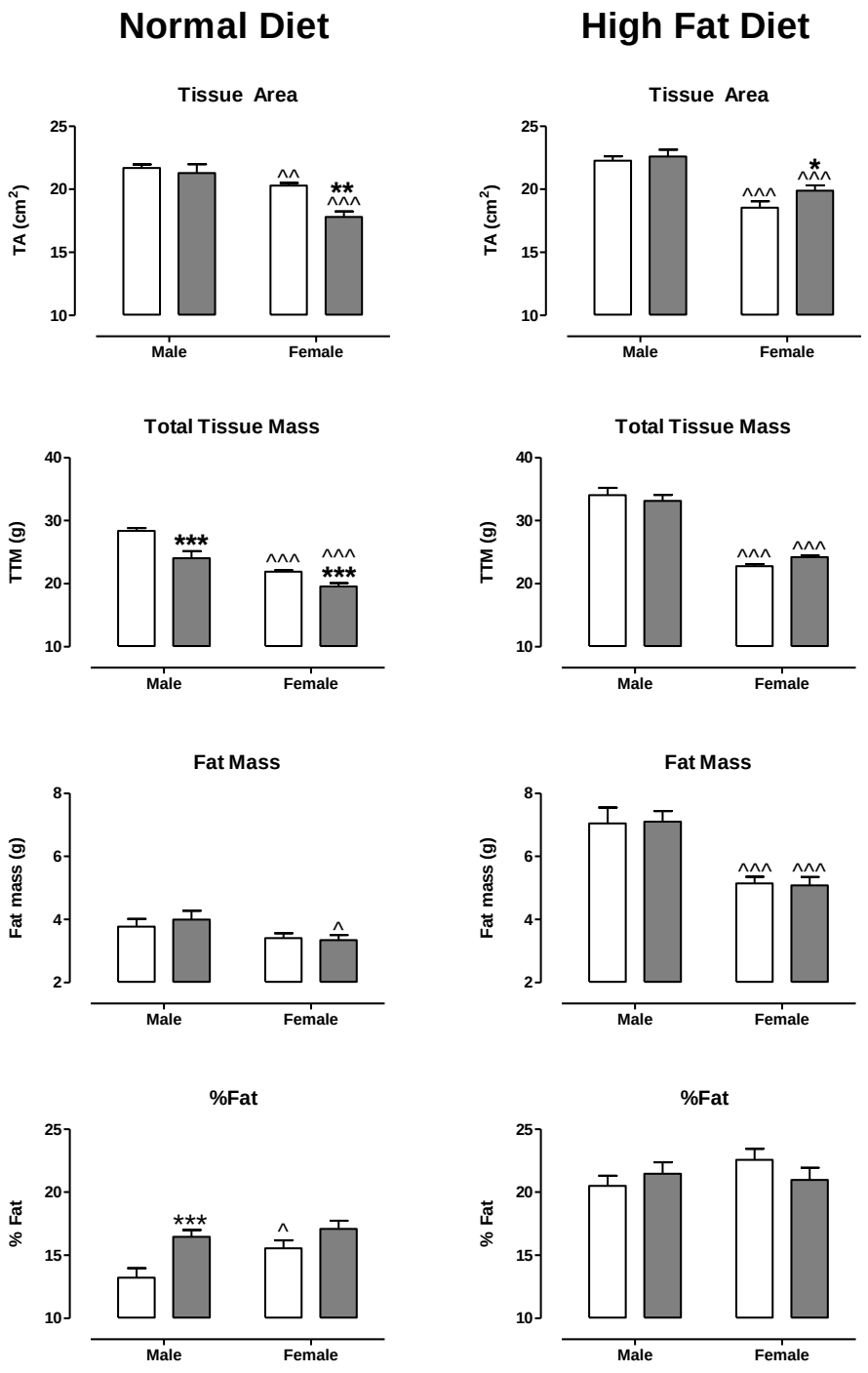

Figure 7.3 – DEXA estimates of the tissue area (A & B), tissue mass (C & D), fat mass (E & F) and %fat (G & H) of wildtype (white bars) and NK1R-/- mice (grey bars) fed a normal and high fat diet. n=8-10, * P<0.05, ** P<0.01, *** P<0.001 NK1R-/- versus wildtype, ^ P<0.05, ^^ P<0.01, ^^^ P<0.001 male versus female. Data show mean ± SEM.

7.3.4 DEXA analysis of bone density (Figure 7.4)

The results of the DEXA bone analysis revealed that, on a normal diet, NK1R-/- mice had a smaller bone area than wildtypes [[RANK]geno: $F_{(1,36)}$=5.95, P=0.020], and this was independent of gender [[RANK]geno*gender: $F_{(1,36)}$=0.01, P=0.943]. However, in mice fed a high fat diet, bone area was determined by both genotype and gender [[RAW]geno*gender: $F_{(1,33)}$=6.30, P=0.017]. Female wildtypes had a smaller bone area than female NK1R-/- mice [WT vs. KO: P=0.001], but no genotype difference was evident in males [WT vs. KO: P=0.957].

Bone mineral content depended on an interaction between genotype and gender in mice fed a normal diet [[RANK]geno*gender: $F_{(1,36)}$=8.04, P=0.008] and mice fed a high fat diet [[RAW]geno*gender: $F_{(1,33)}$=6.85, P=0.013]. Female wildtypes had a lower bone mineral content than male wildtypes in the normal diet [WT male vs. female: P<0.001] and high fat diet groups [WT male vs. female: P<0.001]. In the normal diet group there was a genotype difference in males [WT vs. KO: P<0.001], in the high fat diet group there was a genotype difference in females [WT vs. KO: P=0.016].

As a result of these differences, bone mineral density (BMD: bone mineral content per square centimetre), depended on an interaction between genotype and gender in the normal diet group [[RANK]geno*gender: $F_{(1,36)}$=24.70, P<0.001], but not the high fat diet group [[RAW]geno*gender: $F_{(1,33)}$=2.34, P=0.136]. In mice fed a normal diet, NK1R-/- males had lower BMD than wildtype males [WT vs KO: P<0.001], but the opposing difference in females just missed the criterion for significance [WT vs KO: P=0.056]. In mice fed a high fat diet, female wildtypes had a lower bone mineral density than male wildtypes [WT male vs. female: P<0.001], but no gender difference was present in NK1R-/- mice [KO male vs. female: P=0.227].

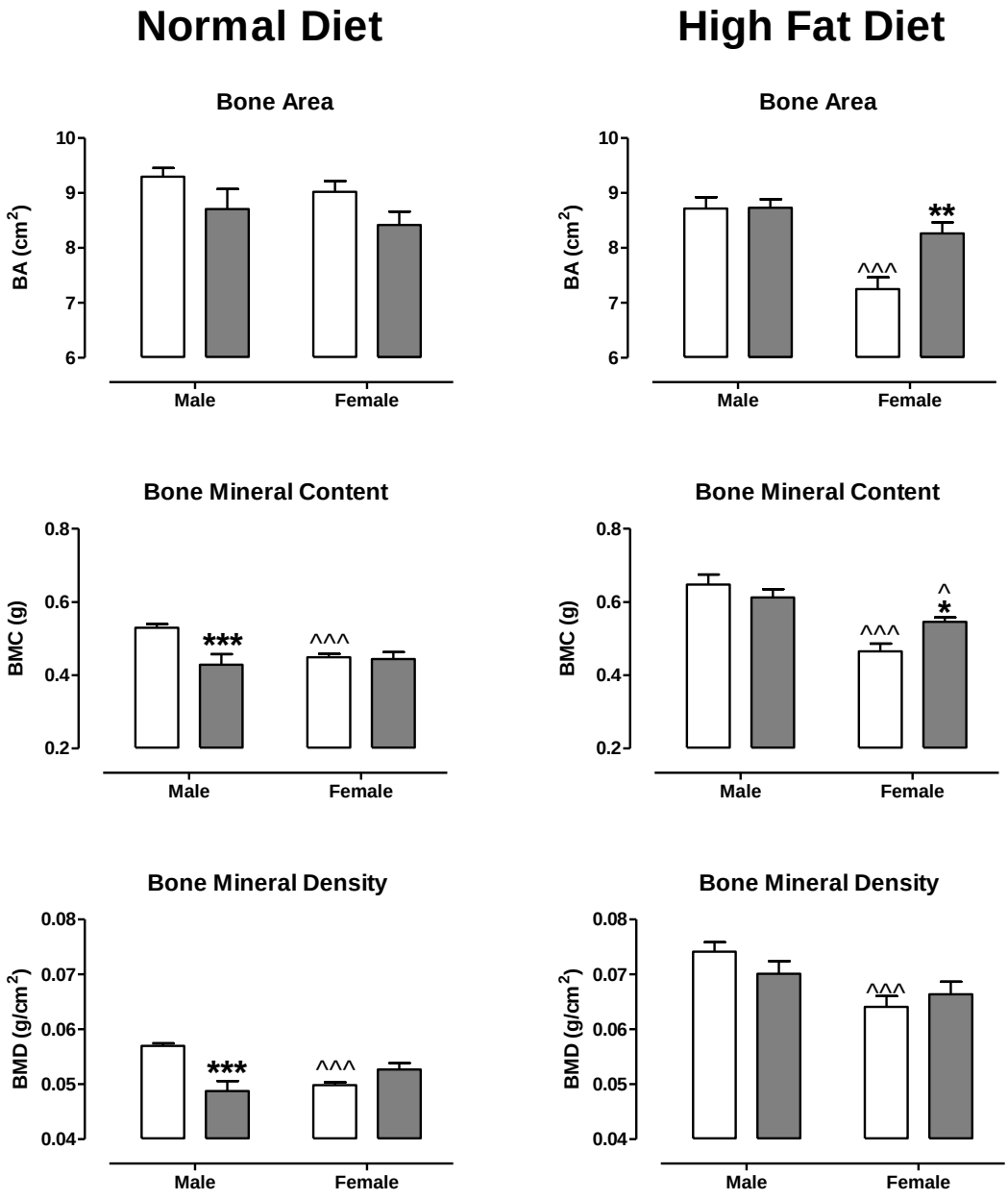

Figure 7.4 – DEXA estimates of the bone area (A & B), bone mineral content (C & D) and bone mineral density (E & F) of wildtype (white bars) and NK1R-/- mice (grey bars) fed a normal and high fat diet. n=8-10, * P<0.05, ** P<0.01, *** P<0.001 NK1R-/- versus wildtype, ^ P<0.05, ^^ P<0.01, ^^^ P<0.001 male versus female. Data show mean ± SEM.

7.3.5 Chemical analysis of body composition (Figure 7.5)

The chemical analysis was divided into four main areas: water, fat, protein and ash.

Water

In mice fed a normal diet, there was an overall difference between the genders [[RANK]gender: $F_{(1,36)}$=6.69, P=0.014] in relative water content, and this did not depend on genotype [[RANK]geno*gender: $F_{(1,36)}$=2.81, P=0.103]. However, female wildtypes had a lower %water content than male wildtypes [P=0.005], but there was no difference in NK1R-/- mice [P=0.524]. The water content of mice which had been on a high fat diet depended on an interaction between genotype and gender [[RAW]geno*gender: $F_{(1,33)}$=10.31, P=0.003]: NK1R-/- males had lower %water than wildtype males [WT vs. KO: P<0.001], but no difference was evident in females [WT vs. KO: P=0.365].

Fat

The differences in %fat were, on the whole, the opposite of those observed for %water. Relative fat content depended on gender in the two genotypes in both the normal diet [[RANK]geno*gender: $F_{(1,36)}$=10.22, P=0.003] and high fat diet [[RAW]geno*gender: $F_{(1,33)}$=10.44, P=0.003] group. In the normal diet group, wildtype females had a higher %fat than wildtype males [male vs. female: P<0.001], but no difference was evident in NK1R-/- mice [male vs. female: P=0.982]. The same difference was apparent in the high fat diet group: a gender difference was evident in wildtypes [WT vs. KO: P<0.001] but not NK1R-/- mice [WT vs. KO: P=0.859]. In the normal diet group a genotype difference (WT>KO) was apparent in females [WT vs. KO: P=0.009], but in contrast, the opposite genotype difference (WT<KO) was evident in males in the high fat diet group [WT vs. KO: P=0.003].

Protein

Protein content depended on gender in both the normal diet group [[RANK]gender: $F_{(1,36)}$=15.33, P<0.001], and high fat diet group [[RANK]gender: $F_{(1,33)}$=53.35, P<0.001], such that females had a lower %protein content than males in both genotypes in the high fat diet group

[WT male vs. female: P<0.001; KO male vs. female: P<0.001], and in wildtypes in the normal group [WT male vs. female: P<0.001].

Ash

Gender was also the main cause of differences in terms of %ash, in mice fed a normal diet [[RANK]gender: $F_{(1,36)}$=29.40, P<0.001], and high fat diet [[RANK]gender: $F_{(1,33)}$=39.49, P<0.001]. This was evident in both genotypes in both groups [male vs. female: normal diet, WT: P=0.006; KO: P<0.001; high fat diet, WT: P<0.001; KO: P<0.001].

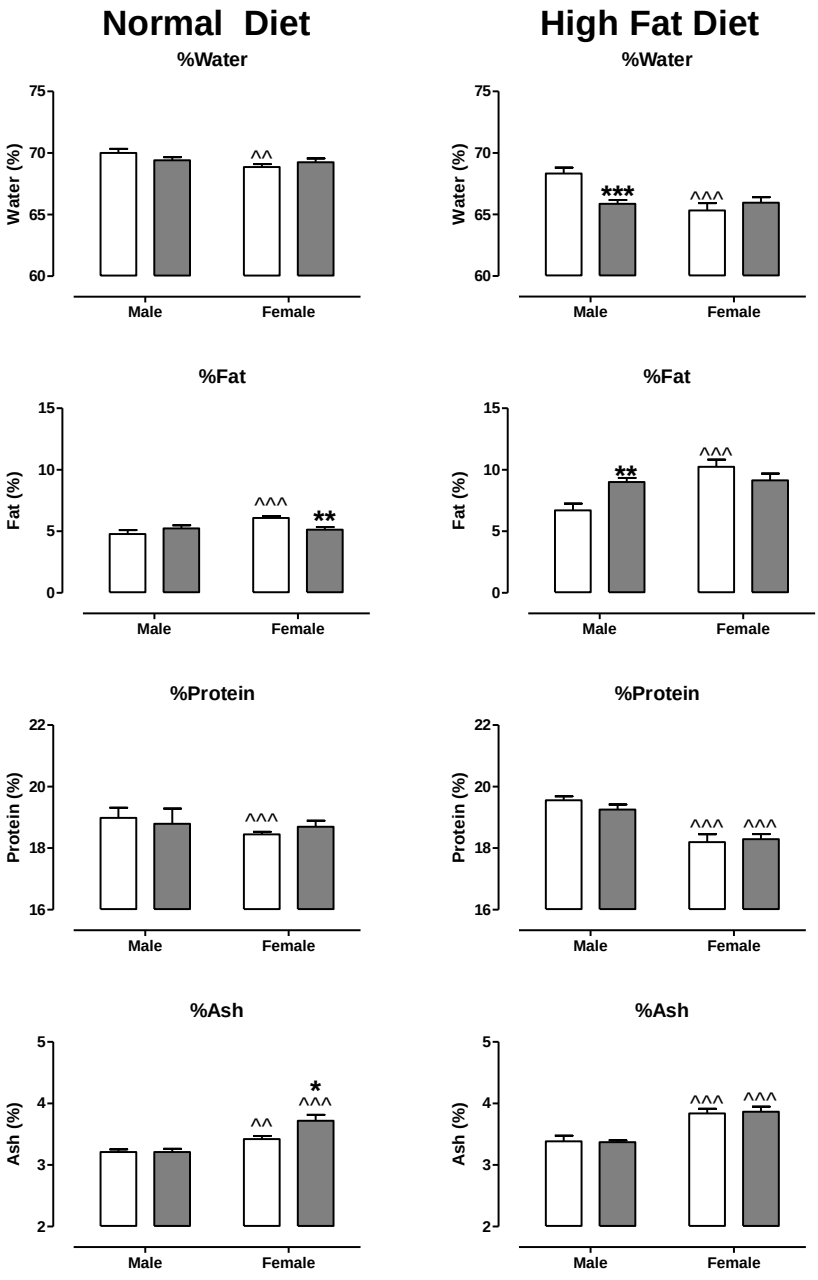

Figure 7.5 – Chemical analysis of %water (A & B), %fat (C & D), %protein (E & F) and %ash (G & H) of wildtype (white bars) and NK1R-/- mice (grey bars) fed a normal or high fat diet. n=8-10, * P<0.05, ** P<0.01, *** P<0.001 NK1R-/- versus wildtype, ^ P<0.05, ^^ P<0.01, ^^^ P<0.001 male versus female.

7.3.6 DEXA analysis over-estimates %fat

The results of the DEXA and chemical analysis were compared using a correlation analysis (Figure 7.6). There was a linear relationship between %fat as calculated by DEXA and by chemical analysis (R^2=0.617, P<0.001). However, DEXA over-estimated %fat by about 9%:

*Body fat (%) = 1.32 * DEXA fat (%) - 9.16*

The relationship between bone mineral content (g) and ash (g) was also linear (R^2=0.792, P<0.001). DEXA underestimated bone mineral content by a negligible amount:

*Body ash (g) = 0.607 * DEXA bone mineral content (g) + 0.039*

As DEXA estimates total tissue mass (TTM) by analysis of fat and bone mass, TTM was also compared with actual mouse weight. There was a linear relationship between TTM and mouse weight (R^2=0.874, P<0.001), but DEXA underestimated tissue mass by 3.63g:

Mouse weight (g) = 1.13 DEXA TTM (g) + 3.63*

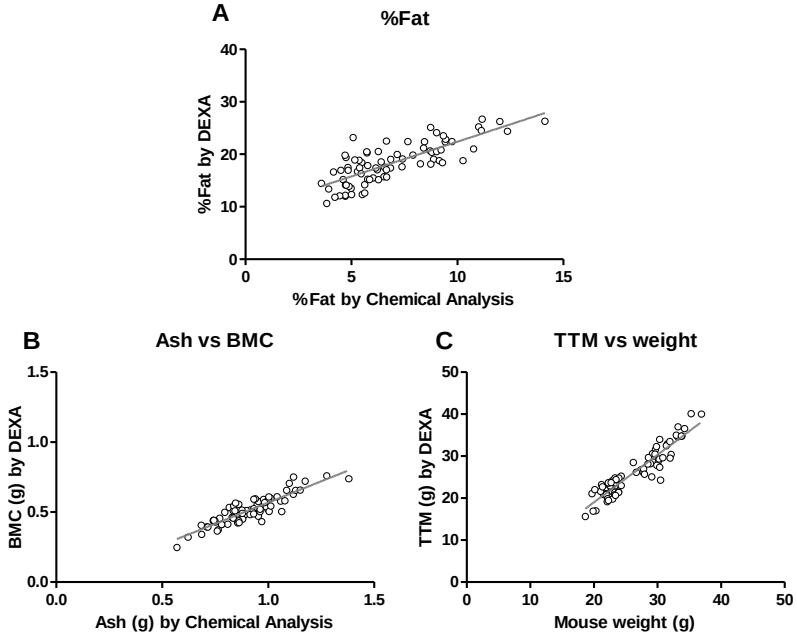

Figure 7.6 – Comparison of %fat (A), bone/ash (B) and mass (C) as measured by DEXA and chemical analysis. Line represents best-fit. n=77.

7.4 Discussion

The aim of the experiments detailed in this Chapter was to investigate whether there are underlying differences in body composition between NK1R-/- mice and wildtypes of both genders. The results confirmed that NK1R-/- mice are smaller, and weigh less, than wildtypes of the same gender. However, NK1R-/- mice were denser than wildtypes in a measure of 'BMI'. In lean animals fed a normal diet, there were small genotype differences in fat content, which depended on gender, when measured by the gold-standard analysis technique (chemical analysis). When mice were fed a high fat diet (HFD), these differences followed the same pattern, but all mice had a higher body fat content. DEXA analysis of fat correlated reasonably well with chemical analysis, but significantly over-estimated fat content. Despite this, DEXA analysis of bone was accurate and precise. Bone density depended on interactions between genotype and gender, and all mice had a higher bone density after access to a HFD.

Analysis of the data collected over the duration of the 5-CSRTT/5C-CPT experiments (see Appendix 6) revealed that NK1R-/- mice needed to eat more, relative to their body weight, than wildtypes, to maintain the same weight. However, there are a number of factors which could have affected the weight of mice in the 5-CSRTT/5C-CPT tasks, such as the amount of milk reward obtained in the task, age and energy expenditure. These factors were effectively accounted for, as the mass of food mice were given depended on their weight. Therefore to investigate body composition, mice were given *ad libitum* access to either a normal diet or a high fat diet.

7.4.1 Body fat

When measured by the gold standard of carcass analysis, body fat depended on interactions between genotype and gender in both the normal diet and high fat diet groups of mice. Although the two diet groups are not directly comparable, mice on a high fat diet had a 3-5% higher fat content than those on a normal diet. Nevertheless, the same pattern was evident in both diet groups: NK1R-/- males had *increased* %fat compared to wildtypes, whereas NK1R-/- females had *decreased* %fat compared to wildtypes. The same pattern could have been evident

because there was no difference between the genotypes or genders in the amount of high fat diet they consumed, relative to body weight.

The literature generally suggests that the substance P (SP)/NK1R system acts to reduce fat storage and weight gain (Miegueu *et al.* 2013). *Tac1*$^{-/-}$ mice, which lack substance P and neurokinin A (as both are alternatively spliced from the same gene: see Chapter 1, section 1.6), show reduced weight gain and reduced circulating leptin and insulin in response to a HFD (Karagiannides *et al.* 2011). Karagiannides *et al* also report that NK1R antagonists reduce weight and adiposity, and improve insulin signaling in obese *ob/ob* mice (Karagiannides *et al.* 2008). Moreover, NK1R antagonists prevent weight gain and fat accumulation in normal mice on a HFD (Karagiannides *et al.* 2008). This is in opposition to the results reported here: NK1R-/- males gained more fat than wildtypes on a HFD, and the opposite genotype difference (WT>NK1R-/-) in female mice on a normal diet was ablated in those on a HFD.

The reason for this difference is not clear, but could relate to a mismatch between functional ablation of NK1Rs from birth, and acute antagonism of NK1Rs (likely at less than 100% receptor occupancy). However, these findings do support the theory that NK1R-/- mice resemble ADHD patients, in so far as these subjects are more prone to increased adiposity/obesity on a 'Western' (high fat) diet. The hypothesis that a subset of ADHD patients with *TACR1* polymorphisms have a higher risk of developing obesity is worth investigating.

Another interesting factor is the difference between male and female mice. The studies examining the effect of NK1R antagonists on weight gain of mice on a HFD discussed above used male mice, only. To date, no study has examined the influence of the SP/NK1R system on the adiposity of male mice compared with females. There is limited literature describing the relationship between the SP/NK1R system, adiposity and gender. However, there are reports of interactions between SP/NK1Rs and gender which could relate to the differences in adiposity observed here. *Tac1* mRNA-expressing neurones are found in the medial preoptic area, arcuate nucleus and ventromedial nucleus of the hypothalamus (Harlan *et al.* 1989; Marksteiner *et al.* 1992; Maeno *et al.* 1993), areas which are strongly implicated in food intake and energy homeostasis (see Williams *et al.* 2001). Importantly, some evidence suggests that expression of this *Tac1* mRNA could be under hormonal control. Exposure of ovariectomised rats to

oestrogen (estradiol benzoate) up-regulates *Tac1* mRNA in the hypothalamus (Brown *et al.* 1990), specifically in the ventromedial nucleus (Akesson 1994). Similarly, although not directly linked to energy homeostasis, estradiol has also been reported to increase NK1R expression in rat pancreatic acinar cells, giving another example of sexual dimorphism in the SP/NK1R system.

A gender difference has also been reported in humans: one PET study using a labelled NK1R antagonist ([^{11}C]GR205171) found that women had a lower density of NK1Rs in the thalamus than men (Engman *et al.* 2012). These studies hint that hormonal control of NK1R gene expression could influence the role of NK1R, not only in energy homeostasis and control of adiposity, but in many their many functions in the CNS and periphery. It could also help to explain why females with ADHD may be more susceptible to comorbid obesity (Kim *et al.* 2011; van Egmond-Frohlich *et al.* 2012; Byrd *et al.* 2013). This is an area which could benefit from further research, as better understanding of the hormonal control of the SP/NK1R system could lead to improved, targeted treatments for ADHD and/or obesity.

7.4.2 Bone density

As with %fat, a similar pattern was observed in both the HFD and normal diet groups: bone mineral density (BMD) was lower in male NK1R-/- mice than male wildtypes, but this was not the case in females. However, in the HFD group, the genotype difference in males was no longer statistically significant. Moreover, all mice which had been on a HFD had a higher BMD than those on a normal diet.

The result that NK1R-/- mice had a lower BMD than wildtypes is noteworthy, as we might expect hyperactive NK1R-/- mice to have an increased BMD, given that exercise is widely accepted to increase bone mass (Behringer *et al.* 2014). The reason for the genotype difference could be due to the influence of NK1Rs on bone metabolism. NK1Rs are present in bone cells, and substance P immuno-reactive (SP-IR) axons innervate bone and surrounding tissues (Goto *et al.* 1998). SP-IR axons appear in development at a stage which coincides with mineralization of long bones (Gajda *et al.* 2005), and thereafter, substance P could act at NK1Rs on osteoblasts to stimulate osteogenesis and bone mineralization (Goto *et al.* 2007, but see Liu *et al.* 2007).

One study also demonstrated that substance P dose-dependently increased the rate of proliferation of chondrocytes (cartilaginous cells), via actions at NK1Rs (Opolka *et al.* 2012).This could explain why, in male NK1R-/- mice at least, bone mineral density was decreased compared to wildtypes.

Despite this evidence for the role of NK1Rs in bone formation and mineralization, little is known about their role in bone disorders and pathologies. Even less evidence is available to link bone pathologies (e.g. osteoporosis) with ADHD, as the two areas are not generally overlapping. However, there is some evidence for an increased risk of bone fractures in ADHD (Chou *et al.* 2014) although whether this relates to physiological changes or behavioural deficits (hyperactivity, inattention or impulsivity) remains unknown. Nevertheless, the results presented here suggest that bone density may be decreased in ADHD, at least in a subset of patients carrying the *TACR1* polymorphism.

Another interesting result was that mice which had been on a HFD had a higher bone density than those on a normal diet. This could in part be explained by the fact that these mice were one week older than the mice on a normal diet, as bone mineral density increases with age, up to a certain point. A high fat diet has actually been reported to lower bone density (Li *et al.* 1990; Ward *et al.* 2003; Lac *et al.* 2008). However, these studies often include other dietary manipulations, such as high sucrose or low mineral content, which can confound the effects of the fat content alone. One study comparing the effects of a high fat diet of different types (e.g. saturated fatty acids versus poly unsaturated fatty acids (PUFAs)) found that the femurs of rats fed a diet rich in PUFAs were stronger than those of rats fed normal chow (Lau *et al.* 2010). This seems logical because the weight gain resulting from the HFD increases load on the bones, thereby increasing bone strength and density. This could be the case here, as mice on a HFD did weigh more than those on a normal diet.

In humans increasing adiposity actually decreases bone density, when weight is corrected for (Dimitri *et al.* 2010; Kawai and Rosen 2010), although the reason for this remains unknown. No studies have, to date, examined the bone density of normal, compared with obese, ADHD patients. This is an important piece of information, not least because there is some evidence that ADHD medications affect bone density (Komatsu *et al.* 2012; Poulton *et al.* 2012). The

question of whether obese ADHD patients should therefore not be prescribed psychostimulants is one worth further investigating.

7.4.3 DEXA versus chemical analysis

The results for percentage body fat differed when measured by the two techniques. Here, we report that DEXA analysis overestimates %fat by approximately 9%. This is strikingly similar to the 10% reported in the literature (Nagy *et al.* 2001; Brommage 2003; Iida-Klein *et al.* 2003; Johnston *et al.* 2005), confirming that all rodent DEXA machines are inaccurate to the same degree. The same studies also questioned the precision of DEXA analysis: here the correlation between %fat by DEXA and %fat by gold standard chemical analysis gave an R^2 value of 0.617, suggesting reasonable precision. However, the lack of absolute precision meant that the conclusions from the DEXA experiments reported here did not match exactly with the conclusions from the chemical analysis. This is likely because the differences in %fat between the experimental groups were small (a maximum of ~4%). It could also be because the mice analysed here were quite lean (less than 10% fat), and perhaps below the threshold at which DEXA can precisely predict %body fat. Brommage (2003) reports a much higher correlation (R^2 = 0.94) when animals are between 3 to 49% body fat, and reports that DEXA would be completely precise in predicting the body fat of a hypothetical animal of 100% body fat. Brommage suggests that DEXA machines were calibrated for this hypothetical, 100% fat animal, and are inaccurately calibrated for low percentage body fat. This means that the lower the body fat, the less accurate and precise DEXA is in predicting body fat content (see Figure 7.7).

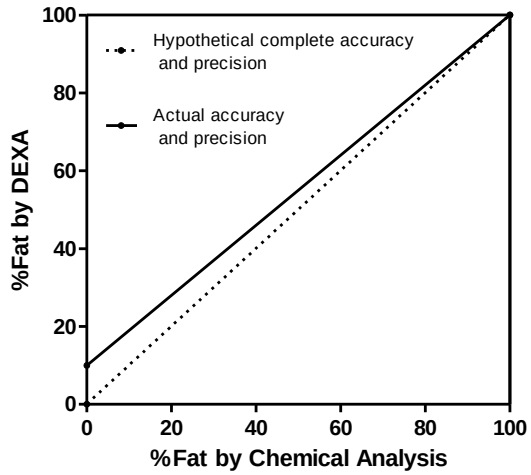

Figure 7.7 – Precision and accuracy of DEXA analysis of %fat. Dotted line represents correlation obtained if DEXA and chemical analysis measurements were identical.

Despite this bone mineral content and ash measured by DEXA and chemical analysis, respectively, did correlate well, suggesting that the result for bone mineral density is accurate and precise. Together, these results suggest that DEXA is a useful tool in the analysis of bone, or analysis of body composition using animals with high fat content. DEXA also has the advantage that it can provide multiple measures over time on the same subject, which chemical analysis cannot.

7.5 Highlights

- Body fat content of mice depends on an interaction between genotype and gender. However, male NK1R-/- mice could reflect the paradox observed in ADHD: these individuals are hyperactive, but actually have a higher body fat content.
- The bone density of NK1R-/- mice is lower than wildtypes, possibly due to the involvement of NK1Rs in bone mineralization and homeostasis.
- The possibility that ADHD patients with *TACR1* polymorphisms are more susceptible to comorbid obesity, and/or bone fractures, could be worth investigating.

- DEXA analysis of fat in rodents is limited by the inaccurate calibration of DEXA machines. Here, all mice were relatively lean, and may have been below the threshold at which DEXA can reliably predict percentage body fat.
- DEXA analysis of bone appears to be accurate and precise.

Chapter 8

General discussion

Chapter 8. General discussion

ADHD is a common childhood psychiatric disorder, characterized by signs of *inattention*, *impulsivity* and *hyperactivity*, which can persist into adulthood. Although ADHD is treatable with psychostimulant drugs (amphetamine and methylphenidate), there remains a need for better tolerated medications with lower abuse potential. Diagnosis of the disorder can be problematic, as it centres around behaviour which can be evident in normal individuals. Moreover, ADHD is often comorbid with a wide variety of other psychiatric and medical disorders, such as anxiety, depression, obesity and substance abuse disorders, which can complicate diagnosis and limit treatment options available.

There have been many preclinical rodent 'models' of ADHD proposed. These models should show good face validity, construct validity and predictive validity, in order to be a useful preclinical tool for research into the disorder. The most well-characterised rat and mouse models, respectively, are spontaneously hypertensive rats (SHRs) and dopamine transporter (DAT) knockout mice. However, neither model is without its limitations: SHRs are limited by a lack of true control strain, and in mice, knockout of the DAT gene is lethal in 32% of animals over the age of 10 weeks (Giros *et al.* 1996).

The abnormal behaviour displayed by NK1R-/- mice (*hyperactivity, inattention* and *impulsivity*) has been proposed to resemble that seen in ADHD (face validity) (Yan *et al.* 2011). In addition, these mice have alterations in dopaminergic, noradrenergic and serotonergic transmission (see Yan *et al.* 2009) and polymorphisms in the *TACR1* gene are associated with ADHD in humans (construct validity) (Sharp *et al.* 2009; Yan *et al.* 2010; Sharp *et al.* 2014). The first aim of this thesis was to test the predictive validity of the NK1R-/- mouse.

The psychostimulants (amphetamine and methylphenidate) alleviate the *hyperactivity* of NK1R-/- mice, but amphetamine did not completely prevent the *impulsivity* or *inattention* displayed by these mice (Yan *et al.* 2010; Yan *et al.* 2011). Here, the aim was to determine whether methylphenidate does alleviate the *inattention* and *impulsivity*, and whether guanfacine and atomoxetine (two non-stimulant alternative ADHD treatments) alleviate all

three behaviours, displayed by NK1R-/- mice. Two rodent analogues of human continuous performance tasks (the 5-CSRTT and the 5C-CPT) were used to test sustained *attention* and response control (*impulsivity*). These tests were used because continuous performance tasks are used to test ADHD-like behaviour in humans, and they are amongst the most informative cognitive tests in rodents.

The light-dark exploration box was used to assess *hyperactivity*, and simultaneously highlight any potential changes in emotionality, which could affect behaviour. To further test the effect of guanfacine, in particular, on emotionality and spatial memory, the elevated plus maze and object recognition tests were also used, respectively.

The second aim of this thesis was to further investigate the body composition of NK1R-/- mice, since the result of the 5-CSRTT and 5C-CPT experiments suggested that these mice have abnormal body weight and food intake. In light of evidence that ADHD and obesity can be comorbid, the body composition of mice maintained on a high fat 'Western'-style diet was also assessed.

8.1 Key findings

In **Chapter 3,** guanfacine was effective in reducing ADHD-like behaviour in NK1R-/- mice. However, this was strongly dependent on dose of the drug: a low dose was sufficient to increase *attentiveness* and reduce *hyperactivity* in NK1R-/- mice, only. At higher doses the drug reduced behavioural measures of arousal in both genotypes, and consequently reduced *impulsivity*, suggesting that this may be how the drug functions in ADHD. The results of **Chapter 4** confirmed that the effects of low dose guanfacine were not secondary to changes in emotionality, as measured by behaviour on the elevated plus maze, but could be a result of improved spatial memory. Spatial working memory is thought to be impaired in ADHD, and guanfacine could alleviate deficits in attentiveness through reversing these impairments. Another α_2-adrenoceptor agonist, medetomidine, also improved spatial memory, but this drug was effective only in wildtypes. The difference between the two drugs is likely due to

α_2-adrenoceptor subtype selectivity, and could be further investigated with the use of an antagonist.

Another noteworthy finding, reported in Chapter 4, was that NK1R-/- mice on the current, mixed background strain (129/Sv/C57Bl6 x MF1), display increased anxiety-like behaviour in the elevated plus maze, as evidenced by reduced time on the open arms: this is supported by the finding that NK1R-/- mice also spend less time in the light zone of the LDEB than wildtypes (reported in Chapters 3 and 5). This behaviour could be a result of an interaction between a loss of functional NK1R and background strain. Interestingly, the results of the object recognition experiments suggest that, when the environment is not aversive, NK1R-/- mice actually spend slightly more time exploring both objects than wildtypes.

Contrary to guanfacine's effects on *attention*, in **Chapter 5** it was discovered that atomoxetine selectively improved *impulsivity* in NK1R-/- mice, but had no effect on *attention*. This effect was not due to a reduction in motivation for the task, but was paralleled by a drug-induced reduction in *hyperactivity* in NK1R-/- mice. Atomoxetine consistently reduces different types of impulsivity in preclinical rodent studies, suggesting that this aspect translates well between rodents and humans. Our results suggest that atomoxetine may be best suited for the hyperactive/impulsive subtype of ADHD.

Together, the results of Chapters 3, 4 and 5 suggested that NK1R-/- mice are more sensitive than wildtypes to manipulations of the noradrenergic system. This could relate to previous reports that noradrenergic signalling is disrupted in these mice (Herpfer *et al.* 2005; Fisher *et al.* 2007). Moreover, an interesting possibility is that ADHD patients, particularly those with *TACR1* polymorphisms, could similarly be more sensitive to non-psychostimulant alternative ADHD treatments, guanfacine and atomoxetine, than healthy controls.

In **Chapter 6**, methylphenidate was tested in an extension of the 5-CSRTT, the 5C-CPT. Methylphenidate was particularly efficacious in attenuating the *perseveration* displayed by NK1R-/- mice. Although NK1R-/- mice did not display impulsivity on the novel 'no-go' trials at baseline, methylphenidate reduced both types of *impulsivity* measured in this task in NK1R-/- mice. The drug also reduced *attention* in wildtypes, but not NK1R-/- mice, at the same dose,

suggesting that overall NK1R-/- mice benefitted from methylphenidate treatment whereas wildtypes did not. This is reminiscent of our previously published experiments in which methylphenidate reduced the hyperactivity of NK1R-/- mice, but increased the activity of wildtypes (Yan *et al.* 2010). These findings together provide good support for the predictive validity of NK1R-/- mice, as methylphenidate remains the first-line treatment for ADHD, and perhaps the most important test of a preclinical model of the disorder.

From the results of the three 5-Choice experiments reported here, it is clear that behavioural variables in these tests should be studied in concert. This is because all these variables are linked: conclusions about the effect of a drug or a genetic modification cannot be drawn from the results of one variable alone. For instance, guanfacine reduced premature responses in both genotypes, but latencies to respond were increased, suggesting that motor activity and/or arousal was blunted. Similarly, methylphenidate appeared to decrease the latency to magazine in NK1R-/- mice, but the drug also reduced perseveration, and so in this case, the likely explanation is that these mice were able to reach the magazine more quickly because they were not 'perseverating'.

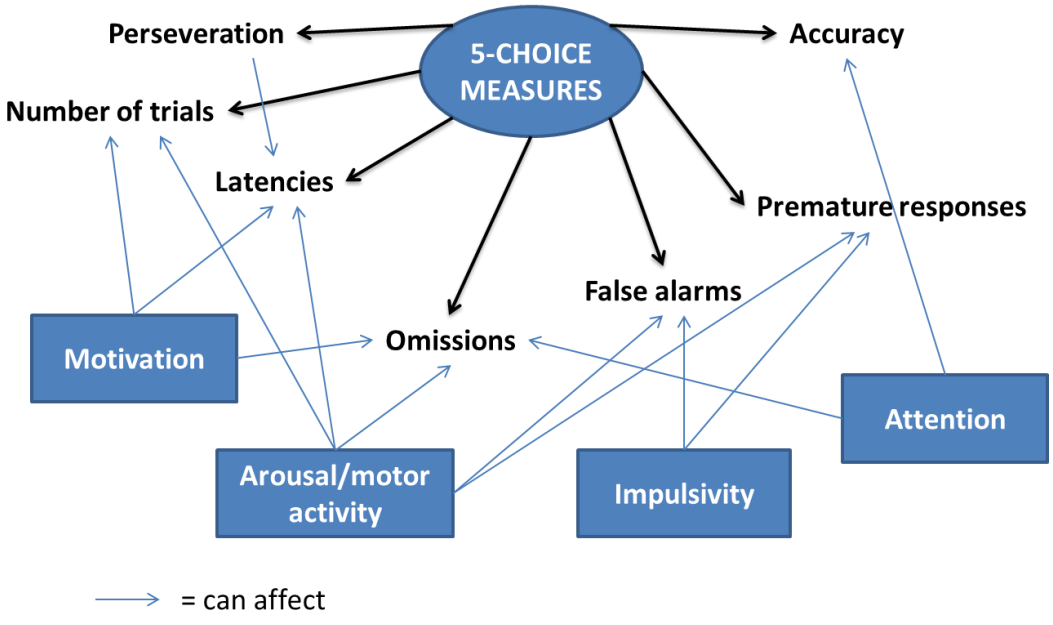

Figure 8.1 – Diagram showing how variables measured in the 5-Choice tasks interact

This use of multiple measurements in the same animal can be advantageous, as they give a broader understanding of the effects of a drug. Overall, the results of Chapters 3 – 6 suggest that the NK1R-/- mouse does have good predictive validity in these measures. However, the dose of drug is an important factor, as NK1R-/- mice may be more sensitive to ADHD treatments: dissociable effects on cognition and motor behaviour can only be detected at doses which are not at either end of the dose-response curve.

The 5-Choice tasks do have some other limitations. As they are operant tasks, mice are largely motivated by an appetitive reward. This means that, although measures of motivation can be taken into account, motivational status is critical in determining behaviour. Importantly, it has been suggested that ADHD may be underpinned by a type of reward-deficiency syndrome, in which the perceived value of rewards is decreased (Luman *et al.* 2010). Moreover, human continuous performance tasks are not motivated by food rewards, so this aspect of the task does not translate well to humans.

Throughout the 5-CSRTT and 5C-CPT tasks, the weight and food intake of mice was closely monitored. The finding that NK1R-/- mice weigh less than wildtypes, but eat more in proportion to their body weight to maintain the same weight, suggested that there might be an interesting underlying difference in the body composition of the two genotypes. **Chapter 7** aimed to further elucidate the underlying differences in the body composition of mice on a normal diet, and on a high fat "Western" diet. The latter was chosen because of reports of an overlap between ADHD and obesity in Western countries (Cortese *et al.* 2013).

The body composition analyses used two different methods; DEXA and chemical carcass analysis. Both have their relative advantages and disadvantages, but chemical analysis is considered the gold standard measure. The chemical carcass analysis reported in Chapter 7 suggested that NK1R-/- mice had lower, or similar, %fat at baseline, but had similar or increased %fat on a high fat diet, compared with wildtypes. These results are contradictory to reports that NK1R antagonists reduce body fat (Karagiannides *et al.* 2008), but are in line with the prediction that NK1R-/- mice resemble ADHD patients, in so far as there is a higher rate of obesity in these patients. The interaction between genotype and gender in %fat of mice is also

notable, given reports that there is a gender difference in obesity rates in ADHD (Kim *et al.* 2011; van Egmond-Frohlich *et al.* 2012; Byrd *et al.* 2013).

The results of Chapter 7 also suggested that NK1R-/- mice have a lower bone density than wildtypes. This is surprising, given that these mice are hyperactive, and exercise generally increases bone density. However, it could be because NK1R-/- mice are smaller, and thus the load on their bones is lower. In concordance with this explanation is the finding that mice fed a high fat diet were heavier, and had a higher bone density, than mice fed a normal diet. Nevertheless, the influence of NK1Rs on bone metabolism cannot be ruled out: there is some evidence to suggest that the actions of substance P on NK1Rs in bone cells increase bone mineralization (Goto *et al.* 2007).

In a comparison of DEXA and chemical analysis, DEXA was found to be reasonably precise, but results were inaccurate: in particular %fat was overestimated by approximately 10%. However, bone measurements (e.g. bone mineral density) did appear to be accurate and precise. These findings support the use of DEXA to measure bone density (its use in the clinic), but suggest that rodent versions of the machines require more accurate calibration before they can reliably be used in a preclinical setting.

8.2 Validity of the NK1R-/- mouse 'model' of ADHD

One of the main aims of this project was to test the predictive validity of the NK1R-/- mouse as a 'model' of ADHD; that is, to test whether the behavioural response of NK1R-/- mice to ADHD treatments resembles that of ADHD patients. However, this approach assumes that NK1R-/- mice express the behavioural deficits expressed by individuals with ADHD at baseline (face validity). Yan and colleagues first reported that NK1R-/- mice expressed hyperactivity, inattention and impulsivity in 2010 and 2011, and this has since been replicated (see Dudley *et al.* 2013; Porter *et al.* 2015). However, upon first inspection of the results reported in this thesis, not all aspects of this phenotype appear to have been replicated in these experiments.

8.2.1 Hyperactivity

In Chapters 3 and 5, the spontaneous locomotor activity of NK1R-/- and wildtype mice was tested acutely in the LDEB. In both experiments, naïve NK1R-/- mice were hyperactive compared to wildtypes, replicating previous findings (Yan *et al.* 2010). This aspect of the phenotype is perhaps the most robust; NK1R-/- mice have now been shown to be hyperactive in a number of paradigms, including a 24 hour measure (Porter *et al.* 2015). Moreover, this hyperactivity is apparent in mice bred from inbred, homozygous parents, and from heterozygous parents (Porter *et al.* 2015), suggesting that it is a direct consequence of a lack of NK1R. Support for this hypothesis comes from the finding that NK1R antagonists can induce hyperactivity in wildtype mice (Yan *et al.* 2010). In the results reported here, it is striking that hyperactivity was apparent in naïve mice, but not in those that had received an injection, in both experiments, suggesting that this behavioural response is also a robust one.

8.2.2 Impulsivity and inattention

Chapters 3 and 5 reported the results of experiments in which NK1R-/- and wildtype mice were tested in the 5-CSRTT; the test in which it was first discovered that NK1R-/- mice express impulsivity and inattention (Yan *et al.* 2011). From these results it appears that this cognitive phenotype was not present in these cohorts of mice. However, there are a few possible explanations for this discrepancy. Firstly, and perhaps most importantly, mice used in these experiments were bred using two different strategies; 1) using the same inbred, homozygous breeding lines as those used for the experiments reported by Yan *et al* (2011), and 2) using heterozygous parents, producing wildtype and NK1R-/- mice that were littermates. Porter *et al* (2015) reported the results of a comparison between the two breeding strategies; it was found that mice bred from heterozygous parents do not display the impulsivity expressed by NK1R-/- mice from inbred, homozygous parents, suggesting that this behaviour is a result of an interaction between a lack of functional NK1R and environment, or that the inbreeding of these mice has resulted in behavioural abnormalities which cannot fully be attributed to a lack of the primary gene of interest.

A second explanation comes from the fact that the results reported here are from mice that had been tested previously (in NI-1), and it is now known that impulsivity and inattention are reduced with repeated testing (Weir *et al.* 2014). This seems to be a likely explanation, as the incidences of premature responses and omissions were particularly low in these experiments. A final explanation could be that this cognitive phenotype is subject to normal fluctuations, i.e. it is present in some groups of animals but not others. However, Porter *et al* (2015) recently reported the results of a 5-CSRTT experiment using the same test procedure as that used by Yan *et al* (2011), in which NK1R-/- mice (from homozygous breeding lines) were both impulsive and inattentive.

It should also be noted that mice were tested for impulsivity and inattention in the 5C-CPT paradigm (reported in Chapter 6), and NK1R-/- mice did not display an increased rate of premature responses, false alarms or omissions in this experiment. However, this was the first time NK1R-/- mice had been tested in this paradigm, and the protocol for the 5C-CPT differed considerably from the 5-CSRTT protocol (see Chapter 2), such that the two are not readily comparable. However, as discussed in Chapter 6, a likely explanation for the lack of impulsive/inattentive phenotype in this experiment is that mice were trained using a VITI schedule, and as such, are likely to have learned the task in a different way, and possibly to a higher degree than in the 5-CSRTT.

Another point to consider is that the effects of NK1R antagonists on impulsivity and inattention are complicated by the effects of these drugs on L-type calcium channels (see Dudley *et al.* 2013; Weir *et al.* 2014). Dudley *et al* (2013) reported that the L-type calcium channel antagonist, nifedipine, exacerbates inattention and improves impulsivity, more potently in wildtypes than NK1R-/- mice performing the 5-CSRTT. This renders it difficult to determine the effects of NK1R antagonists on behaviour that aren't attributable to influences on L-type calcium channel opening (Dudley *et al.* 2013; Weir *et al.* 2014).

8.2.3 Pharmacological phenotype

Despite the apparent lack of impulsive/inattentive phenotype of NK1R-/- mice in the series of experiments reported here, these mice did display a remarkable 'pharmacological phenotype' in most, if not all, experiments. NK1R-/- mice were more sensitive to guanfacine (0.1mg/kg), atomoxetine (3 – 10 mg/kg) and methylphenidate (10 mg/kg) than wildtypes in terms of the effects of these drugs on hyperactivity, impulsivity and/or inattention. This means that, despite the apparent lack of ADHD-like behaviour displayed by NK1R-/- mice in some experiments, the predictive validity of these mice is good, and therefore these animals could be useful in further investigations into potential novel treatments for this disorder.

8.3 Future directions

8.3.1 Studies in NK1R-/- mice

These experiments have detailed the behavioural responses to ADHD treatments in NK1R-/- and wildtype mice. One next logical step would be to determine the neurochemical responses to the same drugs. An amphetamine-induced increase in dopamine release is blunted in the striatum of NK1R-/- mice compared to wildtypes (Yan *et al.* 2010), and based on the behaviour of NK1R-/- mice reported here, it might be hypothesized that these mice also display an abnormal neurochemical response to methylphenidate. Similarly, noradrenergic signalling is disrupted in NK1R-/- mice, and these mice are more sensitive to guanfacine and atomoxetine, suggesting that these drugs would also induce an abnormal noradrenergic response.

To further test the specificity of each of the drugs used here on the ADHD-like behaviour they were found to be most effective in relieving, it might be of interest to test each treatment in NK1R-/- mice in different paradigms examining that behaviour. Atomoxetine was found to be most effective at reducing hyperactive/impulsive behaviours, and so it may be worthwhile testing this drug in paradigms such as the 5C-CPT or SSRT to determine its effects on different types of impulsivity. Likewise, methylphenidate reduced impulsivity more potently in NK1R-/- mice than wildtypes, and so it may be of interest to test this drug at a wider dose range, or

when administered by different routes, as this has been shown to affect behavioural outcomes (e.g. see Botly *et al.* 2008). Guanfacine also appeared to have a more specific effect on attention than hyperactivity/impulsivity, and so paradigms such as the T-maze, 8-arm radial maze or Morris water maze could be used to further expand on these results.

One limitation of the NK1R-/- mouse is that most published reports on the behaviour of these mice have studied animals bred from homozygous inbred lines, rather than wildtype and NK1R-/- littermates. The recent report published by Porter and colleagues (2015) suggests that some aspects of the behaviour of NK1R-/- mice could be attributable to an interaction between genotype and environment/breeding strategy. Whether breeding strategy affects all behaviour in the same way is an important question to answer here, as well as in all studies using genetically modified mice, as early life environment certainly affects behaviour, and this could be particularly important in ADHD (Mill and Petronis 2008). However, although the results reported here revealed that the responses to the drug treatments were not influenced by breeding strategy, future studies should be conducted on mice bred using the two different methods, to enable the best understanding of their behaviour.

The finding that NK1R-/- mice have similar, or increased, %fat compared to wildtypes on a high fat diet is in contrast with previous reports suggest that NK1R antagonists *reduce* fat and weight gain in rodents. This could be further investigated, by chronic use of NK1R antagonists, NK1R antagonists which don't cross the blood-brain barrier or by employing a conditional knockout of the NK1R gene. Background strain could also influence physiology as well as behaviour, so testing NK1R-/- mice bred on different background strains or from heterozygous versus homozygous parents could also help elucidate the influence of NK1Rs on adiposity. It might also be of interest to examine the effects of NK1R antagonists on weight in the clinic. However, this may prove to be difficult as the only such drugs available clinically are indicated as anti-emetics.

8.3.2 Clinical research

The results of the experiments reported here suggest that the *TACR1* polymorphism in humans could render this subset of ADHD patients more sensitive to ADHD treatments. To that end, it might be of interest to conduct a large-scale study on the efficacy of the ADHD treatments, guanfacine, atomoxetine and methylphenidate, in patients with and without *TACR1* polymorphisms. If these patients are more sensitive to such treatments, this could aid a step towards 'personalised medicine', whereby physicians may be better able to tailor treatments to the genotype of the patient.

Similarly, the results of Chapter 7 suggest that the NK1R (*TACR1*) gene could be an important factor in the development of obesity. It would be interesting to investigate whether unmedicated ADHD patients with *TACR1* polymorphisms are more likely to be obese. If this is the case, this might provide a novel therapeutic target for the treatment of ADHD with comorbid obesity in this subset of patients.

8.4 Final conclusions

Overall, the results presented in this thesis further add to the predictive validity of the NK1R-/- mouse as a preclinical tool for the study of ADHD-like behaviour. The results suggest that these mice are more sensitive to ADHD treatment medications, and all of the treatments tested here (guanfacine, atomoxetine and methylphenidate) reduce the incidence of one or more ADHD behaviours in these mice. However, each drug could be more suited to a particular subtype of ADHD, for example, atomoxetine may be most suitable for the hyperactive/impulsive subtype, and guanfacine to the inattentive or combined subtype. Additionally, the results presented here suggest that the NK1R gene may be important in the regulation of food intake and fat storage, and support reports that there is an overlap between ADHD and obesity, which requires further investigation.

References

Abela, A. R. and Y. Chudasama (2014). "Noradrenergic alpha2A-receptor stimulation in the ventral hippocampus reduces impulsive decision-making." ***Psychopharmacology (Berl)*** **231**(3): 521-531.

Ago, Y., M. Umehara, K. Higashino, S. Hasebe, K. Fujita, K. Takuma and T. Matsuda (2014). "Atomoxetine-induced increases in monoamine release in the prefrontal cortex are similar in spontaneously hypertensive rats and Wistar-Kyoto rats." ***Neurochem Res*** **39**(5): 825-832.

Agranat-Meged, A. N., C. Deitcher, G. Goldzweig, L. Leibenson, M. Stein and E. Galili-Weisstub (2005). "Childhood obesity and attention deficit/hyperactivity disorder: a newly described comorbidity in obese hospitalized children." ***Int J Eat Disord*** **37**(4): 357-359.

Akesson, T. R. (1994). "Gonadal steroids regulate immunoreactive tachykinin in the ventromedial nucleus of the rat hypothalamus." ***J Comp Neurol*** **341**(3): 351-356.

Altfas, J. R. (2002). "Prevalence of attention deficit/hyperactivity disorder among adults in obesity treatment." ***BMC Psychiatry*** **2**: 9.

Amara, S. G. and M. J. Kuhar (1993). "Neurotransmitter transporters: recent progress." ***Annu Rev Neurosci*** **16**: 73-93.

Amara, S. G. and M. S. Sonders (1998). "Neurotransmitter transporters as molecular targets for addictive drugs." ***Drug Alcohol Depend*** **51**(1-2): 87-96.

Amini, B., P. B. Yang, A. C. Swann and N. Dafny (2004). "Differential locomotor responses in male rats from three strains to acute methylphenidate." ***Int J Neurosci*** **114**(9): 1063-1084.

Amitai, N. and A. Markou (2010). "Disruption of performance in the five-choice serial reaction time task induced by administration of N-methyl-D-aspartate receptor antagonists: relevance to cognitive dysfunction in schizophrenia." ***Biol Psychiatry*** **68**(1): 5-16.

Amitai, N. and A. Markou (2011). "Comparative effects of different test day challenges on performance in the 5-choice serial reaction time task." ***Behav Neurosci*** **125**(5): 764-774.

Andersen, P. H. (1989). "The dopamine inhibitor GBR 12909: selectivity and molecular mechanism of action." ***Eur J Pharmacol*** **166**(3): 493-504.

Anderson, S. E., P. Cohen, E. N. Naumova and A. Must (2006). "Relationship of childhood behavior disorders to weight gain from childhood into adulthood." ***Ambul Pediatr*** **6**(5): 297-301.

Antshel, K. M., T. M. Hargrave, M. Simonescu, P. Kaul, K. Hendricks and S. V. Faraone (2011). "Advances in understanding and treating ADHD." ***BMC Med*** **9**: 72.

Archer, T., W. Danysz, A. Fredriksson, G. Jonsson, J. Luthman, E. Sundstrom and A. Teiling (1988). "Neonatal 6-hydroxydopamine-induced dopamine depletions: motor activity and performance in maze learning." ***Pharmacol Biochem Behav*** **31**(2): 357-364.

Archer, T. and A. Fredriksson (2003). "An antihypokinesic action of alpha2-adrenoceptors upon MPTP-induced behaviour deficits in mice." ***J Neural Transm*** **110**(2): 183-200.

Arnsten, A. F. (2011). "Catecholamine influences on dorsolateral prefrontal cortical networks." ***Biol Psychiatry*** **69**(12): e89-99.

Arnsten, A. F., J. X. Cai and P. S. Goldman-Rakic (1988). "The alpha-2 adrenergic agonist guanfacine improves memory in aged monkeys without sedative or hypotensive side effects: evidence for alpha-2 receptor subtypes." ***J Neurosci*** **8**(11): 4287-4298.

Arnsten, A. F. and J. D. Jentsch (1997). "The alpha-1 adrenergic agonist, cirazoline, impairs spatial working memory performance in aged monkeys." ***Pharmacol Biochem Behav*** **58**(1): 55-59.

Arnsten, A. F. and L. E. Jin (2012). "Guanfacine for the treatment of cognitive disorders: a century of discoveries at Yale." ***Yale J Biol Med*** **85**(1): 45-58.

Arnsten, A. F. and B. M. Li (2005). "Neurobiology of executive functions: catecholamine influences on prefrontal cortical functions." ***Biol Psychiatry*** **57**(11): 1377-1384.

Aston-Jones, G. and J. D. Cohen (2005). "Adaptive gain and the role of the locus coeruleus-norepinephrine system in optimal performance." ***J Comp Neurol*** **493**(1): 99-110.

Aston-Jones, G., M. T. Shipley, G. Chouvet, M. Ennis, E. van Bockstaele, V. Pieribone, R. Shiekhattar, H. Akaoka, G. Drolet, B. Astier and et al. (1991). "Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology." ***Prog Brain Res*** **88**: 47-75.

Baarendse, P. J. and L. J. Vanderschuren (2012). "Dissociable effects of monoamine reuptake inhibitors on distinct forms of impulsive behavior in rats." ***Psychopharmacology (Berl)*** **219**(2): 313-326.

Balci, F., E. A. Ludvig, J. M. Gibson, B. D. Allen, K. M. Frank, B. J. Kapustinski, T. E. Fedolak and D. Brunner (2008). "Pharmacological manipulations of interval timing using the peak procedure in male C3H mice." ***Psychopharmacology (Berl)*** **201**(1): 67-80.

Bari, A., D. M. Eagle, A. C. Mar, E. S. Robinson and T. W. Robbins (2009). "Dissociable effects of noradrenaline, dopamine, and serotonin uptake blockade on stop task performance in rats." ***Psychopharmacology (Berl)*** **205**(2): 273-283.

Bari, A., A. C. Mar, D. E. Theobald, S. A. Elands, K. C. Oganya, D. M. Eagle and T. W. Robbins (2011). "Prefrontal and monoaminergic contributions to stop-signal task performance in rats." ***J Neurosci*** **31**(25): 9254-9263.

Barron, E., P. B. Yang, A. C. Swann and N. Dafny (2009). "Adolescent and adult male spontaneous hyperactive rats (SHR) respond differently to acute and chronic methylphenidate (Ritalin)." ***Int J Neurosci*** **119**(1): 40-58.

Bassi, G. S., M. J. Nobre, J. E. de Araujo and M. L. Brandao (2007). "Anxiogenic effects of activation of NK-1 receptors of the dorsal periaqueductal gray as assessed by the elevated plus-maze, ultrasound vocalizations and tail-flick tests." ***Neuropeptides*** **41**(6): 365-374.

Beck, L. H., E. D. Bransome, Jr., A. F. Mirsky, H. E. Rosvold and I. Sarason (1956). "A continuous performance test of brain damage." ***J Consult Psychol*** **20**(5): 343-350.

Behringer, M., S. Gruetzner, M. McCourt and J. Mester (2014). "Effects of weight-bearing activities on bone mineral content and density in children and adolescents: a meta-analysis." ***J Bone Miner Res*** **29**(2): 467-478.

Berger, A., A. H. Tran, J. Dida, S. Minkin, N. P. Gerard, J. Yeomans and C. J. Paige (2012). "Diminished pheromone-induced sexual behavior in neurokinin-1 receptor deficient (TACR1(-/-)) mice." ***Genes Brain Behav*** **11**(5): 568-576.

Berridge, C. W., D. M. Devilbiss, M. E. Andrzejewski, A. F. Arnsten, A. E. Kelley, B. Schmeichel, C. Hamilton and R. C. Spencer (2006). "Methylphenidate preferentially increases

catecholamine neurotransmission within the prefrontal cortex at low doses that enhance cognitive function." ***Biol Psychiatry*** **60**(10): 1111-1120.

Berridge, C. W., T. L. Stratford, S. L. Foote and A. E. Kelley (1997). "Distribution of dopamine beta-hydroxylase-like immunoreactive fibers within the shell subregion of the nucleus accumbens." ***Synapse*** **27**(3): 230-241.

Biederman, J., R. J. Baldessarini, V. Wright, D. Knee and J. S. Harmatz (1989). "A double-blind placebo controlled study of desipramine in the treatment of ADD: I. Efficacy." ***J Am Acad Child Adolesc Psychiatry*** **28**(5): 777-784.

Biederman, J. and T. Spencer (1999). "Attention-deficit/hyperactivity disorder (ADHD) as a noradrenergic disorder." ***Biol Psychiatry*** **46**(9): 1234-1242.

Biederman, J., R. D. Melmed, A. Patel, K. McBurnett, J. Konow, A. Lyne and N. Scherer (2008). "A randomized, double-blind, placebo-controlled study of guanfacine extended release in children and adolescents with attention-deficit/hyperactivity disorder." ***Pediatrics*** **121**(1): e73-84.

Bizarro, L., S. Patel, C. Murtagh and I. P. Stolerman (2004). "Differential effects of psychomotor stimulants on attentional performance in rats: nicotine, amphetamine, caffeine and methylphenidate." ***Behav Pharmacol*** **15**(3): 195-206.

Bizot, J. C., N. Chenault, B. Houze, A. Herpin, S. David, S. Pothion and F. Trovero (2007). "Methylphenidate reduces impulsive behaviour in juvenile Wistar rats, but not in adult Wistar, SHR and WKY rats." ***Psychopharmacology (Berl)*** **193**(2): 215-223.

Bjorklund, M., J. Sirvio, J. Puolivali, J. Sallinen, P. Jakala, M. Scheinin, B. K. Kobilka and P. Riekkinen, Jr. (1998). "Alpha2C-adrenoceptor-overexpressing mice are impaired in executing nonspatial and spatial escape strategies." ***Mol Pharmacol*** **54**(3): 569-576.

Bjorklund, M., J. Sirvio, M. Riekkinen, J. Sallinen, M. Scheinin and P. Riekkinen, Jr. (2000). "Overexpression of alpha2C-adrenoceptors impairs water maze navigation." ***Neuroscience*** **95**(2): 481-487.

Bjorklund, M., I. Siverina, T. Heikkinen, H. Tanila, J. Sallinen, M. Scheinin and P. Riekkinen, Jr. (2001). "Spatial working memory improvement by an alpha2-adrenoceptor agonist dexmedetomidine is not mediated through alpha2C-adrenoceptor." ***Prog Neuropsychopharmacol Biol Psychiatry*** **25**(8): 1539-1554.

Blaine, S., E. Claus, N. Harlaar and K. Hutchison (2013). "TACR1 genotypes predict fMRI response to alcohol cues and level of alcohol dependence." ***Alcohol Clin Exp Res*** **37 Suppl 1**: E125-130.

Blier, P. (2006). "Psychopharmacology for the clinician. Treating depression with selective norepinephrine reuptake inhibitors." ***J Psychiatry Neurosci*** **31**(4): 288.

Blier, P., G. Gobbi, N. Haddjeri, L. Santarelli, G. Mathew and R. Hen (2004). "Impact of substance P receptor antagonism on the serotonin and norepinephrine systems: relevance to the antidepressant/anxiolytic response." ***J Psychiatry Neurosci*** **29**(3): 208-218.

Bloch, M. H., K. E. Panza, A. Landeros-Weisenberger and J. F. Leckman (2009). "Meta-analysis: treatment of attention-deficit/hyperactivity disorder in children with comorbid tic disorders." ***J Am Acad Child Adolesc Psychiatry*** **48**(9): 884-893.

Blondeau, C. and F. Dellu-Hagedorn (2007). "Dimensional analysis of ADHD subtypes in rats." ***Biol Psychiatry*** **61**(12): 1340-1350.

Bobb, A. J., A. M. Addington, E. Sidransky, M. C. Gornick, J. P. Lerch, D. K. Greenstein, L. S. Clasen, W. S. Sharp, G. Inoff-Germain, F. Wavrant-De Vrieze, M. Arcos-Burgos, R. E. Straub, J. A. Hardy, F. X. Castellanos and J. L. Rapoport (2005). "Support for association between ADHD and two candidate genes: NET1 and DRD1." ***Am J Med Genet B Neuropsychiatr Genet*** **134B**(1): 67-72.

Both, C., G. Kojda and C. Lange-Asschenfeldt (2014). "Pharmacotherapy of generalized anxiety disorder: focus and update on pregabalin." ***Expert Rev Neurother*** **14**(1): 29-38.

Botly, L. C., C. L. Burton, Z. Rizos and P. J. Fletcher (2008). "Characterization of methylphenidate self-administration and reinstatement in the rat." ***Psychopharmacology (Berl)*** **199**(1): 55-66.

Broadbent, N. J., L. R. Squire and R. E. Clark (2004). "Spatial memory, recognition memory, and the hippocampus." ***Proc Natl Acad Sci U S A*** **101**(40): 14515-14520.

Brommage, R. (2003). "Validation and calibration of DEXA body composition in mice." ***Am J Physiol Endocrinol Metab*** **285**(3): E454-459.

Brown, E. R., R. E. Harlan and J. E. Krause (1990). "Gonadal steroid regulation of substance P (SP) and SP-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus." ***Endocrinology*** **126**(1): 330-340.

Brunello, N., J. Mendlewicz, S. Kasper, B. Leonard, S. Montgomery, J. Nelson, E. Paykel, M. Versiani and G. Racagni (2002). "The role of noradrenaline and selective noradrenaline reuptake inhibition in depression." ***Eur Neuropsychopharmacol*** **12**(5): 461-475.

Brunner, D. and R. Hen (1997). "Insights into the neurobiology of impulsive behavior from serotonin receptor knockout mice." ***Ann N Y Acad Sci*** **836**: 81-105.

Bruno, K. J., C. S. Freet, R. C. Twining, K. Egami, P. S. Grigson and E. J. Hess (2007). "Abnormal latent inhibition and impulsivity in coloboma mice, a model of ADHD." ***Neurobiol Dis*** **25**(1): 206-216.

Bruno, K. J. and E. J. Hess (2006). "The alpha(2C)-adrenergic receptor mediates hyperactivity of coloboma mice, a model of attention deficit hyperactivity disorder." ***Neurobiol Dis*** **23**(3): 679-688.

Brus, R., P. Nowak, R. Szkilnik, U. Mikolajun and R. M. Kostrzewa (2004). "Serotoninergics attenuate hyperlocomotor activity in rats. Potential new therapeutic strategy for hyperactivity." ***Neurotox Res*** **6**(4): 317-325.

Buck, S. H., E. Burcher, C. W. Shults, W. Lovenberg and T. L. O'Donohue (1984). "Novel pharmacology of substance K-binding sites: a third type of tachykinin receptor." ***Science*** **226**(4677): 987-989.

Bylund, D. B., D. C. Eikenberg, J. P. Hieble, S. Z. Langer, R. J. Lefkowitz, K. P. Minneman, P. B. Molinoff, R. R. Ruffolo, Jr. and U. Trendelenburg (1994). "International Union of Pharmacology nomenclature of adrenoceptors." ***Pharmacol Rev*** **46**(2): 121-136.

Bymaster, F. P., J. S. Katner, D. L. Nelson, S. K. Hemrick-Luecke, P. G. Threlkeld, J. H. Heiligenstein, S. M. Morin, D. R. Gehlert and K. W. Perry (2002). "Atomoxetine increases extracellular levels of norepinephrine and dopamine in prefrontal cortex of rat: a potential mechanism for efficacy in attention deficit/hyperactivity disorder." ***Neuropsychopharmacology*** **27**(5): 699-711.

Byrd, H. C., C. Curtin and S. E. Anderson (2013). "Attention-deficit/hyperactivity disorder and obesity in US males and females, age 8-15 years: National Health and Nutrition Examination Survey 2001-2004." ***Pediatr Obes*** **8**(6): 445-453.

Cao, A. H., L. Yu, Y. W. Wang, J. M. Wang, L. J. Yang and G. F. Lei (2012). "Effects of methylphenidate on attentional set-shifting in a genetic model of attention-deficit/hyperactivity disorder." ***Behav Brain Funct*** **8**(1): 10.

Carboni, E., A. Silvagni, V. Valentini and G. Di Chiara (2003). "Effect of amphetamine, cocaine and depolarization by high potassium on extracellular dopamine in the nucleus accumbens shell of SHR rats. An in vivo microdyalisis study." ***Neurosci Biobehav Rev*** **27**(7): 653-659.

Carboni, E., G. L. Tanda, R. Frau and G. Di Chiara (1990). "Blockade of the noradrenaline carrier increases extracellular dopamine concentrations in the prefrontal cortex: evidence that dopamine is taken up in vivo by noradrenergic terminals." ***J Neurochem*** **55**(3): 1067-1070.

Cardinal, R. N., C. A. Winstanley, T. W. Robbins and B. J. Everitt (2004). "Limbic corticostriatal systems and delayed reinforcement." ***Ann N Y Acad Sci*** **1021**: 33-50.

Carli, M., T. W. Robbins, J. L. Evenden and B. J. Everitt (1983). "Effects of lesions to ascending noradrenergic neurones on performance of a 5-choice serial reaction task in rats; implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal." ***Behav Brain Res*** **9**(3): 361-380.

Carlson, S., H. Tanila, P. Rama, E. Mecke and A. Pertovaara (1992). "Effects of medetomidine, an alpha-2 adrenoceptor agonist, and atipamezole, an alpha-2 antagonist, on spatial memory performance in adult and aged rats." ***Behav Neural Biol*** **58**(2): 113-119.

Cassaday, H. J., A. J. Nelson and M. A. Pezze (2014). "From attention to memory along the dorsal-ventral axis of the medial prefrontal cortex: some methodological considerations." ***Front Syst Neurosci*** **8**: 160.

Castellanos, F. X. and M. T. Acosta (2004). "[The neuroanatomy of attention deficit/hyperactivity disorder]." ***Rev Neurol*** **38 Suppl 1**: S131-136.

Castellanos, F. X., P. P. Lee, W. Sharp, N. O. Jeffries, D. K. Greenstein, L. S. Clasen, J. D. Blumenthal, R. S. James, C. L. Ebens, J. M. Walter, A. Zijdenbos, A. C. Evans, J. N. Giedd and J. L. Rapoport (2002). "Developmental trajectories of brain volume abnormalities in children and adolescents with attention-deficit/hyperactivity disorder." ***JAMA*** **288**(14): 1740-1748.

Chang, M. M., S. E. Leeman and H. D. Niall (1971). "Amino-acid sequence of substance P." ***Nat New Biol*** **232**(29): 86-87.

Chappell, P. B., M. A. Riddle, L. Scahill, K. A. Lynch, R. Schultz, A. Arnsten, J. F. Leckman and D. J. Cohen (1995). "Guanfacine treatment of comorbid attention-deficit hyperactivity disorder and Tourette's syndrome: preliminary clinical experience." ***J Am Acad Child Adolesc Psychiatry*** **34**(9): 1140-1146.

Cheeta, S., S. Tucci, J. Sandhu, A. R. Williams, N. M. Rupniak and S. E. File (2001). "Anxiolytic actions of the substance P (NK1) receptor antagonist L-760735 and the 5-HT1A agonist 8-OH-DPAT in the social interaction test in gerbils." ***Brain Res*** **915**(2): 170-175.

Chen, L. W., L. C. Wei, H. L. Liu and Z. R. Rao (2000). "Noradrenergic neurons expressing substance P receptor (NK1) in the locus coeruleus complex: a double immunofluorescence study in the rat." ***Brain Res*** **873**(1): 155-159.

Childress, A. C. (2012). "Guanfacine extended release as adjunctive therapy to psychostimulants in children and adolescents with attention-deficit/hyperactivity disorder." ***Adv Ther*** **29**(5): 385-400.

Chou, I. C., C. C. Lin, F. C. Sung and C. H. Kao (2014). "Attention-deficit-hyperactivity disorder increases risk of bone fracture: a population-based cohort study." ***Dev Med Child Neurol*** **56**(11): 1111-1116.

Clark, R. A., M. Shoaib, K. N. Hewitt, S. C. Stanford and S. T. Bate (2012). "A comparison of InVivoStat with other statistical software packages for analysis of data generated from animal experiments." ***J Psychopharmacol*** **26**(8): 1136-1142.

Coghill, D. R., S. M. Rhodes and K. Matthews (2007). "The neuropsychological effects of chronic methylphenidate on drug-naive boys with attention-deficit/hyperactivity disorder." ***Biol Psychiatry*** **62**(9): 954-962.

Cohen, D. J., J. G. Young, J. A. Nathanson and B. A. Shaywitz (1979). "Clonidine in Tourette's syndrome." ***Lancet*** **2**(8142): 551-553.

Cohn, L. M. and G. C. Caliendo (1997). "Guanfacine use in children with attention deficit hyperactivity disorder." ***Ann Pharmacother*** **31**(7-8): 918-919.

Cole, B. J. and T. W. Robbins (1987). "Amphetamine impairs the discriminative performance of rats with dorsal noradrenergic bundle lesions on a 5-choice serial reaction time task: new evidence for central dopaminergic-noradrenergic interactions." ***Psychopharmacology (Berl)*** **91**(4): 458-466.

Commons, K. G. (2010). "Neuronal pathways linking substance P to drug addiction and stress." ***Brain Res*** **1314**: 175-182.

Connor, D. F., D. J. Grasso, M. D. Slivinsky, G. S. Pearson and A. Banga (2013). "An open-label study of guanfacine extended release for traumatic stress related symptoms in children and adolescents." ***J Child Adolesc Psychopharmacol*** **23**(4): 244-251.

Cortese, S. (2012). "The neurobiology and genetics of Attention-Deficit/Hyperactivity Disorder (ADHD): what every clinician should know." ***Eur J Paediatr Neurol*** **16**(5): 422-433.

Cortese, S., M. Angriman, C. Maffeis, P. Isnard, E. Konofal, M. Lecendreux, D. Purper-Ouakil, B. Vincenzi, B. D. Bernardina and M. C. Mouren (2008). "Attention-deficit/hyperactivity disorder (ADHD) and obesity: a systematic review of the literature." ***Crit Rev Food Sci Nutr*** **48**(6): 524-537.

Cortese, S., S. V. Faraone, S. Bernardi, S. Wang and C. Blanco (2013). "Adult attention-deficit hyperactivity disorder and obesity: epidemiological study." ***Br J Psychiatry*** **203**(1): 24-34.

Crawley, J. N. (1981). "Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines." ***Pharmacol Biochem Behav*** **15**(5): 695-699.

Crawley, J. N., J. K. Belknap, A. Collins, J. C. Crabbe, W. Frankel, N. Henderson, R. J. Hitzemann, S. C. Maxson, L. L. Miner, A. J. Silva, J. M. Wehner, A. Wynshaw-Boris and R. Paylor (1997). "Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies." ***Psychopharmacology (Berl)*** **132**(2): 107-124.

Curtin, C., L. G. Bandini, E. C. Perrin, D. J. Tybor and A. Must (2005). "Prevalence of overweight in children and adolescents with attention deficit hyperactivity disorder and autism spectrum disorders: a chart review." ***BMC Pediatr*** **5**: 48.

Cusack, B., A. Nelson and E. Richelson (1994). "Binding of antidepressants to human brain receptors: focus on newer generation compounds." ***Psychopharmacology (Berl)*** **114**(4): 559-565.

Dableh, L. J., K. Yashpal, J. Rochford and J. L. Henry (2005). "Antidepressant-like effects of neurokinin receptor antagonists in the forced swim test in the rat." ***Eur J Pharmacol*** **507**(1-3): 99-105.

Dalley, J. W., T. D. Fryer, L. Brichard, E. S. Robinson, D. E. Theobald, K. Laane, Y. Pena, E. R. Murphy, Y. Shah, K. Probst, I. Abakumova, F. I. Aigbirhio, H. K. Richards, Y. Hong, J. C. Baron, B. J. Everitt and T. W. Robbins (2007). "Nucleus accumbens D2/3 receptors predict trait impulsivity and cocaine reinforcement." ***Science*** **315**(5816): 1267-1270.

Dalley, J. W. and J. P. Roiser (2012). "Dopamine, serotonin and impulsivity." ***Neuroscience*** **215**: 42-58.

Dalley, J. W. and S. C. Stanford (1995). "Incremental changes in extracellular noradrenaline availability in the frontal cortex induced by naturalistic environmental stimuli: a microdialysis study in the freely moving rat." ***J Neurochem*** **65**(6): 2644-2651.

Damsa, C., A. Bumb, F. Bianchi-Demicheli, P. Vidailhet, R. Sterck, A. Andreoli and S. Beyenburg (2004). ""Dopamine-dependent" side effects of selective serotonin reuptake inhibitors: a clinical review." ***J Clin Psychiatry*** **65**(8): 1064-1068.

Davis, J. A. and T. J. Gould (2007). "Atomoxetine reverses nicotine withdrawal-associated deficits in contextual fear conditioning." ***Neuropsychopharmacology*** **32**(9): 2011-2019.

Day, M., J. B. Pan, M. J. Buckley, E. Cronin, P. R. Hollingsworth, W. D. Hirst, R. Navarra, J. P. Sullivan, M. W. Decker and G. B. Fox (2007). "Differential effects of ciproxifan and nicotine on impulsivity and attention measures in the 5-choice serial reaction time test." ***Biochem Pharmacol*** **73**(8): 1123-1134.

de Felipe, C., J. F. Herrero, J. A. O'Brien, J. A. Palmer, C. A. Doyle, A. J. Smith, J. M. Laird, C. Belmonte, F. Cervero and S. P. Hunt (1998). "Altered nociception, analgesia and aggression in mice lacking the receptor for substance P." ***Nature*** **392**(6674): 394-397.

de Graaf, R., R. C. Kessler, J. Fayyad, M. ten Have, J. Alonso, M. Angermeyer, G. Borges, K. Demyttenaere, I. Gasquet, G. de Girolamo, J. M. Haro, R. Jin, E. G. Karam, J. Ormel and J. Posada-Villa (2008). "The prevalence and effects of adult attention-deficit/hyperactivity disorder (ADHD) on the performance of workers: results from the WHO World Mental Health Survey Initiative." ***Occup Environ Med*** **65**(12): 835-842.

De Sousa, A. and G. Kalra (2012). "Drug therapy of attention deficit hyperactivity disorder: current trends." ***Mens Sana Monogr*** **10**(1): 45-69.

Decamp, E., K. Clark and J. S. Schneider (2011). "Effects of the alpha-2 adrenoceptor agonist guanfacine on attention and working memory in aged non-human primates." ***Eur J Neurosci*** **34**(6): 1018-1022.

Del'Guidice, T., M. Lemasson, A. Etievant, S. Manta, L. A. Magno, G. Escoffier, F. S. Roman and J. M. Beaulieu (2014). "Dissociations between cognitive and motor effects of psychostimulants and atomoxetine in hyperactive DAT-KO mice." ***Psychopharmacology (Berl)*** **231**(1): 109-122.

Del Campo, N., S. R. Chamberlain, B. J. Sahakian and T. W. Robbins (2011). "The roles of dopamine and noradrenaline in the pathophysiology and treatment of attention-deficit/hyperactivity disorder." ***Biol Psychiatry*** **69**(12): e145-157.

Delfs, J. M., Y. Zhu, J. P. Druhan and G. S. Aston-Jones (1998). "Origin of noradrenergic afferents to the shell subregion of the nucleus accumbens: anterograde and retrograde tract-tracing studies in the rat." ***Brain Res*** **806**(2): 127-140.

Dell'Osso, B., M. C. Palazzo, L. Oldani and A. C. Altamura (2011). "The noradrenergic action in antidepressant treatments: pharmacological and clinical aspects." ***CNS Neurosci Ther*** **17**(6): 723-732.

Devoto, P. and G. Flore (2006). "On the origin of cortical dopamine: is it a co-transmitter in noradrenergic neurons?" ***Curr Neuropharmacol*** **4**(2): 115-125.

Devoto, P., G. Flore, L. Pani and G. L. Gessa (2001). "Evidence for co-release of noradrenaline and dopamine from noradrenergic neurons in the cerebral cortex." ***Mol Psychiatry*** **6**(6): 657-664.

Devoto, P., G. Flore, P. Saba, M. P. Castelli, A. P. Piras, W. Luesu, M. C. Viaggi, M. G. Ennas and G. L. Gessa (2008). "6-Hydroxydopamine lesion in the ventral tegmental area fails to reduce extracellular dopamine in the cerebral cortex." ***J Neurosci Res*** **86**(7): 1647-1658.

Di Chiara, G., G. L. Tanda, R. Frau and E. Carboni (1992). "Heterologous monoamine reuptake: lack of transmitter specificity of neuron-specific carriers." ***Neurochem Int*** **20 Suppl**: 231S-235S.

Diana, G. (2002). "Does hypertension alone lead to cognitive decline in spontaneously hypertensive rats?" ***Behav Brain Res*** **134**(1-2): 113-121.

Diaz-Granados, J. L., P. L. Greene and A. Amsel (1994). "Selective activity enhancement and persistence in weanling rats after hippocampal X-irradiation in infancy: possible relevance for ADHD." ***Behav Neural Biol*** **61**(3): 251-259.

Dimitri, P., J. K. Wales and N. Bishop (2010). "Fat and bone in children: differential effects of obesity on bone size and mass according to fracture history." ***J Bone Miner Res*** **25**(3): 527-536.

Dommett, E. J. (2014). "Using the five-choice serial reaction time task to examine the effects of atomoxetine and methylphenidate in the male spontaneously hypertensive rat." ***Pharmacol Biochem Behav***.

Dommett, E. J. (2014). "Using the five-choice serial reaction time task to examine the effects of atomoxetine and methylphenidate in the male spontaneously hypertensive rat." ***Pharmacol Biochem Behav*** **124C**: 196-203.

Dornan, W. A. and C. W. Malsbury (1989). "Peptidergic control of male rat sexual behavior: the effects of intracerebral injections of substance P and cholecystokinin." ***Physiol Behav*** **46**(3): 547-556.

Dubach, U. C., R. Huwyler, P. Radielovic and M. Singeisen (1977). "A new centrally action antihypertensive agent guanfacine (BS 100-141)." ***Arzneimittelforschung*** **27**(3): 674-676.

Dudley, J. A., R. K. Weir, T. C. Yan, E. M. Grabowska, A. J. Grimme, S. Amini, D. N. Stephens, S. P. Hunt and S. C. Stanford (2013). "Antagonism of L-type Ca(v) channels with nifedipine differentially affects performance of wildtype and NK1R-/- mice in the 5-Choice Serial Reaction-Time Task." ***Neuropharmacology*** **64**(1): 329-336.

Durcan, M. J., R. G. Lister and M. Linnoila (1989). "Behavioral effects of alpha 2 adrenoceptor antagonists and their interactions with ethanol in tests of locomotion, exploration and anxiety in mice." ***Psychopharmacology (Berl)*** **97**(2): 189-193.

Durston, S., H. E. Hulshoff Pol, H. G. Schnack, J. K. Buitelaar, M. P. Steenhuis, R. B. Minderaa, R. S. Kahn and H. van Engeland (2004). "Magnetic resonance imaging of boys with attention-deficit/hyperactivity disorder and their unaffected siblings." ***J Am Acad Child Adolesc Psychiatry*** **43**(3): 332-340.

Eagle, D. M., A. Bari and T. W. Robbins (2008). "The neuropsychopharmacology of action inhibition: cross-species translation of the stop-signal and go/no-go tasks." ***Psychopharmacology (Berl)*** **199**(3): 439-456.

Eagle, D. M., O. Lehmann, D. E. Theobald, Y. Pena, R. Zakaria, R. Ghosh, J. W. Dalley and T. W. Robbins (2009). "Serotonin depletion impairs waiting but not stop-signal reaction time in rats: implications for theories of the role of 5-HT in behavioral inhibition." ***Neuropsychopharmacology*** **34**(5): 1311-1321.

Eagle, D. M. and T. W. Robbins (2003). "Inhibitory control in rats performing a stop-signal reaction-time task: effects of lesions of the medial striatum and d-amphetamine." ***Behav Neurosci*** **117**(6): 1302-1317.

Eagle, D. M., M. R. Tufft, H. L. Goodchild and T. W. Robbins (2007). "Differential effects of modafinil and methylphenidate on stop-signal reaction time task performance in the rat, and interactions with the dopamine receptor antagonist cis-flupenthixol." ***Psychopharmacology (Berl)*** **192**(2): 193-206.

Eason, M. G. and S. B. Liggett (1996). "Chimeric mutagenesis of putative G-protein coupling domains of the alpha2A-adrenergic receptor. Localization of two redundant and fully competent gi coupling domains." ***J Biol Chem*** **271**(22): 12826-12832.

Ebner, K., N. M. Rupniak, A. Saria and N. Singewald (2004). "Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats." ***Proc Natl Acad Sci U S A*** **101**(12): 4280-4285.

Ebner, K. and N. Singewald (2006). "The role of substance P in stress and anxiety responses." ***Amino Acids*** **31**(3): 251-272.

Ebner, K. and N. Singewald (2007). "Stress-induced release of substance P in the locus coeruleus modulates cortical noradrenaline release." ***Naunyn Schmiedebergs Arch Pharmacol*** **376**(1-2): 73-82.

Economidou, D., D. E. Theobald, T. W. Robbins, B. J. Everitt and J. W. Dalley (2012). "Norepinephrine and dopamine modulate impulsivity on the five-choice serial reaction time task through opponent actions in the shell and core sub-regions of the nucleus accumbens." ***Neuropsychopharmacology*** **37**(9): 2057-2066.

Elliott, P. J. (1988). "Place aversion induced by the substance P analogue, dimethyl-C7, is not state dependent: implication of substance P in aversion." ***Exp Brain Res*** **73**(2): 354-356.

Engman, J., F. Ahs, T. Furmark, C. Linnman, A. Pissiota, L. Appel, O. Frans, B. Langstrom and M. Fredrikson (2012). "Age, sex and NK1 receptors in the human brain -- a positron emission tomography study with [(1)(1)C]GR205171." ***Eur Neuropsychopharmacol*** **22**(8): 562-568.

Ennaceur, A. and J. Delacour (1988). "A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data." ***Behav Brain Res*** **31**(1): 47-59.

Ennis, M. and G. Aston-Jones (1988). "Activation of locus coeruleus from nucleus paragigantocellularis: a new excitatory amino acid pathway in brain." ***J Neurosci*** **8**(10): 3644-3657.

Ennis, M. and G. Aston-Jones (1989). "GABA-mediated inhibition of locus coeruleus from the dorsomedial rostral medulla." ***J Neurosci*** **9**(8): 2973-2981.

Erenberg, G. (2005). "The relationship between tourette syndrome, attention deficit hyperactivity disorder, and stimulant medication: a critical review." ***Semin Pediatr Neurol*** **12**(4): 217-221.

Erermis, S., N. Cetin, M. Tamar, N. Bukusoglu, F. Akdeniz and D. Goksen (2004). "Is obesity a risk factor for psychopathology among adolescents?" ***Pediatr Int*** **46**(3): 296-301.

Esbenshade, T. A., K. E. Browman, R. S. Bitner, M. Strakhova, M. D. Cowart and J. D. Brioni (2008). "The histamine H3 receptor: an attractive target for the treatment of cognitive disorders." ***Br J Pharmacol*** **154**(6): 1166-1181.

Evenden, J. L. (1999). "Varieties of impulsivity." ***Psychopharmacology (Berl)*** **146**(4): 348-361.

Faraone, S. V., J. Biederman, T. J. Spencer and M. Aleardi (2006). "Comparing the efficacy of medications for ADHD using meta-analysis." ***MedGenMed*** **8**(4): 4.

Faraone, S. V. and S. J. Glatt (2010). "A comparison of the efficacy of medications for adult attention-deficit/hyperactivity disorder using meta-analysis of effect sizes." ***J Clin Psychiatry*** **71**(6): 754-763.

Faraone, S. V., K. McBurnett, F. R. Sallee, J. Steeber and F. A. Lopez (2013). "Guanfacine extended release: a novel treatment for attention-deficit/hyperactivity disorder in children and adolescents." ***Clin Ther*** **35**(11): 1778-1793.

Feola, T. W., H. de Wit and J. B. Richards (2000). "Effects of d-amphetamine and alcohol on a measure of behavioral inhibition in rats." ***Behav Neurosci*** **114**(4): 838-848.

Ferraro, L., K. Fuxe, S. Tanganelli, M. C. Tomasini, F. A. Rambert and T. Antonelli (2002). "Differential enhancement of dialysate serotonin levels in distinct brain regions of the awake rat by modafinil: possible relevance for wakefulness and depression." ***J Neurosci Res*** **68**(1): 107-112.

Ferguson, S. A. (1996). "Neuroanatomical and functional alterations resulting from early postnatal cerebellar insults in rodents." ***Pharmacol Biochem Behav*** **55**(4): 663-671.

Fernando, A. B., D. Economidou, D. E. Theobald, M. F. Zou, A. H. Newman, M. Spoelder, D. Caprioli, M. Moreno, L. Hipolito, A. T. Aspinall, T. W. Robbins and J. W. Dalley (2012). "Modulation of high impulsivity and attentional performance in rats by selective direct and indirect dopaminergic and noradrenergic receptor agonists." ***Psychopharmacology (Berl)*** **219**(2): 341-352.

Fernstrom, J. D. and S. Choi (2008). "The development of tolerance to drugs that suppress food intake." ***Pharmacol Ther*** **117**(1): 105-122.

File, S. E. (1997). "Anxiolytic action of a neurokinin1 receptor antagonist in the social interaction test." ***Pharmacol Biochem Behav*** **58**(3): 747-752.

File, S. E. (2000). "NKP608, an NK1 receptor antagonist, has an anxiolytic action in the social interaction test in rats." ***Psychopharmacology (Berl)*** **152**(1): 105-109.

Filipek, P. A., M. Semrud-Clikeman, R. J. Steingard, P. F. Renshaw, D. N. Kennedy and J. Biederman (1997). "Volumetric MRI analysis comparing subjects having attention-deficit hyperactivity disorder with normal controls." ***Neurology*** **48**(3): 589-601.

Fisher, A. S., R. J. Stewart, T. Yan, S. P. Hunt and S. C. Stanford (2007). "Disruption of noradrenergic transmission and the behavioural response to a novel environment in NK1R-/- mice." ***Eur J Neurosci*** **25**(4): 1195-1204.

Fleckenstein, A. E., T. J. Volz, E. L. Riddle, J. W. Gibb and G. R. Hanson (2007). "New insights into the mechanism of action of amphetamines." ***Annu Rev Pharmacol Toxicol*** **47**: 681-698.

Fleming, J. P., L. D. Levy and R. D. Levitan (2005). "Symptoms of attention deficit hyperactivity disorder in severely obese women." ***Eat Weight Disord*** **10**(1): e10-13.

Flood, D. G., E. Zuvich, M. J. Marino and M. Gasior (2010). "The effects of d-amphetamine, methylphenidate, sydnocarb, and caffeine on prepulse inhibition of the startle reflex in DBA/2 mice." ***Psychopharmacology (Berl)*** **211**(3): 325-336.

Foote, S. L., G. Aston-Jones and F. E. Bloom (1980). "Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal." ***Proc Natl Acad Sci U S A*** **77**(5): 3033-3037.

Forrest, L. R., Y. W. Zhang, M. T. Jacobs, J. Gesmonde, L. Xie, B. H. Honig and G. Rudnick (2008). "Mechanism for alternating access in neurotransmitter transporters." ***Proc Natl Acad Sci U S A*** **105**(30): 10338-10343.

Franowicz, J. S. and A. F. Arnsten (1998). "The alpha-2a noradrenergic agonist, guanfacine, improves delayed response performance in young adult rhesus monkeys." ***Psychopharmacology (Berl)*** **136**(1): 8-14.

Franowicz, J. S. and A. F. Arnsten (2002). "Actions of alpha-2 noradrenergic agonists on spatial working memory and blood pressure in rhesus monkeys appear to be mediated by the same receptor subtype." ***Psychopharmacology (Berl)*** **162**(3): 304-312.

Franowicz, J. S., L. E. Kessler, C. M. Borja, B. K. Kobilka, L. E. Limbird and A. F. Arnsten (2002). "Mutation of the alpha2A-adrenoceptor impairs working memory performance and annuls cognitive enhancement by guanfacine." ***J Neurosci*** **22**(19): 8771-8777.

Froger, N., A. M. Gardier, R. Moratalla, I. Alberti, I. Lena, C. Boni, C. De Felipe, N. M. Rupniak, S. P. Hunt, C. Jacquot, M. Hamon and L. Lanfumey (2001). "5-hydroxytryptamine (5-HT)1A autoreceptor adaptive changes in substance P (neurokinin 1) receptor knock-out mice mimic antidepressant-induced desensitization." ***J Neurosci*** **21**(20): 8188-8197.

Fulton, S., P. Pissios, R. P. Manchon, L. Stiles, L. Frank, E. N. Pothos, E. Maratos-Flier and J. S. Flier (2006). "Leptin regulation of the mesoaccumbens dopamine pathway." ***Neuron*** **51**(6): 811-822.

Gadd, C. A., P. Murtra, C. De Felipe and S. P. Hunt (2003). "Neurokinin-1 receptor-expressing neurons in the amygdala modulate morphine reward and anxiety behaviors in the mouse." ***J Neurosci*** **23**(23): 8271-8280.

Gainetdinov, R. R. and M. G. Caron (2001). "Genetics of childhood disorders: XXIV. ADHD, part 8: hyperdopaminergic mice as an animal model of ADHD." ***J Am Acad Child Adolesc Psychiatry*** **40**(3): 380-382.

Gainetdinov, R. R., W. C. Wetsel, S. R. Jones, E. D. Levin, M. Jaber and M. G. Caron (1999). "Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity." ***Science*** **283**(5400): 397-401.

Gajda, M., J. A. Litwin, T. Cichocki, J. P. Timmermans and D. Adriaensen (2005). "Development of sensory innervation in rat tibia: co-localization of CGRP and substance P with growth-associated protein 43 (GAP-43)." ***J Anat*** **207**(2): 135-144.

Gamaro, G. D., M. E. Prediger, J. Lopes, M. G. Bassani and C. Dalmaz (2008). "Fluoxetine alters feeding behavior and leptin levels in chronically-stressed rats." ***Pharmacol Biochem Behav*** **90**(3): 312-317.

Gamo, N. J., M. Wang and A. F. Arnsten (2010). "Methylphenidate and atomoxetine enhance prefrontal function through alpha2-adrenergic and dopamine D1 receptors." ***J Am Acad Child Adolesc Psychiatry*** **49**(10): 1011-1023.

Gasior, M., J. Bergman, M. J. Kallman and C. A. Paronis (2005). "Evaluation of the reinforcing effects of monoamine reuptake inhibitors under a concurrent schedule of food and i.v. drug delivery in rhesus monkeys." ***Neuropsychopharmacology*** **30**(4): 758-764.

Gattu, M., J. R. Pauly, K. L. Boss, J. B. Summers and J. J. Buccafusco (1997). "Cognitive impairment in spontaneously hypertensive rats: role of central nicotinic receptors. I." ***Brain Res*** **771**(1): 89-103.

Gattu, M., A. V. Terry, Jr., J. R. Pauly and J. J. Buccafusco (1997). "Cognitive impairment in spontaneously hypertensive rats: role of central nicotinic receptors. Part II." ***Brain Res*** **771**(1): 104-114.

Gehlert, D. R., S. L. Gackenheimer and D. W. Robertson (1993). "Localization of rat brain binding sites for [3H]tomoxetine, an enantiomerically pure ligand for norepinephrine reuptake sites." ***Neurosci Lett*** **157**(2): 203-206.

Geller, D. A. (2006). "Obsessive-compulsive and spectrum disorders in children and adolescents." ***Psychiatr Clin North Am*** **29**(2): 353-370.

Gentsch, C., M. Cutler, A. Vassout, S. Veenstra and F. Brugger (2002). "Anxiolytic effect of NKP608, a NK1-receptor antagonist, in the social investigation test in gerbils." ***Behav Brain Res*** **133**(2): 363-368.

George, D. T., J. Gilman, J. Hersh, A. Thorsell, D. Herion, C. Geyer, X. Peng, W. Kielbasa, R. Rawlings, J. E. Brandt, D. R. Gehlert, J. T. Tauscher, S. P. Hunt, D. Hommer and M. Heilig (2008). "Neurokinin 1 receptor antagonism as a possible therapy for alcoholism." ***Science*** **319**(5869): 1536-1539.

Gerard, N. P., L. A. Garraway, R. L. Eddy, Jr., T. B. Shows, H. Iijima, J. L. Paquet and C. Gerard (1991). "Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones." ***Biochemistry*** **30**(44): 10640-10646.

Gerasimov, M. R., M. Franceschi, N. D. Volkow, A. Gifford, S. J. Gatley, D. Marsteller, P. E. Molina and S. L. Dewey (2000). "Comparison between intraperitoneal and oral methylphenidate administration: A microdialysis and locomotor activity study." ***J Pharmacol Exp Ther*** **295**(1): 51-57.

Gerrard, P. and R. Malcolm (2007). "Mechanisms of modafinil: A review of current research." ***Neuropsychiatr Dis Treat*** **3**(3): 349-364.

Ghanizadeh, A. (2014). "A systematic review of reboxetine for treating patients with attention deficit hyperactivity disorder." ***Nord J Psychiatry***: 1-8.

Gibson, A. P., T. L. Bettinger, N. C. Patel and M. L. Crismon (2006). "Atomoxetine versus stimulants for treatment of attention deficit/hyperactivity disorder." ***Ann Pharmacother*** **40**(6): 1134-1142.

Giros, B., M. Jaber, S. R. Jones, R. M. Wightman and M. G. Caron (1996). "Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter." ***Nature*** **379**(6566): 606-612.

Giros, B., Y. M. Wang, S. Suter, S. B. McLeskey, C. Pifl and M. G. Caron (1994). "Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters." ***J Biol Chem*** **269**(23): 15985-15988.

Gizer, I. R., C. Ficks and I. D. Waldman (2009). "Candidate gene studies of ADHD: a meta-analytic review." ***Hum Genet*** **126**(1): 51-90.

Glennon, R. A. (1999). "Arylalkylamine drugs of abuse: an overview of drug discrimination studies." ***Pharmacol Biochem Behav*** **64**(2): 251-256.

Glue, P. and D. Nutt (1987). "Clonidine in alcohol withdrawal: a pilot study of differential symptom responses following i.v. clonidine." ***Alcohol Alcohol*** **22**(2): 161-166.

Godfrey, J. (2009). "Safety of therapeutic methylphenidate in adults: a systematic review of the evidence." ***J Psychopharmacol*** **23**(2): 194-205.

Gonzalez-Lima, F. and A. G. Sadile (2000). "Network operations revealed by brain metabolic mapping in a genetic model of hyperactivity and attention deficit: the naples high- and low-excitability rats." ***Neurosci Biobehav Rev*** **24**(1): 157-160.

Goto, T., K. Nakao, K. K. Gunjigake, M. A. Kido, S. Kobayashi and T. Tanaka (2007). "Substance P stimulates late-stage rat osteoblastic bone formation through neurokinin-1 receptors." ***Neuropeptides*** **41**(1): 25-31.

Goto, T., T. Yamaza, M. A. Kido and T. Tanaka (1998). "Light- and electron-microscopic study of the distribution of axons containing substance P and the localization of neurokinin-1 receptor in bone." ***Cell Tissue Res*** **293**(1): 87-93.

Gould, T. J., M. Rukstalis and M. C. Lewis (2005). "Atomoxetine and nicotine enhance prepulse inhibition of acoustic startle in C57BL/6 mice." ***Neurosci Lett*** **377**(2): 85-90.

Gray, J. and N. McNaughton (2003). The neuropsychology of anxiety: An enquiry into the function of the septo-hippocampal system, Oxford University Press.

Gresch, P. J., A. F. Sved, M. J. Zigmond and J. M. Finlay (1995). "Local influence of endogenous norepinephrine on extracellular dopamine in rat medial prefrontal cortex." ***J Neurochem*** **65**(1): 111-116.

Griffin, W. C., 3rd, R. W. McGovern, G. H. Bell, P. K. Randall, L. D. Middaugh and K. S. Patrick (2013). "Interactive effects of methylphenidate and alcohol on discrimination, conditioned place preference and motor coordination in C57BL/6J mice." ***Psychopharmacology (Berl)*** **225**(3): 613-625.

Gu, H., S. C. Wall and G. Rudnick (1994). "Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence." ***J Biol Chem*** **269**(10): 7124-7130.

Guiard, B. P., N. Froger, M. Hamon, A. M. Gardier and L. Lanfumey (2005). "Sustained pharmacological blockade of NK1 substance P receptors causes functional desensitization of dorsal raphe 5-HT 1A autoreceptors in mice." ***J Neurochem*** **95**(6): 1713-1723.

Guiard, B. P., J. P. Guilloux, C. Reperant, S. P. Hunt, M. Toth and A. M. Gardier (2007). "Substance P neurokinin 1 receptor activation within the dorsal raphe nucleus controls serotonin release in the mouse frontal cortex." ***Mol Pharmacol*** **72**(6): 1411-1418.

Guo, T., C. Yang, L. Guo and K. Liu (2012). "A comparative study of the effects of ABT-418 and methylphenidate on spatial memory in an animal model of ADHD." ***Neurosci Lett*** **528**(1): 11-15.

Haller, J. and M. Alicki (2012). "Current animal models of anxiety, anxiety disorders, and anxiolytic drugs." ***Curr Opin Psychiatry*** **25**(1): 59-64.

Hamdi, A., J. Porter and C. Prasad (1992). "Decreased striatal D2 dopamine receptors in obese Zucker rats: changes during aging." ***Brain Res*** **589**(2): 338-340.

Handley, S. L. and S. Mithani (1984). "Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour." ***Naunyn Schmiedebergs Arch Pharmacol*** **327**(1): 1-5.

Harlan, R. E., M. M. Garcia and J. E. Krause (1989). "Cellular localization of substance P- and neurokinin A-encoding preprotachykinin mRNA in the female rat brain." ***J Comp Neurol*** **287**(2): 179-212.

Harley, C. W. (1987). "A role for norepinephrine in arousal, emotion and learning?: limbic modulation by norepinephrine and the Kety hypothesis." ***Prog Neuropsychopharmacol Biol Psychiatry*** **11**(4): 419-458.

Harrison, A. A., B. J. Everitt and T. W. Robbins (1997). "Central 5-HT depletion enhances impulsive responding without affecting the accuracy of attentional performance: interactions with dopaminergic mechanisms." ***Psychopharmacology (Berl)*** **133**(4): 329-342.

Harrison, A. A., B. J. Everitt and T. W. Robbins (1999). "Central serotonin depletion impairs both the acquisition and performance of a symmetrically reinforced go/no-go conditional visual discrimination." ***Behav Brain Res*** **100**(1-2): 99-112.

Hazell, P. L., M. R. Kohn, R. Dickson, R. J. Walton, R. E. Granger and G. W. Wyk (2011). "Core ADHD symptom improvement with atomoxetine versus methylphenidate: a direct comparison meta-analysis." ***J Atten Disord*** **15**(8): 674-683.

Heal, D. J., S. C. Cheetham and S. L. Smith (2009). "The neuropharmacology of ADHD drugs in vivo: insights on efficacy and safety." ***Neuropharmacology*** **57**(7-8): 608-618.

Heal, D. J., M. R. Prow and W. R. Buckett (1989). "Clonidine-induced hypoactivity and mydriasis in mice are respectively mediated via pre- and postsynaptic alpha 2-adrenoceptors in the brain." ***Eur J Pharmacol*** **170**(1-2): 19-28.

Heal, D. J., M. R. Prow, S. A. Butler and W. R. Buckett (1995). "Mediation of mydriasis in conscious rats by central postsynaptic alpha 2-adrenoceptors." ***Pharmacol Biochem Behav*** **50**(2): 219-224.

Heal, D. J., S. L. Smith, R. S. Kulkarni and H. L. Rowley (2008). "New perspectives from microdialysis studies in freely-moving, spontaneously hypertensive rats on the pharmacology of drugs for the treatment of ADHD." ***Pharmacol Biochem Behav*** **90**(2): 184-197.

Hediger, M. A., M. F. Romero, J. B. Peng, A. Rolfs, H. Takanaga and E. A. Bruford (2004). "The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction." ***Pflugers Arch*** **447**(5): 465-468.

Helms, C. M., J. M. Reeves and S. H. Mitchell (2006). "Impact of strain and D-amphetamine on impulsivity (delay discounting) in inbred mice." ***Psychopharmacology (Berl)*** **188**(2): 144-151.

Herkenham, M. (1987). "Mismatches between neurotransmitter and receptor localizations in brain: observations and implications." ***Neuroscience*** **23**(1): 1-38.

Herpfer, I., S. P. Hunt and S. C. Stanford (2005). "A comparison of neurokinin 1 receptor knock-out (NK1-/-) and wildtype mice: exploratory behaviour and extracellular noradrenaline concentration in the cerebral cortex of anaesthetised subjects." ***Neuropharmacology*** **48**(5): 706-719.

Hershey, A. D. and J. E. Krause (1990). "Molecular characterization of a functional cDNA encoding the rat substance P receptor." ***Science*** **247**(4945): 958-962.

Hervey, A. S., J. N. Epstein and J. F. Curry (2004). "Neuropsychology of adults with attention-deficit/hyperactivity disorder: a meta-analytic review." ***Neuropsychology*** **18**(3): 485-503.

Hess, E. J., K. A. Collins, N. G. Copeland, N. A. Jenkins and M. C. Wilson (1994). "Deletion map of the coloboma (Cm) locus on mouse chromosome 2." ***Genomics*** **21**(1): 257-261.

Hess, E. J., K. A. Collins and M. C. Wilson (1996). "Mouse model of hyperkinesis implicates SNAP-25 in behavioral regulation." ***J Neurosci*** **16**(9): 3104-3111.

Heyser, C. J., M. C. Wilson and L. H. Gold (1995). "Coloboma hyperactive mutant exhibits delayed neurobehavioral developmental milestones." ***Brain Res Dev Brain Res*** **89**(2): 264-269.

Highfield, D. A., D. Hu and A. Amsel (1998). "Alleviation of x-irradiation-based deficit in memory-based learning by D-amphetamine: suggestions for attention deficit-hyperactivity disorder." ***Proc Natl Acad Sci U S A*** **95**(10): 5785-5788.

Hill, D. E., R. A. Yeo, R. A. Campbell, B. Hart, J. Vigil and W. Brooks (2003). "Magnetic resonance imaging correlates of attention-deficit/hyperactivity disorder in children." ***Neuropsychology*** **17**(3): 496-506.

Hirota, T., S. Schwartz and C. U. Correll (2014). "Alpha-2 agonists for attention-deficit/hyperactivity disorder in youth: a systematic review and meta-analysis of monotherapy and add-on trials to stimulant therapy." ***J Am Acad Child Adolesc Psychiatry*** **53**(2): 153-173.

Horner, W. E., D. E. Johnson, A. W. Schmidt and H. Rollema (2007). "Methylphenidate and atomoxetine increase histamine release in rat prefrontal cortex." ***Eur J Pharmacol*** **558**(1-3): 96-97.

Hubel, R., J. Jass, A. Marcus and R. G. Laessle (2006). "Overweight and basal metabolic rate in boys with attention-deficit/hyperactivity disorder." ***Eat Weight Disord*** **11**(3): 139-146.

Hudson, A. L., E. S. Robinson, M. D. Lalies, R. J. Tyacke, H. C. Jackson and D. J. Nutt (1999). "In vitro and in vivo approaches to the characterization of the alpha2-adrenoceptor." ***J Auton Pharmacol*** **19**(6): 311-320.

Humby, T., F. M. Laird, W. Davies and L. S. Wilkinson (1999). "Visuospatial attentional functioning in mice: interactions between cholinergic manipulations and genotype." ***Eur J Neurosci*** **11**(8): 2813-2823.

Hunt, R. D., R. B. Minderaa and D. J. Cohen (1985). "Clonidine benefits children with attention deficit disorder and hyperactivity: report of a double-blind placebo-crossover therapeutic trial." ***J Am Acad Child Psychiatry*** **24**(5): 617-629.

Hynd, G. W., M. Semrud-Clikeman, A. R. Lorys, E. S. Novey, D. Eliopulos and H. Lyytinen (1991). "Corpus callosum morphology in attention deficit-hyperactivity disorder: morphometric analysis of MRI." ***J Learn Disabil*** **24**(3): 141-146.

Iida-Klein, A., S. S. Lu, K. Yokoyama, D. W. Dempster, J. W. Nieves and R. Lindsay (2003). "Precision, accuracy, and reproducibility of dual X-ray absorptiometry measurements in mice in vivo." ***J Clin Densitom*** **6**(1): 25-33.

Iversen, L. L., M. R. Hanley, B. E. Sandberg, C. M. Lee, R. D. Pinnock and S. P. Watson (1982). "Substance P receptors in the nervous system and possible receptor subtypes." ***Ciba Found Symp***(91): 186-205.

Jackson, H. C., A. M. Needham, L. J. Hutchins, S. E. Mazurkiewicz and D. J. Heal (1997). "Comparison of the effects of sibutramine and other monoamine reuptake inhibitors on food intake in the rat." ***Br J Pharmacol*** **121**(8): 1758-1762.

Jakala, P., M. Riekkinen, J. Sirvio, E. Koivisto and P. Riekkinen, Jr. (1999). "Clonidine, but not guanfacine, impairs choice reaction time performance in young healthy volunteers." ***Neuropsychopharmacology*** **21**(4): 495-502.

Jakala, P., J. Sirvio, P. Riekkinen, Jr., A. Haapalanna and P. Riekkinen (1992). "Effects of atipamezole, an alpha 2-adrenoceptor antagonist, on the performance of rats in a five-choice serial reaction time task." ***Pharmacol Biochem Behav*** **42**(4): 903-907.

James, W. P., I. D. Caterson, W. Coutinho, N. Finer, L. F. Van Gaal, A. P. Maggioni, C. Torp-Pedersen, A. M. Sharma, G. M. Shepherd, R. A. Rode and C. L. Renz (2010). "Effect of sibutramine on cardiovascular outcomes in overweight and obese subjects." ***N Engl J Med*** **363**(10): 905-917.

Janak, P. H., M. S. Bowers and L. H. Corbit (2012). "Compound stimulus presentation and the norepinephrine reuptake inhibitor atomoxetine enhance long-term extinction of cocaine-seeking behavior." ***Neuropsychopharmacology*** **37**(4): 975-985.

Jarrott, B., W. J. Louis and R. J. Summers (1983). "Characterization of central alpha-adrenoceptors using 3H-clonidine and its derivatives." ***Chest*** **83**(2 Suppl): 339-340.

Jentsch, J. D. (2005). "Impaired visuospatial divided attention in the spontaneously hypertensive rat." ***Behav Brain Res*** **157**(2): 323-330.

Jentsch, J. D. and L. A. Anzivino (2004). "A low dose of the alpha2 agonist clonidine ameliorates the visual attention and spatial working memory deficits produced by phencyclidine administration to rats." ***Psychopharmacology (Berl)*** **175**(1): 76-83.

Jerie, P. (1980). "Clinical experience with guanfacine in long-term treatment of hypertension." ***Br J Clin Pharmacol*** **10 Suppl 1**: 37S-47S.

Johnston, S. L., W. L. Peacock, L. M. Bell, M. Lonchampt and J. R. Speakman (2005). "PIXImus DXA with different software needs individual calibration to accurately predict fat mass." ***Obes Res*** **13**(9): 1558-1565.

Jones, M. D. and E. J. Hess (2003). "Norepinephrine regulates locomotor hyperactivity in the mouse mutant coloboma." ***Pharmacol Biochem Behav*** **75**(1): 209-216.

Jones, S. R., R. R. Gainetdinov, R. M. Wightman and M. G. Caron (1998). "Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter." ***J Neurosci*** **18**(6): 1979-1986.

Kaczmarczyk, M. M., A. S. Machaj, G. S. Chiu, M. A. Lawson, S. J. Gainey, J. M. York, D. D. Meling, S. A. Martin, K. A. Kwakwa, A. F. Newman, J. A. Woods, K. W. Kelley, Y. Wang, M. J. Miller and G. G. Freund (2013). "Methylphenidate prevents high-fat diet (HFD)-induced learning/memory impairment in juvenile mice." ***Psychoneuroendocrinology***.

Kang, J. G., C. Y. Park, J. H. Kang, Y. W. Park and S. W. Park (2010). "Randomized controlled trial to investigate the effects of a newly developed formulation of phentermine diffuse-controlled release for obesity." ***Diabetes Obes Metab*** **12**(10): 876-882.

Kantak, K. M., T. Singh, K. A. Kerstetter, K. A. Dembro, M. M. Mutebi, R. C. Harvey, C. F. Deschepper and L. P. Dwoskin (2008). "Advancing the spontaneous hypertensive rat model of attention deficit/hyperactivity disorder." ***Behav Neurosci*** **122**(2): 340-357.

Karagiannides, I., K. Bakirtzi, E. Kokkotou, D. Stavrakis, K. G. Margolis, T. Thomou, N. Giorgadze, J. L. Kirkland and C. Pothoulakis (2011). "Role of substance P in the regulation of

glucose metabolism via insulin signaling-associated pathways." ***Endocrinology*** **152**(12): 4571-4580.

Karagiannides, I., D. Torres, Y. H. Tseng, C. Bowe, E. Carvalho, D. Espinoza, C. Pothoulakis and E. Kokkotou (2008). "Substance P as a novel anti-obesity target." ***Gastroenterology*** **134**(3): 747-755.

Kauser, H., S. Sahu, S. Kumar and U. Panjwani (2014). "Guanfacine ameliorates hypobaric hypoxia induced spatial working memory deficits." ***Physiol Behav*** **123**: 187-192.

Kawahara, Y., H. Kawahara and B. H. Westerink (1999). "Tonic regulation of the activity of noradrenergic neurons in the locus coeruleus of the conscious rat studied by dual-probe microdialysis." ***Brain Res*** **823**(1-2): 42-48.

Kawai, M. and C. J. Rosen (2010). "Bone: adiposity and bone accrual-still an established paradigm?" ***Nat Rev Endocrinol*** **6**(2): 63-64.

Keck, T. M., K. L. Suchland, C. C. Jimenez and D. K. Grandy (2013). "Dopamine D4 receptor deficiency in mice alters behavioral responses to anxiogenic stimuli and the psychostimulant methylphenidate." ***Pharmacol Biochem Behav*** **103**(4): 831-841.

Keller, M., S. Montgomery, W. Ball, M. Morrison, D. Snavely, G. Liu, R. Hargreaves, J. Hietala, C. Lines, K. Beebe and S. Reines (2006). "Lack of efficacy of the substance p (neurokinin1 receptor) antagonist aprepitant in the treatment of major depressive disorder." ***Biol Psychiatry*** **59**(3): 216-223.

Kessler, R. C., L. A. Adler, R. Barkley, J. Biederman, C. K. Conners, S. V. Faraone, L. L. Greenhill, S. Jaeger, K. Secnik, T. Spencer, T. B. Ustun and A. M. Zaslavsky (2005). "Patterns and predictors of attention-deficit/hyperactivity disorder persistence into adulthood: results from the national comorbidity survey replication." ***Biol Psychiatry*** **57**(11): 1442-1451.

Kim, J., B. Mutyala, S. Agiovlasitis and B. Fernhall (2011). "Health behaviors and obesity among US children with attention deficit hyperactivity disorder by gender and medication use." ***Prev Med*** **52**(3-4): 218-222.

Kim, K. K., H. J. Cho, H. C. Kang, B. B. Youn and K. R. Lee (2006). "Effects on weight reduction and safety of short-term phentermine administration in Korean obese people." ***Yonsei Med J*** **47**(5): 614-625.

Kim, S., I. Bobeica, N. J. Gamo, A. F. Arnsten and D. Lee (2012). "Effects of alpha-2A adrenergic receptor agonist on time and risk preference in primates." ***Psychopharmacology (Berl)*** **219**(2): 363-375.

Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, R. J. Lefkowitz and J. W. Regan (1987). "Cloning, sequencing, and expression of the gene coding for the human platelet alpha 2-adrenergic receptor." ***Science*** **238**(4827): 650-656.

Koda, K., Y. Ago, Y. Cong, Y. Kita, K. Takuma and T. Matsuda (2010). "Effects of acute and chronic administration of atomoxetine and methylphenidate on extracellular levels of noradrenaline, dopamine and serotonin in the prefrontal cortex and striatum of mice." ***J Neurochem*** **114**(1): 259-270.

Koike, H., D. Ibi, H. Mizoguchi, T. Nagai, A. Nitta, K. Takuma, T. Nabeshima, Y. Yoneda and K. Yamada (2009). "Behavioral abnormality and pharmacologic response in social isolation-reared mice." ***Behav Brain Res*** **202**(1): 114-121.

Kolar, D., A. Keller, M. Golfinopoulos, L. Cumyn, C. Syer and L. Hechtman (2008). "Treatment of adults with attention-deficit/hyperactivity disorder." ***Neuropsychiatr Dis Treat*** **4**(2): 389-403.

Kollins, S. H., F. A. Lopez, B. D. Vince, J. M. Turnbow, K. Farrand, A. Lyne, S. B. Wigal and T. Roth (2011). "Psychomotor functioning and alertness with guanfacine extended release in subjects with attention-deficit/hyperactivity disorder." ***J Child Adolesc Psychopharmacol*** **21**(2): 111-120.

Komatsu, D. E., P. K. Thanos, M. N. Mary, H. A. Janda, C. M. John, L. Robison, M. Ananth, J. M. Swanson, N. D. Volkow and M. Hadjiargyrou (2012). "Chronic exposure to methylphenidate impairs appendicular bone quality in young rats." ***Bone*** **50**(6): 1214-1222.

Kooij, J. J., M. Huss, P. Asherson, R. Akehurst, K. Beusterien, A. French, R. Sasane and P. Hodgkins (2012). "Distinguishing comorbidity and successful management of adult ADHD." ***J Atten Disord*** **16**(5 Suppl): 3S-19S.

Krain, A. L. and F. X. Castellanos (2006). "Brain development and ADHD." ***Clin Psychol Rev*** **26**(4): 433-444.

Kramer, M. S., N. Cutler, J. Feighner, R. Shrivastava, J. Carman, J. J. Sramek, S. A. Reines, G. Liu, D. Snavely, E. Wyatt-Knowles, J. J. Hale, S. G. Mills, M. MacCoss, C. J. Swain, T. Harrison, R. G. Hill, F. Hefti, E. M. Scolnick, M. A. Cascieri, G. G. Chicchi, S. Sadowski, A. R. Williams, L. Hewson, D. Smith, E. J. Carlson, R. J. Hargreaves and N. M. Rupniak (1998). "Distinct mechanism for antidepressant activity by blockade of central substance P receptors." ***Science*** **281**(5383): 1640-1645.

Kuczenski, R. and D. S. Segal (1997). "Effects of methylphenidate on extracellular dopamine, serotonin, and norepinephrine: comparison with amphetamine." ***J Neurochem*** **68**(5): 2032-2037.

Kumari, V., P. Cotter, P. J. Corr, J. A. Gray and S. A. Checkley (1996). "Effect of clonidine on the human acoustic startle reflex." ***Psychopharmacology (Berl)*** **123**(4): 353-360.

Laarakker, M. C., J. R. Raai, H. A. van Lith and F. Ohl (2010). "The role of the alpha 2A-adrenoceptor in mouse stress-coping behaviour." ***Psychoneuroendocrinology*** **35**(4): 490-502.

Lac, G., H. Cavalie, E. Ebal and O. Michaux (2008). "Effects of a high fat diet on bone of growing rats. Correlations between visceral fat, adiponectin and bone mass density." ***Lipids Health Dis*** **7**: 16.

Lange, K. W., S. Reichl, K. M. Lange, L. Tucha and O. Tucha (2010). "The history of attention deficit hyperactivity disorder." ***Atten Defic Hyperact Disord*** **2**(4): 241-255.

Langen, B. and R. Dost (2011). "Comparison of SHR, WKY and Wistar rats in different behavioural animal models: effect of dopamine D1 and alpha2 agonists." ***Atten Defic Hyperact Disord*** **3**(1): 1-12.

Langer, S. Z. (1974). "Presynaptic regulation of catecholamine release." ***Biochem Pharmacol*** **23**(13): 1793-1800.

Larsen, K. E., E. A. Fon, T. G. Hastings, R. H. Edwards and D. Sulzer (2002). "Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis." ***J Neurosci*** **22**(20): 8951-8960.

Lau, B. Y., V. A. Fajardo, L. McMeekin, S. M. Sacco, W. E. Ward, B. D. Roy, S. J. Peters and P. J. Leblanc (2010). "Influence of high-fat diet from differential dietary sources on bone

mineral density, bone strength, and bone fatty acid composition in rats." ***Appl Physiol Nutr Metab*** **35**(5): 598-606.

Laufer, R., C. Gilon, M. Chorev and Z. Selinger (1986). "Characterization of a neurokinin B receptor site in rat brain using a highly selective radioligand." ***J Biol Chem*** **261**(22): 10257-10263.

Le, A. D., D. Funk, W. Juzytsch, K. Coen, B. M. Navarre, C. Cifani and Y. Shaham (2011). "Effect of prazosin and guanfacine on stress-induced reinstatement of alcohol and food seeking in rats." ***Psychopharmacology (Berl)*** **218**(1): 89-99.

Lee, C. M., L. L. Iversen, M. R. Hanley and B. E. Sandberg (1982). "The possible existence of multiple receptors for substance P." ***Naunyn Schmiedebergs Arch Pharmacol*** **318**(4): 281-287.

Leger, M., A. Quiedeville, V. Bouet, B. Haelewyn, M. Boulouard, P. Schumann-Bard and T. Freret (2013). "Object recognition test in mice." ***Nat Protoc*** **8**(12): 2531-2537.

Lejeune, F., A. Gobert and M. J. Millan (2002). "The selective neurokinin (NK)(1) antagonist, GR205,171, stereospecifically enhances mesocortical dopaminergic transmission in the rat: a combined dialysis and electrophysiological study." ***Brain Res*** **935**(1-2): 134-139.

Lembeck, F. (1953). "[Central transmission of afferent impulses. III. Incidence and significance of the substance P in the dorsal roots of the spinal cord]." ***Naunyn Schmiedebergs Arch Exp Pathol Pharmakol*** **219**(3): 197-213.

Leonard, B. E., D. McCartan, J. White and D. J. King (2004). "Methylphenidate: a review of its neuropharmacological, neuropsychological and adverse clinical effects." ***Hum Psychopharmacol*** **19**(3): 151-180.

Leonard, J. (1959). "Five-choice serial reaction apparatus." ***Medical Research Council, Applied Psychology Unit Reports***.

Levy, F. (1991). "The dopamine theory of attention deficit hyperactivity disorder (ADHD)." ***Aust N Z J Psychiatry*** **25**(2): 277-283.

Li, K. C., R. F. Zernicke, R. J. Barnard and A. F. Li (1990). "Effects of a high fat-sucrose diet on cortical bone morphology and biomechanics." ***Calcif Tissue Int*** **47**(5): 308-313.

Ligneau, X., J. Lin, G. Vanni-Mercier, M. Jouvet, J. L. Muir, C. R. Ganellin, H. Stark, S. Elz, W. Schunack and J. Schwartz (1998). "Neurochemical and behavioral effects of ciproxifan, a potent histamine H3-receptor antagonist." ***J Pharmacol Exp Ther*** **287**(2): 658-666.

Lister, R. G. (1987). "The use of a plus-maze to measure anxiety in the mouse." ***Psychopharmacology (Berl)*** **92**(2): 180-185.

Liu, D., L. S. Jiang and L. Y. Dai (2007). "Substance P and its receptors in bone metabolism." ***Neuropeptides*** **41**(5): 271-283.

Liu, L. L., J. Yang, G. F. Lei, G. J. Wang, Y. W. Wang and R. P. Sun (2008). "Atomoxetine increases histamine release and improves learning deficits in an animal model of attention-deficit hyperactivity disorder: the spontaneously hypertensive rat." ***Basic Clin Pharmacol Toxicol*** **102**(6): 527-532.

Liu, Y. P., Y. L. Lin, C. H. Chuang, Y. C. Kao, S. T. Chang and C. S. Tung (2009). "Alpha adrenergic modulation on effects of norepinephrine transporter inhibitor reboxetine in five-choice serial reaction time task." ***J Biomed Sci*** **16**: 72.

Loonam, T. M., P. A. Noailles, J. Yu, J. P. Zhu and J. A. Angulo (2003). "Substance P and cholecystokinin regulate neurochemical responses to cocaine and methamphetamine in the striatum." ***Life Sci*** **73**(6): 727-739.

Loughlin, S. E., S. L. Foote and J. H. Fallon (1982). "Locus coeruleus projections to cortex: topography, morphology and collateralization." ***Brain Res Bull*** **9**(1-6): 287-294.

Ludolph, A. G., P. T. Udvardi, U. Schaz, C. Henes, O. Adolph, H. U. Weigt, J. M. Fegert, T. M. Boeckers and K. J. Fohr (2010). "Atomoxetine acts as an NMDA receptor blocker in clinically relevant concentrations." ***Br J Pharmacol*** **160**(2): 283-291.

Ludolph, A. G., V. Roessner, A. Munchau and K. Muller-Vahl (2012). "Tourette syndrome and other tic disorders in childhood, adolescence and adulthood." ***Dtsch Arztebl Int*** **109**(48): 821-288.

Luman, M., G. Tripp and A. Scheres (2010). "Identifying the neurobiology of altered reinforcement sensitivity in ADHD: a review and research agenda." ***Neurosci Biobehav Rev*** **34**(5): 744-754.

Luthman, J., A. Fredriksson, T. Lewander, G. Jonsson and T. Archer (1989). "Effects of d-amphetamine and methylphenidate on hyperactivity produced by neonatal 6-hydroxydopamine treatment." ***Psychopharmacology (Berl)*** **99**(4): 550-557.

Madras, B. K., G. M. Miller and A. J. Fischman (2005). "The dopamine transporter and attention-deficit/hyperactivity disorder." ***Biol Psychiatry*** **57**(11): 1397-1409.

Madras, B. K., Z. Xie, Z. Lin, A. Jassen, H. Panas, L. Lynch, R. Johnson, E. Livni, T. J. Spencer, A. A. Bonab, G. M. Miller and A. J. Fischman (2006). "Modafinil occupies dopamine and norepinephrine transporters in vivo and modulates the transporters and trace amine activity in vitro." ***J Pharmacol Exp Ther*** **319**(2): 561-569.

Maeno, H., H. Kiyama and M. Tohyama (1993). "Distribution of the substance P receptor (NK-1 receptor) in the central nervous system." ***Brain Res Mol Brain Res*** **18**(1-2): 43-58.

Maggi, C. A. and T. W. Schwartz (1997). "The dual nature of the tachykinin NK1 receptor." ***Trends Pharmacol Sci*** **18**(10): 351-355.

Mancini, C., M. Van Ameringen, J. M. Oakman and D. Figueiredo (1999). "Childhood attention deficit/hyperactivity disorder in adults with anxiety disorders." ***Psychol Med*** **29**(3): 515-525.

Mantyh, P. W. (2002). "Neurobiology of substance P and the NK1 receptor." ***J Clin Psychiatry*** **63 Suppl 11**: 6-10.

Marcondes, F. K., K. J. Miguel, L. L. Melo and R. C. Spadari-Bratfisch (2001). "Estrous cycle influences the response of female rats in the elevated plus-maze test." ***Physiol Behav*** **74**(4-5): 435-440.

Mao, Z. M., A. F. Arnsten and B. M. Li (1999). "Local infusion of an alpha-1 adrenergic agonist into the prefrontal cortex impairs spatial working memory performance in monkeys." ***Biol Psychiatry*** **46**(9): 1259-1265.

Marksteiner, J., G. Sperk and J. E. Krause (1992). "Distribution of neurons expressing neurokinin B in the rat brain: immunohistochemistry and in situ hybridization." ***J Comp Neurol*** **317**(4): 341-356.

Marrs, W., J. Kuperman, T. Avedian, R. H. Roth and J. D. Jentsch (2005). "Alpha-2 adrenoceptor activation inhibits phencyclidine-induced deficits of spatial working memory in rats." ***Neuropsychopharmacology*** **30**(8): 1500-1510.

Martin, A. R., A. D. Carides, J. D. Pearson, K. Horgan, M. Elmer, C. Schmidt, B. Cai, S. P. Chawla and S. M. Grunberg (2003). "Functional relevance of antiemetic control. Experience using the FLIE questionnaire in a randomised study of the NK-1 antagonist aprepitant." ***Eur J Cancer*** **39**(10): 1395-1401.

Martinussen, R., J. Hayden, S. Hogg-Johnson and R. Tannock (2005). "A meta-analysis of working memory impairments in children with attention-deficit/hyperactivity disorder." ***J Am Acad Child Adolesc Psychiatry*** **44**(4): 377-384.

Masse, F., M. Hascoet, E. Dailly and M. Bourin (2006). "Effect of noradrenergic system on the anxiolytic-like effect of DOI (5-HT2A/2C agonists) in the four-plate test." ***Psychopharmacology (Berl)*** **183**(4): 471-481.

Maubach, K. A., K. Martin, G. Chicchi, T. Harrison, A. Wheeldon, C. J. Swain, M. J. Cumberbatch, N. M. Rupniak and G. R. Seabrook (2002). "Chronic substance P (NK1) receptor antagonist and conventional antidepressant treatment increases burst firing of monoamine neurones in the locus coeruleus." ***Neuroscience*** **109**(3): 609-617.

McCormick, D. A. (1989). "Cholinergic and noradrenergic modulation of thalamocortical processing." ***Trends Neurosci*** **12**(6): 215-221.

McLean, S. (2005). "Do substance P and the NK1 receptor have a role in depression and anxiety?" ***Curr Pharm Des*** **11**(12): 1529-1547.

McNaughton, N. and J. A. Gray (2000). "Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety." ***J Affect Disord*** **61**(3): 161-176.

McQuade, R., D. Creton and S. C. Stanford (1999). "Effect of novel environmental stimuli on rat behaviour and central noradrenaline function measured by in vivo microdialysis." ***Psychopharmacology (Berl)*** **145**(4): 393-400.

Menon, P., R. Deane, A. Sagare, S. M. Lane, T. J. Zarcone, M. R. O'Dell, C. Yan, B. V. Zlokovic and B. C. Berk (2010). "Impaired spine formation and learning in GPCR kinase 2 interacting protein-1 (GIT1) knockout mice." ***Brain Res*** **1317**: 218-226.

Michaelides, M., P. K. Thanos, R. Kim, J. Cho, M. Ananth, G. J. Wang and N. D. Volkow (2012). "PET imaging predicts future body weight and cocaine preference." ***Neuroimage*** **59**(2): 1508-1513.

Miegueu, P., D. H. St-Pierre, M. Lapointe, P. Poursharifi, H. Lu, A. Gupta and K. Cianflone (2013). "Substance P decreases fat storage and increases adipocytokine production in 3T3-L1 adipocytes." ***Am J Physiol Gastrointest Liver Physiol*** **304**(4): G420-427.

Mill, J. and A. Petronis (2008). "Pre- and peri-natal environmental risks for attention-deficit hyperactivity disorder (ADHD): the potential role of epigenetic processes in mediating susceptibility." ***J Child Psychol Psychiatry*** **49**(10): 1020-1030.

Milstein, J. A., O. Lehmann, D. E. Theobald, J. W. Dalley and T. W. Robbins (2007). "Selective depletion of cortical noradrenaline by anti-dopamine beta-hydroxylase-saporin impairs attentional function and enhances the effects of guanfacine in the rat." ***Psychopharmacology (Berl)*** **190**(1): 51-63.

Mook, D. M., J. Jeffrey and A. Neuringer (1993). "Spontaneously hypertensive rats (SHR) readily learn to vary but not repeat instrumental responses." ***Behav Neural Biol*** **59**(2): 126-135.

Moore, R. Y. and F. E. Bloom (1979). "Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems." ***Annu Rev Neurosci*** **2**: 113-168.

Moran-Gates, T., K. Zhang, R. J. Baldessarini and F. I. Tarazi (2005). "Atomoxetine blocks motor hyperactivity in neonatal 6-hydroxydopamine-lesioned rats: implications for treatment of attention-deficit hyperactivity disorder." ***Int J Neuropsychopharmacol*** **8**(3): 439-444.

Morcuende, S., C. A. Gadd, M. Peters, A. Moss, E. A. Harris, A. Sheasby, A. S. Fisher, C. De Felipe, P. W. Mantyh, N. M. Rupniak, K. P. Giese and S. P. Hunt (2003). "Increased neurogenesis and brain-derived neurotrophic factor in neurokinin-1 receptor gene knockout mice." ***Eur J Neurosci*** **18**(7): 1828-1836.

Morgan, C. A., 3rd, S. M. Southwick, C. Grillon, M. Davis, J. H. Krystal and D. S. Charney (1993). "Yohimbine-facilitated acoustic startle reflex in humans." ***Psychopharmacology (Berl)*** **110**(3): 342-346.

Moron, J. A., A. Brockington, R. A. Wise, B. A. Rocha and B. T. Hope (2002). "Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines." ***J Neurosci*** **22**(2): 389-395.

Mostofsky, S. H., K. L. Cooper, W. R. Kates, M. B. Denckla and W. E. Kaufmann (2002). "Smaller prefrontal and premotor volumes in boys with attention-deficit/hyperactivity disorder." ***Biol Psychiatry*** **52**(8): 785-794.

Mozaffarian, D., T. Hao, E. B. Rimm, W. C. Willett and F. B. Hu (2011). "Changes in diet and lifestyle and long-term weight gain in women and men." ***N Engl J Med*** **364**(25): 2392-2404.

Murai, T., S. Okuda, T. Tanaka and H. Ohta (2007). "Characteristics of object location memory in mice: Behavioral and pharmacological studies." ***Physiol Behav*** **90**(1): 116-124.

Murtra, P., A. M. Sheasby, S. P. Hunt and C. De Felipe (2000). "Rewarding effects of opiates are absent in mice lacking the receptor for substance P." ***Nature*** **405**(6783): 180-183.

Myers, M. M., R. E. Musty and E. D. Hendley (1982). "Attenuation of hyperactivity in the spontaneously hypertensive rat by amphetamine." ***Behav Neural Biol*** **34**(1): 42-54.

Nagy, T. R., C. W. Prince and J. Li (2001). "Validation of peripheral dual-energy X-ray absorptiometry for the measurement of bone mineral in intact and excised long bones of rats." ***J Bone Miner Res*** **16**(9): 1682-1687.

Nakaya, Y., T. Kaneko, R. Shigemoto, S. Nakanishi and N. Mizuno (1994). "Immunohistochemical localization of substance P receptor in the central nervous system of the adult rat." ***J Comp Neurol*** **347**(2): 249-274.

Navarra, R., R. Graf, Y. Huang, S. Logue, T. Comery, Z. Hughes and M. Day (2008). "Effects of atomoxetine and methylphenidate on attention and impulsivity in the 5-choice serial reaction time test." ***Prog Neuropsychopharmacol Biol Psychiatry*** **32**(1): 34-41.

Nelson, D. L. and D. R. Gehlert (2006). "Central nervous system biogenic amine targets for control of appetite and energy expenditure." ***Endocrine*** **29**(1): 49-60.

Newcorn, J. H., M. A. Stein, A. C. Childress, S. Youcha, C. White, G. Enright and J. Rubin (2013). "Randomized, double-blind trial of guanfacine extended release in children with attention-deficit/hyperactivity disorder: morning or evening administration." ***J Am Acad Child Adolesc Psychiatry*** **52**(9): 921-930.

Nicholas, A. P., V. Pieribone and T. Hokfelt (1993). "Distributions of mRNAs for alpha-2 adrenergic receptor subtypes in rat brain: an in situ hybridization study." ***J Comp Neurol*** **328**(4): 575-594.

Niijima, F., H. Saito, S. Murai, Y. Arai, O. Nakagawasai, K. Tan-No, H. Watanabe, H. Hiraga and T. Tadano (2010). "Effects of atomoxetine on levels of monoamines and related substances in discrete brain regions in mice intermittently deprived of rapid eye movement sleep." ***Biol Pharm Bull*** **33**(4): 617-621.

Nutt, D. J. (1989). "Altered central alpha 2-adrenoceptor sensitivity in panic disorder." ***Arch Gen Psychiatry*** **46**(2): 165-169.

Nutt, D. J. (1990). "The pharmacology of human anxiety." ***Pharmacol Ther*** **47**(2): 233-266.

Nutt, D. J., K. Fone, P. Asherson, D. Bramble, P. Hill, K. Matthews, K. A. Morris, P. Santosh, E. Sonuga-Barke, E. Taylor, M. Weiss and S. Young (2007). "Evidence-based guidelines for management of attention-deficit/hyperactivity disorder in adolescents in transition to adult services and in adults: recommendations from the British Association for Psychopharmacology." ***J Psychopharmacol*** **21**(1): 10-41.

O'Keeffe, S. M., J. Thome and A. N. Coogan (2012). "The noradrenaline reuptake inhibitor atomoxetine phase-shifts the circadian clock in mice." ***Neuroscience*** **201**: 219-230.

O'Leary, T. P., R. K. Gunn and R. E. Brown (2013). "What are we measuring when we test strain differences in anxiety in mice?" ***Behav Genet*** **43**(1): 34-50.

O'Neill, J., L. J. Fitten, D. W. Siembieda, F. Ortiz and E. Halgren (2000). "Effects of guanfacine on three forms of distraction in the aging macaque." ***Life Sci*** **67**(8): 877-885.

Oades, R. D. (2007). "Role of the serotonin system in ADHD: treatment implications." ***Expert Rev Neurother*** **7**(10): 1357-1374.

Okamoto, K. and K. Aoki (1963). "Development of a strain of spontaneously hypertensive rats." ***Jpn Circ J*** **27**: 282-293.

Opolka, A., R. H. Straub, A. Pasoldt, J. Grifka and S. Grassel (2012). "Substance P and norepinephrine modulate murine chondrocyte proliferation and apoptosis." ***Arthritis Rheum*** **64**(3): 729-739.

Otsuka, M., S. Konishi and T. Takahashi (1975). "Hypothalamic substance P as a candidate for transmitter of primary afferent neurons." ***Fed Proc*** **34**(10): 1922-1928.

Otsuka, M. and K. Yoshioka (1993). "Neurotransmitter functions of mammalian tachykinins." ***Physiol Rev*** **73**(2): 229-308.

Paine, T. A., H. C. Tomasiewicz, K. Zhang and W. A. Carlezon, Jr. (2007). "Sensitivity of the five-choice serial reaction time task to the effects of various psychotropic drugs in Sprague-Dawley rats." ***Biol Psychiatry*** **62**(6): 687-693.

Parker, M. O., A. J. Brock, A. Sudwarts and C. H. Brennan (2014). "Atomoxetine reduces anticipatory responding in a 5-choice serial reaction time task for adult zebrafish." ***Psychopharmacology (Berl)*** **231**(13): 2671-2679.

Parker, M. O., D. Ife, J. Ma, M. Pancholi, F. Smeraldi, C. Straw and C. H. Brennan (2013). "Development and automation of a test of impulse control in zebrafish." ***Front Syst Neurosci*** **7**: 65.

Passetti, F., J. W. Dalley and T. W. Robbins (2003). "Double dissociation of serotonergic and dopaminergic mechanisms on attentional performance using a rodent five-choice reaction time task." ***Psychopharmacology (Berl)*** **165**(2): 136-145.

Pastor, P. N. and C. A. Reuben (2008). "Diagnosed attention deficit hyperactivity disorder and learning disability: United States, 2004-2006." ***Vital Health Stat 10***(237): 1-14.

Paterson, N. E., J. Ricciardi, C. Wetzler and T. Hanania (2011). "Sub-optimal performance in the 5-choice serial reaction time task in rats was sensitive to methylphenidate, atomoxetine and d-amphetamine, but unaffected by the COMT inhibitor tolcapone." ***Neurosci Res*** **69**(1): 41-50.

Pellow, S., P. Chopin and S. E. File (1985). "Are the anxiogenic effects of yohimbine mediated by its action at benzodiazepine receptors?" ***Neurosci Lett*** **55**(1): 5-9.

Pellow, S., P. Chopin, S. E. File and M. Briley (1985). "Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat." ***J Neurosci Methods*** **14**(3): 149-167.

Pliszka, S. R. (2005). "The neuropsychopharmacology of attention-deficit/hyperactivity disorder." ***Biol Psychiatry*** **57**(11): 1385-1390.

Polanczyk, G., M. S. de Lima, B. L. Horta, J. Biederman and L. A. Rohde (2007). "The worldwide prevalence of ADHD: a systematic review and metaregression analysis." ***Am J Psychiatry*** **164**(6): 942-948.

Porter, A.J., K. Pillidge, Y.C. Tsai, J.A. Dudley, S.P. Hunt, S.N. Peirson, L.A. Brown and S.C. Stanford (2015). "A lack of functional NK1 receptors explains most, but not all, abnormal behaviors of NK1R-/- mice. " ***Genes Brain Behav*** (accepted) DOI:10.1111/gbb.12195.

Porter, A. J., K. Pillidge, E. M. Grabowska and S. C. Stanford (2015). "The angiotensin converting enzyme inhibitor, captopril, prevents the hyperactivity and impulsivity of neurokinin-1 receptor gene 'knockout' mice: Sex differences and implications for the treatment of attention deficit hyperactivity disorder." ***Eur Neuropsychopharmacol*** **25**(4): 512-521.

Postle, B. R. (2006). "Distraction-spanning sustained activity during delayed recognition of locations." ***Neuroimage*** **30**(3): 950-962.

Poulton, A., J. Briody, T. McCorquodale, E. Melzer, M. Herrmann, L. A. Baur and G. Duque (2012). "Weight loss on stimulant medication: how does it affect body composition and bone metabolism? - A prospective longitudinal study." ***Int J Pediatr Endocrinol*** **2012**(1): 30.

Preti, A. (2002). "Tomoxetine (Eli Lilly & Co)." ***Curr Opin Investig Drugs*** **3**(2): 272-277.

Pringsheim, T. and T. Steeves (2011). "Pharmacological treatment for Attention Deficit Hyperactivity Disorder (ADHD) in children with comorbid tic disorders." ***Cochrane Database Syst Rev***(4): CD007990.

Quartara, L., M. Altamura, S. Evangelista and C. A. Maggi (2009). "Tachykinin receptor antagonists in clinical trials." ***Expert Opin Investig Drugs*** **18**(12): 1843-1864.

Quartara, L. and C. A. Maggi (1997). "The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation." ***Neuropeptides*** **31**(6): 537-563.

Rago, L., E. MacDonald, V. Saano and M. M. Airaksinen (1991). "The effect of medetomidine on GABA and benzodiazepine receptors in vivo: lack of anxiolytic but some evidence of possible stress-protective activity." ***Pharmacol Toxicol*** **69**(2): 81-86.

Ralph-Williams, R. J., M. P. Paulus, X. Zhuang, R. Hen and M. A. Geyer (2003). "Valproate attenuates hyperactive and perseverative behaviors in mutant mice with a dysregulated dopamine system." ***Biol Psychiatry*** **53**(4): 352-359.

Ratti, E., K. Bellew, P. Bettica, H. Bryson, S. Zamuner, G. Archer, L. Squassante, A. Bye, D. Trist, K. R. Krishnan and S. Fernandes (2011). "Results from 2 randomized, double-blind, placebo-controlled studies of the novel NK1 receptor antagonist casopitant in patients with major depressive disorder." ***J Clin Psychopharmacol*** **31**(6): 727-733.

Redmond, D. E., Jr. and Y. H. Huang (1979). "Current concepts. II. New evidence for a locus coeruleus-norepinephrine connection with anxiety." ***Life Sci*** **25**(26): 2149-2162.

Redmond, D. E., Jr., Y. H. Huang, D. R. Snyder and J. W. Maas (1976). "Behavioral effects of stimulation of the nucleus locus coeruleus in the stump-tailed monkey Macaca arctoides." ***Brain Res*** **116**(3): 502-510.

Renoldi, G. and R. W. Invernizzi (2006). "Blockade of tachykinin NK1 receptors attenuates stress-induced rise of extracellular noradrenaline and dopamine in the rat and gerbil medial prefrontal cortex." ***J Neurosci Res*** **84**(5): 961-968.

Rhodes, J. S. and T. Garland (2003). "Differential sensitivity to acute administration of Ritalin, apomorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior." ***Psychopharmacology (Berl)*** **167**(3): 242-250.

Ripley, T. L., C. A. Gadd, C. De Felipe, S. P. Hunt and D. N. Stephens (2002). "Lack of self-administration and behavioural sensitisation to morphine, but not cocaine, in mice lacking NK1 receptors." ***Neuropharmacology*** **43**(8): 1258-1268.

Robbins, T. W. (2002). "The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry." ***Psychopharmacology (Berl)*** **163**(3-4): 362-380.

Robbins, T. W. and A. F. Arnsten (2009). "The neuropsychopharmacology of fronto-executive function: monoaminergic modulation." ***Annu Rev Neurosci*** **32**: 267-287.

Robbins, T. W. and A. C. Roberts (2007). "Differential regulation of fronto-executive function by the monoamines and acetylcholine." ***Cereb Cortex*** **17 Suppl 1**: i151-160.

Robinson, E. S. (2012). "Blockade of noradrenaline re-uptake sites improves accuracy and impulse control in rats performing a five-choice serial reaction time tasks." ***Psychopharmacology (Berl)*** **219**(2): 303-312.

Robinson, E. S., D. M. Eagle, A. C. Mar, A. Bari, G. Banerjee, X. Jiang, J. W. Dalley and T. W. Robbins (2008). "Similar effects of the selective noradrenaline reuptake inhibitor atomoxetine on three distinct forms of impulsivity in the rat." ***Neuropsychopharmacology*** **33**(5): 1028-1037.

Roche, M., D. M. Kerr, S. P. Hunt and J. P. Kelly (2012). "Neurokinin-1 receptor deletion modulates behavioural and neurochemical alterations in an animal model of depression." ***Behav Brain Res*** **228**(1): 91-98.

Rodgers, R. J. and J. C. Cole (1993). "Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice." ***Physiol Behav*** **54**(4): 729-736.

Rodgers, R. J. and N. J. Johnson (1995). "Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety." ***Pharmacol Biochem Behav*** **52**(2): 297-303.

Rodgers, R. J. and A. Dalvi (1997). "Anxiety, defence and the elevated plus-maze." ***Neurosci Biobehav Rev*** **21**(6): 801-810.

Rodgers, R. J., C. Gentsch, D. Hoyer, E. Bryant, A. J. Green, K. Z. Kolokotroni and J. L. Martin (2004). "The NK1 receptor antagonist NKP608 lacks anxiolytic-like activity in Swiss-Webster mice exposed to the elevated plus-maze." ***Behav Brain Res*** **154**(1): 183-192.

Rosvold, H. E., A. F. Mirsky, I. Sarason, L. H. Beck and E. D. Bransome, Jr. (1956). "A continuous performance test of brain damage." ***J Consult Psychol*** **20**(5): 343-350.

Roush, E. D. and M. M. Kwatra (1998). "Human substance P receptor expressed in Chinese hamster ovary cells directly activates G(alpha q/11), G(alpha s), G(alpha o)." ***FEBS Lett*** **428**(3): 291-294.

Rudnick, G. and J. Clark (1993). "From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters." ***Biochim Biophys Acta*** **1144**(3): 249-263.

Ruffolo, R. R., Jr. and J. P. Hieble (1994). "Alpha-adrenoceptors." ***Pharmacol Ther*** **61**(1-2): 1-64.

Ruocco, L. A., U. A. Carnevale, C. Treno, A. G. Sadile, D. Melisi, C. Arra, M. Ibba, C. Schirru and E. Carboni (2010). "Prepuberal subchronic methylphenidate and atomoxetine induce

different long-term effects on adult behaviour and forebrain dopamine, norepinephrine and serotonin in Naples high-excitability rats." ***Behav Brain Res*** **210**(1): 99-106.

Rupniak, N. M., E. J. Carlson, J. K. Webb, T. Harrison, R. D. Porsolt, S. Roux, C. de Felipe, S. P. Hunt, B. Oates and A. Wheeldon (2001). "Comparison of the phenotype of NK1R-/- mice with pharmacological blockade of the substance P (NK1) receptor in assays for antidepressant and anxiolytic drugs." ***Behav Pharmacol*** **12**(6-7): 497-508.

Rupniak, N. M. (2003). "Animal models of depression: challenges from a drug development perspective." ***Behav Pharmacol*** **14**(5-6): 385-390.

Rupniak, N. M., E. C. Carlson, T. Harrison, B. Oates, E. Seward, S. Owen, C. de Felipe, S. Hunt and A. Wheeldon (2000). "Pharmacological blockade or genetic deletion of substance P (NK(1)) receptors attenuates neonatal vocalisation in guinea-pigs and mice." ***Neuropharmacology*** **39**(8): 1413-1421.

Rupniak, N. M., J. K. Webb, A. Fisher, D. Smith and S. Boyce (2003). "The substance P (NK1) receptor antagonist L-760735 inhibits fear conditioning in gerbils." ***Neuropharmacology*** **44**(4): 516-523.

Sacchetti, G., M. Bernini, A. Bianchetti, S. Parini, R. W. Invernizzi and R. Samanin (1999). "Studies on the acute and chronic effects of reboxetine on extracellular noradrenaline and other monoamines in the rat brain." ***Br J Pharmacol*** **128**(6): 1332-1338.

Sadile, A. G., C. Lamberti, B. Siegfried and H. Welzl (1993). "Circadian activity, nociceptive thresholds, nigrostriatal and mesolimbic dopaminergic activity in the Naples High- and Low-Excitability rat lines." ***Behav Brain Res*** **55**(1): 17-27.

Sagvolden, T. (2000). "Behavioral validation of the spontaneously hypertensive rat (SHR) as an animal model of attention-deficit/hyperactivity disorder (AD/HD)." ***Neurosci Biobehav Rev*** **24**(1): 31-39.

Sagvolden, T. (2006). "The alpha-2A adrenoceptor agonist guanfacine improves sustained attention and reduces overactivity and impulsiveness in an animal model of Attention-Deficit/Hyperactivity Disorder (ADHD)." ***Behav Brain Funct*** **2**: 41.

Sagvolden, T., E. B. Johansen, G. Woien, S. I. Walaas, J. Storm-Mathisen, L. H. Bergersen, O. Hvalby, V. Jensen, H. Aase, V. A. Russell, P. R. Killeen, T. Dasbanerjee, F. A. Middleton and S. V. Faraone (2009). "The spontaneously hypertensive rat model of ADHD--the importance of selecting the appropriate reference strain." ***Neuropharmacology*** **57**(7-8): 619-626.

de Saint Hilaire, Z., M. Orosco, C. Rouch, G. Blanc and S. Nicolaidis (2001). "Variations in extracellular monoamines in the prefrontal cortex and medial hypothalamus after modafinil administration: a microdialysis study in rats." ***Neuroreport*** **12**(16): 3533-3537.

Sallee, F. R., S. H. Kollins and T. L. Wigal (2012). "Efficacy of guanfacine extended release in the treatment of combined and inattentive only subtypes of attention-deficit/hyperactivity disorder." ***J Child Adolesc Psychopharmacol*** **22**(3): 206-214.

Sallee, F. R., A. Lyne, T. Wigal and J. J. McGough (2009). "Long-term safety and efficacy of guanfacine extended release in children and adolescents with attention-deficit/hyperactivity disorder." ***J Child Adolesc Psychopharmacol*** **19**(3): 215-226.

Sallinen, J., A. Haapalinna, T. Viitamaa, B. K. Kobilka and M. Scheinin (1998). "D-amphetamine and L-5-hydroxytryptophan-induced behaviours in mice with genetically-altered expression of the alpha2C-adrenergic receptor subtype." ***Neuroscience*** **86**(3): 959-965.

Sandau, U. S., Z. Alderman, G. Corfas, S. R. Ojeda and J. Raber (2012). "Astrocyte-specific disruption of SynCAM1 signaling results in ADHD-like behavioral manifestations." ***PLoS One*** **7**(4): e36424.

Santarelli, L., G. Gobbi, P. C. Debs, E. T. Sibille, P. Blier, R. Hen and M. J. Heath (2001). "Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function." ***Proc Natl Acad Sci U S A*** **98**(4): 1912-1917.

Sara, S. J. (2009). "The locus coeruleus and noradrenergic modulation of cognition." ***Nat Rev Neurosci*** **10**(3): 211-223.

Sara, S. J. and M. Segal (1991). "Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: implications for cognition." ***Prog Brain Res*** **88**: 571-585.

Sartori, S. B., R. Landgraf and N. Singewald (2011). "The clinical implications of mouse models of enhanced anxiety." ***Future Neurol*** **6**(4): 531-571.

Saudou, F., D. A. Amara, A. Dierich, M. LeMeur, S. Ramboz, L. Segu, M. C. Buhot and R. Hen (1994). "Enhanced aggressive behavior in mice lacking 5-HT1B receptor." ***Science*** **265**(5180): 1875-1878.

Saunders, C., J. V. Ferrer, L. Shi, J. Chen, G. Merrill, M. E. Lamb, L. M. Leeb-Lundberg, L. Carvelli, J. A. Javitch and A. Galli (2000). "Amphetamine-induced loss of human dopamine transporter activity: an internalization-dependent and cocaine-sensitive mechanism." ***Proc Natl Acad Sci U S A*** **97**(12): 6850-6855.

Saunders, C. and L. E. Limbird (1999). "Localization and trafficking of alpha2-adrenergic receptor subtypes in cells and tissues." ***Pharmacol Ther*** **84**(2): 193-205.

Schank, J. R. (2014). "The neurokinin-1 receptor in addictive processes." ***J Pharmacol Exp Ther*** **351**(1): 2-8.

Schatzberg, A. and C. Nemeroff (2009). The American Psychiatric Publishing Textbook of Psychopharmacology, Fourth Edition, American Psychiatric Publishing.

Scheinin, H., R. Virtanen, E. MacDonald, R. Lammintausta and M. Scheinin (1989). "Medetomidine--a novel alpha 2-adrenoceptor agonist: a review of its pharmacodynamic effects." ***Prog Neuropsychopharmacol Biol Psychiatry*** **13**(5): 635-651.

Scheinin, M., J. W. Lomasney, D. M. Hayden-Hixson, U. B. Schambra, M. G. Caron, R. J. Lefkowitz and R. T. Fremeau, Jr. (1994). "Distribution of alpha 2-adrenergic receptor subtype gene expression in rat brain." ***Brain Res Mol Brain Res*** **21**(1-2): 133-149.

Schneider, E. H., D. Neumann and R. Seifert (2014). "Modulation of behavior by the histaminergic system: lessons from H(1)R-and H(2)R-deficient mice." ***Neurosci Biobehav Rev*** **42**: 252-266.

Schroeter, S., S. Apparsundaram, R. G. Wiley, L. H. Miner, S. R. Sesack and R. D. Blakely (2000). "Immunolocalization of the cocaine- and antidepressant-sensitive l-norepinephrine transporter." ***J Comp Neurol*** **420**(2): 211-232.

Seneviratne, C., N. Ait-Daoud, J. Z. Ma, G. Chen, B. A. Johnson and M. D. Li (2009). "Susceptibility locus in neurokinin-1 receptor gene associated with alcohol dependence." ***Neuropsychopharmacology*** **34**(11): 2442-2449.

Sepede, G., M. Corbo, F. Fiori and G. Martinotti (2012). "Reboxetine in clinical practice: a review." ***Clin Ter*** **163**(4): e255-262.

Sesack, S. R., V. A. Hawrylak, M. A. Guido and A. I. Levey (1998). "Cellular and subcellular localization of the dopamine transporter in rat cortex." ***Adv Pharmacol*** **42**: 171-174.

Sharp, S. I., A. McQuillin and H. M. Gurling (2009). "Genetics of attention-deficit hyperactivity disorder (ADHD)." ***Neuropharmacology*** **57**(7-8): 590-600.

Sharp, S. I., A. McQuillin, M. Marks, S. P. Hunt, S. C. Stanford, G. J. Lydall, M. Y. Morgan, P. Asherson, D. Curtis and H. M. Gurling (2014). "Genetic association of the tachykinin receptor 1 TACR1 gene in bipolar disorder, attention deficit hyperactivity disorder, and the alcohol dependence syndrome." ***Am J Med Genet B Neuropsychiatr Genet*** **165B**(4): 373-380.

Shaywitz, B. A., J. H. Klopper and J. W. Gordon (1978). "Methylphenidate in 6-hydroxydopamine-treated developing rat pups. Effects on activity and maze performance." ***Arch Neurol*** **35**(7): 463-469.

Simon, V., P. Czobor, S. Balint, A. Meszaros and I. Bitter (2009). "Prevalence and correlates of adult attention-deficit hyperactivity disorder: meta-analysis." ***Br J Psychiatry*** **194**(3): 204-211.

Sirvio, J., M. Mazurkiewicz, A. Haapalinna, P. Riekkinen, Jr., H. Lahtinen and P. J. Riekkinen, Sr. (1994). "The effects of selective alpha-2 adrenergic agents on the performance of rats in a 5-choice serial reaction time task." ***Brain Res Bull*** **35**(5-6): 451-455.

Sirvio, J., P. Riekkinen, Jr., T. Ekonsalo, R. Lammintausta and P. J. Riekkinen (1992). "The effects of dexmedetomidine, an alpha 2 agonist, on learning and memory, assessed using passive avoidance and water maze tasks in rats." ***Neuropharmacology*** **31**(2): 163-168.

Sobanski, E. (2006). "Psychiatric comorbidity in adults with attention-deficit/hyperactivity disorder (ADHD)." ***Eur Arch Psychiatry Clin Neurosci*** **256 Suppl 1**: i26-31.

Sontag, T. A., O. Tucha, S. Walitza and K. W. Lange (2010). "Animal models of attention deficit/hyperactivity disorder (ADHD): a critical review." ***Atten Defic Hyperact Disord*** **2**(1): 1-20.

Sontag, T. A., A. B. Fuermaier, J. Hauser, I. Kaunzinger, O. Tucha and K. W. Lange (2013). "Spatial memory in spontaneously hypertensive rats (SHR)." ***PLoS One*** **8**(8): e74660.

Sonuga-Barke, E. J. (2002). "Psychological heterogeneity in AD/HD--a dual pathway model of behaviour and cognition." ***Behav Brain Res*** **130**(1-2): 29-36.

Sonuga-Barke, E. J. (2005). "Causal models of attention-deficit/hyperactivity disorder: from common simple deficits to multiple developmental pathways." ***Biol Psychiatry*** **57**(11): 1231-1238.

Sorkina, T., S. Doolen, E. Galperin, N. R. Zahniser and A. Sorkin (2003). "Oligomerization of dopamine transporters visualized in living cells by fluorescence resonance energy transfer microscopy." ***J Biol Chem*** **278**(30): 28274-28283.

Sowell, E. R., P. M. Thompson, S. E. Welcome, A. L. Henkenius, A. W. Toga and B. S. Peterson (2003). "Cortical abnormalities in children and adolescents with attention-deficit hyperactivity disorder." ***Lancet*** **362**(9397): 1699-1707.

Spencer, T., J. Biederman, T. Wilens, R. Doyle, C. Surman, J. Prince, E. Mick, M. Aleardi, K. Herzig and S. Faraone (2005). "A large, double-blind, randomized clinical trial of methylphenidate in the treatment of adults with attention-deficit/hyperactivity disorder." ***Biol Psychiatry*** **57**(5): 456-463.

Stanford, S. C. (2007). "The Open Field Test: reinventing the wheel." ***J Psychopharmacol*** **21**(2): 134-135.

Stanford, S. C. (2007). "Open fields (unlike wheels) can be any shape but still miss the target." ***J Psychopharmacol*** **21**(2): 144.

Steinberg, R., R. Alonso, L. Rouquier, C. Desvignes, J. C. Michaud, A. Cudennec, M. Jung, J. Simiand, G. Griebel, X. Emonds-Alt, G. Le Fur and P. Soubrie (2002). "SSR240600 [(R)-2-(1-[2-[4-[2-[3,5-bis(trifluoromethyl)phenyl]acetyl]-2-(3,4-dichlorophenyl) -2-morpholinyl]ethyl]-4-piperidinyl)-2-methylpropanamide], a centrally active nonpeptide antagonist of the tachykinin neurokinin 1 receptor: II. Neurochemical and behavioral characterization." ***J Pharmacol Exp Ther*** **303**(3): 1180-1188.

Sulzer, D., T. K. Chen, Y. Y. Lau, H. Kristensen, S. Rayport and A. Ewing (1995). "Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport." ***J Neurosci*** **15**(5 Pt 2): 4102-4108.

Sulzer, D. and R. Edwards (2000). "Vesicles: equal in neurotransmitter concentration but not in volume." ***Neuron*** **28**(1): 5-7.

Sulzer, D., E. Pothos, H. M. Sung, N. T. Maidment, B. G. Hoebel and S. Rayport (1992). "Weak base model of amphetamine action." ***Ann N Y Acad Sci*** **654**: 525-528.

Sulzer, D., M. S. Sonders, N. W. Poulsen and A. Galli (2005). "Mechanisms of neurotransmitter release by amphetamines: a review." ***Prog Neurobiol*** **75**(6): 406-433.

Sun, H., P. J. Cocker, F. D. Zeeb and C. A. Winstanley (2012). "Chronic atomoxetine treatment during adolescence decreases impulsive choice, but not impulsive action, in adult rats and alters markers of synaptic plasticity in the orbitofrontal cortex." ***Psychopharmacology (Berl)*** **219**(2): 285-301.

Suplicy, H., C. L. Boguszewski, C. M. dos Santos, M. do Desterro de Figueiredo, D. R. Cunha and R. Radominski (2014). "A comparative study of five centrally acting drugs on the pharmacological treatment of obesity." ***Int J Obes (Lond)*** **38**(8): 1097-1103.

Swanson, J. M., P. Flodman, J. Kennedy, M. A. Spence, R. Moyzis, S. Schuck, M. Murias, J. Moriarity, C. Barr, M. Smith and M. Posner (2000). "Dopamine genes and ADHD." ***Neurosci Biobehav Rev*** **24**(1): 21-25.

Swanson, C. J., K. W. Perry, S. Koch-Krueger, J. Katner, K. A. Svensson and F. P. Bymaster (2006). "Effect of the attention deficit/hyperactivity disorder drug atomoxetine on extracellular concentrations of norepinephrine and dopamine in several brain regions of the rat." ***Neuropharmacology*** **50**(6): 755-760.

Swanson, J. M., G. A. Sunohara, J. L. Kennedy, R. Regino, E. Fineberg, T. Wigal, M. Lerner, L. Williams, G. J. LaHoste and S. Wigal (1998). "Association of the dopamine receptor D4 (DRD4) gene with a refined phenotype of attention deficit hyperactivity disorder (ADHD): a family-based approach." ***Mol Psychiatry*** **3**(1): 38-41.

Szot, P., M. Lester, M. L. Laughlin, R. D. Palmiter, L. C. Liles and D. Weinshenker (2004). "The anticonvulsant and proconvulsant effects of alpha2-adrenoreceptor agonists are mediated by distinct populations of alpha2A-adrenoreceptors." ***Neuroscience*** **126**(3): 795-803.

Takamatsu, I., A. Iwase, M. Ozaki, T. Kazama, K. Wada and M. Sekiguchi (2008). "Dexmedetomidine reduces long-term potentiation in mouse hippocampus." ***Anesthesiology*** **108**(1): 94-102.

Tamburella, A., V. Micale, C. Mazzola, S. Salomone and F. Drago (2012). "The selective norepinephrine reuptake inhibitor atomoxetine counteracts behavioral impairments in trimethyltin-intoxicated rats." ***Eur J Pharmacol***.

Tanila, H., K. Mustonen, J. Sallinen, M. Scheinin and P. Riekkinen, Jr. (1999). "Role of alpha2C-adrenoceptor subtype in spatial working memory as revealed by mice with targeted disruption of the alpha2C-adrenoceptor gene." ***Eur J Neurosci*** **11**(2): 599-603.

Teixeira, R. M., A. R. Santos, S. J. Ribeiro, J. B. Calixto, G. A. Rae and T. C. De Lima (1996). "Effects of central administration of tachykinin receptor agonists and antagonists on plus-maze behavior in mice." ***Eur J Pharmacol*** **311**(1): 7-14.

Thanos, P. K., M. Michaelides, Y. K. Piyis, G. J. Wang and N. D. Volkow (2008). "Food restriction markedly increases dopamine D2 receptor (D2R) in a rat model of obesity as assessed with in-vivo muPET imaging ([11C] raclopride) and in-vitro ([3H] spiperone) autoradiography." ***Synapse*** **62**(1): 50-61.

Thorsell, A., J. R. Schank, E. Singley, S. P. Hunt and M. Heilig (2010). "Neurokinin-1 receptors (NK1R:s), alcohol consumption, and alcohol reward in mice." ***Psychopharmacology (Berl)*** **209**(1): 103-111.

Tomasi, D., N. D. Volkow, G. J. Wang, R. Wang, F. Telang, E. C. Caparelli, C. Wong, M. Jayne and J. S. Fowler (2011). "Methylphenidate enhances brain activation and deactivation responses to visual attention and working memory tasks in healthy controls." ***Neuroimage*** **54**(4): 3101-3110.

Tomlinson, A., B. Grayson, S. Marsh, M. K. Harte, S. A. Barnes, K. M. Marshall and J. C. Neill (2014). "Pay attention to impulsivity: Modelling low attentive and high impulsive subtypes of adult ADHD in the 5-choice continuous performance task (5C-CPT) in female rats." ***Eur Neuropsychopharmacol***.

Tomlinson, A., B. Grayson, S. Marsh, M. K. Harte, S. A. Barnes, K. M. Marshall and J. C. Neill (2014). "Pay attention to impulsivity: Modelling low attentive and high impulsive subtypes of adult ADHD in the 5-choice continuous performance task (5C-CPT) in female rats." ***Eur Neuropsychopharmacol*** **24**(8): 1371-1380.

Tsuchida, R., M. Kubo, M. Kuroda, Y. Shibasaki, N. Shintani, M. Abe, K. Koves, H. Hashimoto and A. Baba (2009). "An antihyperkinetic action by the serotonin 1A-receptor agonist osemozotan co-administered with psychostimulants or the non-stimulant atomoxetine in mice." ***J Pharmacol Sci*** **109**(3): 396-402.

Uhde, T. W., M. B. Stein, B. J. Vittone, L. J. Siever, J. P. Boulenger, E. Klein and T. A. Mellman (1989). "Behavioral and physiologic effects of short-term and long-term administration of clonidine in panic disorder." ***Arch Gen Psychiatry*** **46**(2): 170-177.

Uhlen, S. and J. E. Wikberg (1991). "Delineation of rat kidney alpha 2A- and alpha 2B-adrenoceptors with [3H]RX821002 radioligand binding: computer modelling reveals that guanfacine is an alpha 2A-selective compound." ***Eur J Pharmacol*** **202**(2): 235-243.

Uhlen, S., Y. Xia, V. Chhajlani, C. C. Felder and J. E. Wikberg (1992). "[3H]-MK 912 binding delineates two alpha 2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d alpha 2-adrenoceptor." ***Br J Pharmacol*** **106**(4): 986-995.

Umehara, M., Y. Ago, T. Kawanai, K. Fujita, N. Hiramatsu, K. Takuma and T. Matsuda (2013). "Methylphenidate and venlafaxine attenuate locomotion in spontaneously hypertensive rats, an animal model of attention-deficit/hyperactivity disorder, through alpha2-adrenoceptor activation." ***Behav Pharmacol*** **24**(4): 328-331.

Uzsoki, B., M. Toth and I. Hernadi (2011). "Novelty response of rats determines the effect of prefrontal alpha-2 adrenoceptor modulation on anxiety." ***Neurosci Lett*** **499**(3): 219-223.

van den Bergh, F. S., E. Bloemarts, J. S. Chan, L. Groenink, B. Olivier and R. S. Oosting (2006). "Spontaneously hypertensive rats do not predict symptoms of attention-deficit hyperactivity disorder." ***Pharmacol Biochem Behav*** **83**(3): 380-390.

van der Kooij, M. A. and J. C. Glennon (2007). "Animal models concerning the role of dopamine in attention-deficit hyperactivity disorder." ***Neurosci Biobehav Rev*** **31**(4): 597-618.

Van der Laan, J. W., W. Van Veenendaal, P. Voorthuis, G. Weick and F. C. Hillen (1985). "The effects of centrally acting adrenergic agonists on temperature and on explorative and motor behaviour. Relation with effects on quasi-morphine withdrawal behaviour." ***Eur J Pharmacol*** **107**(3): 367-373.

van Egmond-Frohlich, A. W., K. Widhalm and M. de Zwaan (2012). "Association of symptoms of attention-deficit/hyperactivity disorder with childhood overweight adjusted for confounding parental variables." ***Int J Obes (Lond)*** **36**(7): 963-968.

van Gaalen, M. M., R. van Koten, A. N. Schoffelmeer and L. J. Vanderschuren (2006). "Critical involvement of dopaminergic neurotransmission in impulsive decision making." ***Biol Psychiatry*** **60**(1): 66-73.

Vance, A., M. Ferrin, J. Winther and R. Gomez (2013). "Examination of spatial working memory performance in children and adolescents with attention deficit hyperactivity disorder, combined type (ADHD-CT) and anxiety." ***J Abnorm Child Psychol*** **41**(6): 891-900.

Varty, G. B., M. E. Cohen-Williams, C. A. Morgan, U. Pylak, R. A. Duffy, J. E. Lachowicz, G. J. Carey and V. L. Coffin (2002). "The gerbil elevated plus-maze II: anxiolytic-like effects of selective neurokinin NK1 receptor antagonists." ***Neuropsychopharmacology*** **27**(3): 371-379.

Vassout, A., S. Veenstra, K. Hauser, S. Ofner, F. Brugger, W. Schilling and C. Gentsch (2000). "NKP608: a selective NK-1 receptor antagonist with anxiolytic-like effects in the social interaction and social exploration test in rats." ***Regul Pept*** **96**(1-2): 7-16.

Verbeeck, W., S. Tuinier and G. E. Bekkering (2009). "Antidepressants in the treatment of adult attention-deficit hyperactivity disorder: a systematic review." ***Adv Ther*** **26**(2): 170-184.

Verleye, M. and F. Bernet (1983). "Behavioral effects of lesions of the central noradrenergic bundles in the rat." ***Pharmacol Biochem Behav*** **19**(3): 407-414.

Von Euler, U. S. and J. H. Gaddum (1931). "An unidentified depressor substance in certain tissue extracts." ***J Physiol*** **72**(1): 74-87.

Votava, M., L. Hess, J. Sliva, M. Krsiak and V. Agova (2005). "Dexmedetomidine selectively suppresses dominant behaviour in aggressive and sociable mice." ***Eur J Pharmacol*** **523**(1-3): 79-85.

Wang, G. J., N. D. Volkow, J. Logan, N. R. Pappas, C. T. Wong, W. Zhu, N. Netusil and J. S. Fowler (2001). "Brain dopamine and obesity." ***Lancet*** **357**(9253): 354-357.

Wang, G. J., N. D. Volkow, P. K. Thanos and J. S. Fowler (2004). "Similarity between obesity and drug addiction as assessed by neurofunctional imaging: a concept review." ***J Addict Dis*** **23**(3): 39-53.

Wang, R., L. B. Macmillan, R. T. Fremeau, Jr., M. A. Magnuson, J. Lindner and L. E. Limbird (1996). "Expression of alpha 2-adrenergic receptor subtypes in the mouse brain: evaluation of spatial and temporal information imparted by 3 kb of 5' regulatory

sequence for the alpha 2A AR-receptor gene in transgenic animals." ***Neuroscience*** **74**(1): 199-218.

Ward, A. S., S. D. Comer, M. Haney, M. W. Fischman and R. W. Foltin (1999). "Fluoxetine-maintained obese humans: effect on food intake and body weight." ***Physiol Behav*** **66**(5): 815-821.

Ward, W. E., S. Kim and W. Robert Bruce (2003). "A western-style diet reduces bone mass and biomechanical bone strength to a greater extent in male compared with female rats during development." ***Br J Nutr*** **90**(3): 589-595.

Waring, M. E. and K. L. Lapane (2008). "Overweight in children and adolescents in relation to attention-deficit/hyperactivity disorder: results from a national sample." ***Pediatrics*** **122**(1): e1-6.

Weir, R., J. Dudley, T. Yan, E. Grabowska, Y. Pena-Oliver, T. Ripley, D. Stephens, S. Stanford and S. Hunt (2014). "The influence of test experience and NK1 receptor antagonists on the performance of NK1R-/- and wild type mice in the 5-Choice Serial Reaction-Time Task." ***J Psychopharmacol***.

Wender, P. H., L. E. Wolf and J. Wasserstein (2001). "Adults with ADHD. An overview." ***Ann N Y Acad Sci*** **931**: 1-16.

Wilcock, J. and P. L. Broadhurst (1967). "Strain differences in emotionality: open-field and conditioned avoidance behavior in the rat." ***J Comp Physiol Psychol*** **63**(2): 335-338.

Wilens, T. E., L. A. Adler, J. Adams, S. Sgambati, J. Rotrosen, R. Sawtelle, L. Utzinger and S. Fusillo (2008). "Misuse and diversion of stimulants prescribed for ADHD: a systematic review of the literature." ***J Am Acad Child Adolesc Psychiatry*** **47**(1): 21-31.

Wilens, T. E., J. Biederman, J. Prince, T. J. Spencer, S. V. Faraone, R. Warburton, D. Schleifer, M. Harding, C. Linehan and D. Geller (1996). "Six-week, double-blind, placebo-controlled study of desipramine for adult attention deficit hyperactivity disorder." ***Am J Psychiatry*** **153**(9): 1147-1153.

Wilens, T. E., O. Bukstein, M. Brams, A. J. Cutler, A. Childress, T. Rugino, A. Lyne, K. Grannis and S. Youcha (2012). "A controlled trial of extended-release guanfacine and psychostimulants for attention-deficit/hyperactivity disorder." ***J Am Acad Child Adolesc Psychiatry*** **51**(1): 74-85 e72.

Wilens, T. E., S. V. Faraone, J. Biederman and S. Gunawardene (2003). "Does stimulant therapy of attention-deficit/hyperactivity disorder beget later substance abuse? A meta-analytic review of the literature." ***Pediatrics*** **111**(1): 179-185.

Wilens, T. E., J. H. Newcorn, C. J. Kratochvil, H. Gao, C. K. Thomason, A. K. Rogers, P. D. Feldman and L. R. Levine (2006). "Long-term atomoxetine treatment in adolescents with attention-deficit/hyperactivity disorder." ***J Pediatr*** **149**(1): 112-119.

Willcutt, E. G. (2012). "The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review." ***Neurotherapeutics*** **9**(3): 490-499.

Williams, G., C. Bing, X. J. Cai, J. A. Harrold, P. J. King and X. H. Liu (2001). "The hypothalamus and the control of energy homeostasis: different circuits, different purposes." ***Physiol Behav*** **74**(4-5): 683-701.

Winstanley, C. A., Y. Chudasama, J. W. Dalley, D. E. Theobald, J. C. Glennon and T. W. Robbins (2003). "Intra-prefrontal 8-OH-DPAT and M100907 improve visuospatial attention and decrease impulsivity on the five-choice serial reaction time task in rats." ***Psychopharmacology (Berl)*** **167**(3): 304-314.

Winstanley, C. A., J. W. Dalley, D. E. Theobald and T. W. Robbins (2003). "Global 5-HT depletion attenuates the ability of amphetamine to decrease impulsive choice on a delay-discounting task in rats." ***Psychopharmacology (Berl)*** **170**(3): 320-331.

Winstanley, C. A., J. W. Dalley, D. E. Theobald and T. W. Robbins (2004). "Fractionating impulsivity: contrasting effects of central 5-HT depletion on different measures of impulsive behavior." ***Neuropsychopharmacology*** **29**(7): 1331-1343.

Winstanley, C. A., D. E. Theobald, J. W. Dalley and T. W. Robbins (2005). "Interactions between serotonin and dopamine in the control of impulsive choice in rats: therapeutic implications for impulse control disorders." ***Neuropsychopharmacology*** **30**(4): 669-682.

Won, H., W. Mah, E. Kim, J. W. Kim, E. K. Hahm, M. H. Kim, S. Cho, J. Kim, H. Jang, S. C. Cho, B. N. Kim, M. S. Shin, J. Seo, J. Jeong, S. Y. Choi, D. Kim and C. Kang (2011). "GIT1 is associated with ADHD in humans and ADHD-like behaviors in mice." ***Nat Med*** **17**(5): 566-572.

Wong, D. T., L. R. Reid and P. G. Threlkeld (1988). "Suppression of food intake in rats by fluoxetine: comparison of enantiomers and effects of serotonin antagonists." ***Pharmacol Biochem Behav*** **31**(2): 475-479.

Wong, E. H., M. S. Sonders, S. G. Amara, P. M. Tinholt, M. F. Piercey, W. P. Hoffmann, D. K. Hyslop, S. Franklin, R. D. Porsolt, A. Bonsignori, N. Carfagna and R. A. McArthur (2000). "Reboxetine: a pharmacologically potent, selective, and specific norepinephrine reuptake inhibitor." ***Biol Psychiatry*** **47**(9): 818-829.

Wooters, T. E. and M. T. Bardo (2011). "Methylphenidate and fluphenazine, but not amphetamine, differentially affect impulsive choice in spontaneously hypertensive, Wistar-Kyoto and Sprague-Dawley rats." ***Brain Res*** **1396**: 45-53.

Wultz, B., T. Sagvolden, E. I. Moser and M. B. Moser (1990). "The spontaneously hypertensive rat as an animal model of attention-deficit hyperactivity disorder: effects of methylphenidate on exploratory behavior." ***Behav Neural Biol*** **53**(1): 88-102.

Wyss, J. M., G. Fisk and T. van Groen (1992). "Impaired learning and memory in mature spontaneously hypertensive rats." ***Brain Res*** **592**(1-2): 135-140.

Xin, L., E. B. Geller, L. Y. Liu-Chen, C. Chen and M. W. Adler (1997). "Substance P release in the rat periaqueductal gray and preoptic anterior hypothalamus after noxious cold stimulation: effect of selective mu and kappa opioid agonists." ***J Pharmacol Exp Ther*** **282**(2): 1055-1063.

Yamamoto, B. K. and S. Novotney (1998). "Regulation of extracellular dopamine by the norepinephrine transporter." ***J Neurochem*** **71**(1): 274-280.

Yamashita, M., Y. Sakakibara, F. S. Hall, Y. Numachi, S. Yoshida, H. Kobayashi, O. Uchiumi, G. R. Uhl, Y. Kasahara and I. Sora (2013). "Impaired cliff avoidance reaction in dopamine transporter knockout mice." ***Psychopharmacology (Berl)***.

Yan, T. C., J. A. Dudley, R. K. Weir, E. M. Grabowska, Y. Pena-Oliver, T. L. Ripley, S. P. Hunt, D. N. Stephens and S. C. Stanford (2011). "Performance deficits of NK1 receptor knockout mice in the 5-choice serial reaction-time task: effects of d-amphetamine, stress and time of day." ***PLoS One*** **6**(3): e17586.

Yan, T. C., S. P. Hunt and S. C. Stanford (2009). "Behavioural and neurochemical abnormalities in mice lacking functional tachykinin-1 (NK1) receptors: a model of attention deficit hyperactivity disorder." ***Neuropharmacology*** **57**(7-8): 627-635.

Yan, T. C., A. McQuillin, A. Thapar, P. Asherson, S. P. Hunt, S. C. Stanford and H. Gurling (2010). "NK1 (TACR1) receptor gene 'knockout' mouse phenotype predicts genetic association with ADHD." ***J Psychopharmacol*** **24**(1): 27-38.

Yokota, Y., Y. Sasai, K. Tanaka, T. Fujiwara, K. Tsuchida, R. Shigemoto, A. Kakizuka, H. Ohkubo and S. Nakanishi (1989). "Molecular characterization of a functional cDNA for rat substance P receptor." ***J Biol Chem*** **264**(30): 17649-17652.

Young, J. W., K. Finlayson, C. Spratt, H. M. Marston, N. Crawford, J. S. Kelly and J. Sharkey (2004). "Nicotine improves sustained attention in mice: evidence for involvement of the alpha7 nicotinic acetylcholine receptor." ***Neuropsychopharmacology*** **29**(5): 891-900.

Young, J. W., G. A. Light, H. M. Marston, R. Sharp and M. A. Geyer (2009). "The 5-choice continuous performance test: evidence for a translational test of vigilance for mice." ***PLoS One*** **4**(1): e4227.

Young, J. W., S. B. Powell, C. N. Scott, X. Zhou and M. A. Geyer (2011). "The effect of reduced dopamine D4 receptor expression in the 5-choice continuous performance task: Separating response inhibition from premature responding." ***Behav Brain Res*** **222**(1): 183-192.

Young, J. W., J. van Enkhuizen, C. A. Winstanley and M. A. Geyer (2011). "Increased risk-taking behavior in dopamine transporter knockdown mice: further support for a mouse model of mania." ***J Psychopharmacol*** **25**(7): 934-943.

Zerbe, R. L., H. Rowe, G. G. Enas, D. Wong, N. Farid and L. Lemberger (1985). "Clinical pharmacology of tomoxetine, a potential antidepressant." ***J Pharmacol Exp Ther*** **232**(1): 139-143.

Zhang, L., S. Chang, Z. Li, K. Zhang, Y. Du, J. Ott and J. Wang (2012). "ADHDgene: a genetic database for attention deficit hyperactivity disorder." ***Nucleic Acids Res*** **40**(Database issue): D1003-1009.

Zhu, J., X. Zhang, Y. Xu, T. J. Spencer, J. Biederman and P. G. Bhide (2012). "Prenatal nicotine exposure mouse model showing hyperactivity, reduced cingulate cortex volume, reduced dopamine turnover, and responsiveness to oral methylphenidate treatment." ***J Neurosci*** **32**(27): 9410-9418.

Zhuang, X., R. S. Oosting, S. R. Jones, R. R. Gainetdinov, G. W. Miller, M. G. Caron and R. Hen (2001). "Hyperactivity and impaired response habituation in hyperdopaminergic mice." ***Proc Natl Acad Sci U S A*** **98**(4): 1982-1987.

Zocchi, A., G. Varnier, R. Arban, C. Griffante, L. Zanetti, L. Bettelini, M. Marchi, P. A. Gerrard and M. Corsi (2003). "Effects of antidepressant drugs and GR 205171, an neurokinin-1 (NK1) receptor antagonist, on the response in the forced swim test and on monoamine extracellular levels in the frontal cortex of the mouse." ***Neurosci Lett*** **345**(2): 73-76.

Appendix 1. Genotyping

Genotyping was completed in 3 stages; DNA extraction, amplification and visualization.

DNA extraction

- Ear notches taken with a 2mm ear punch provided the DNA for the sample.
- 75µl of alkaline lysis reagent (25mM NaOH, 0.2mM disodium EDTA in ultra-pure water) was added to each DNA sample
- Samples were heated at 95°C for 30 min, then cooled to 4°C
- 75µl of neutralizing reagent (40mM Tris-HCl in ultra-pure water) was added to each sample
- Samples were frozen at -20°C until needed

DNA amplification (PCR)

Reagent	Volume (µl) per sample
Thermopillic DNA Polymerase 10X, Mg^{2+}-free Reaction Buffer (Promega)	2.6
dNTP (Promega)	0.6
25mM $MgCl_2$	1.7
NK1-F primer (**5'-CTGTGGACTCTAATCTCTTCC-3')**	1.4
NK1-R primer (**5'-ACAGCTGTCATGGAGTAGATAC-3')**	1.4
NeoF primer (**5'-GCAGCGATCGCCTTCTATC-3')**	1.4
UPH20, nuclease-free H_2O	9.9
Taq DNA polymerase	0.1

- Master mix was made up on ice, using volumes scaled up for the number of samples.
- 19µl of master mix was added to 5µl of sample

- 3 control samples were prepared (one wildtype, one NK1R-/- and one nuclease-free water only)
- Samples were then run on a PCR thermocylcer (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) which ran the following program;

Step	Temperature (°C)	Duration
1	95	5min
2	60	30s
3	72	30s
4	94	30s
5	Steps 2-4 are cycled 35 times	
6	60	30s
7	72	5min
8	Samples held at 4°C	

Gel electrophoresis & visualization

- 5µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) is added to each sample
- 15µl of sample is loaded into wells in a 2% agarose (2g agarose in 100ml 0.5M Tris-borate-EDTA (TBE) buffer (National Diagnostics, Hull, UK)) containing 8µl ethidium bromide
- 5µl 1kB ladder (Bioline, London, UK) was loaded into the first well in each row
- The tank was filled with 500ml TBE buffer and samples were run at 110-120mV using a FEC105 Voltage Power Pack for approximately 1 h
- The gel was visualized and photographed with an ultraviolet transilluminator plate (UVP Ltd, Cambridge, UK)

Appendix 2. Doses of guanfacine

Reference	Route	Dose	Pre-treatment time	Species/strain	Type of experiment	Effective Doses
(Masse *et al.* 2006)	i.p.	0.06 and 0.125mg/kg	45min	Mouse/Swiss	Anxiety – four plate test	0.06, 0.126mg/kg
(Szot *et al.* 2004)	i.p.	0.1mg/kg	30min	Mouse/129/SvEv x C57Bl6	Epilepsy/seizure activity	0.1mg/kg
(Archer and Fredriksson 2003)	s.c.	0.1, 0.3, 1 and 3mg/kg	None	Mouse/C57Bl6	Parkinsonian locomotor behaviour	1mg/kg
(Franowicz *et al.* 2002)	i.p.	0.0001, 0.001, 0.01, 0.1, 1, or 10mg/kg	1hr	Mouse/C57Bl6	Cognition/working memory – T-maze	1mg/kg
(Langen and Dost 2011)	i.p.	0.3 and 1mg/kg	30min	Rat/ SHR/WKY	Elevated plus maze, open field	0.3, 1mg/kg
(Le *et al.* 2011)	i.p.	0.125, 0.25, and 0.5 mg/kg	1hr	Rat/Wistar	Alcohol self-administration	0.5mg/kg
(Sagvolden 2006)	i.p.	0.075, 0.15, 0.3 and 0.6 mg/kg	30min	Rat/ SHR/WKY	ADHD behaviour – 2 lever choice test	0.3, 0.6mg/kg
(Jentsch 2005)	i.p.	0.01, 0.05 or 0.1mg/kg	20min	Rat/ SHR/WKY	ADHD behaviour – lateralized reaction time task/visuospatial attention	None

Date: October 2011. Key words: "guanfacine" and "rat" or "mouse". Search engine: Pubmed

Appendix 3. Doses of medetomidine

Reference	Route	Dose	Pre-treatment time	Species/strain	Type of experiment	Effective Doses
(Bjorklund *et al.* 2001)	s.c.	5 – 10 μg/kg	20 min	Mouse/ C57Bl6	Memory (T maze / 8 arm radial maze)	5μg/kg
(Tanila *et al.* 1999)	s.c.	1, 3, 5μg/kg	20 min	Mouse/ C57Bl6	Memory (T maze)	5μg/kg
(Sallinen *et al.* 1998)	s.c.	10μg/kg	20 min	Mouse/ C57Bl6	Locomotor activity	10μg/kg
(Sirvio *et al.* 1992)	s.c.	0.3, 0.9, 3, 9μg/kg	30 min	Rat/ Wistar	Memory (step through passive avoidance task & water maze)	0.3, 0.9, 3, 9μg/kg
(Laarakker *et al.* 2010)	i.p.	5μg/kg	30 min	Mouse/ B6 and A/J	Stress (forced swim)	5μg/kg
(Rago *et al.* 1991)	s.c. / i.p.	0.5 - 10μg/kg	15 / 30 min	Rat/ Wistar & mouse/ NMRI	Anxiety (EPM)	None
(Votava *et al.* 2005)	i.p.	5, 10, 20. 40μg/kg	30 min	Mouse/ ICR albino	Locomotor activity	20 & 40μg/kg

Date: November 2013. Key words: "medetomidine" or "dexmedetomidine" and "rat" or "mouse". Search engine: Pubmed

Appendix 4. Doses of atomoxetine

Reference	Route	Dose	Pre-treatment time	Species/strain	Type of experiment	Effective Doses
(Koda *et al.* 2010)	i.p.	1 or 3mg/kg	None	Mouse/ ICR	Locomotor activity	None
(Tsuchida *et al.* 2009)	i.p.	1mg/kg	None	Mouse/ Slc:ICR	Locomotor activity	None
(Bruno and Hess 2006)	i.p.	1 – 20mg/kg	None	Mouse/ Coloboma & C3H/HeSnJ control	Locomotor activity	All doses
(O'Keeffe *et al.* 2012)	i.p.	3mg/kg	30 min	Mouse/ C57BL/6	Circadian rhythms	3mg/kg
(Balci *et al.* 2008)	i.p.	1, 3 and 10mg/kg	30 min	Mouse/ C3H	Interval timing	3 and 10mg/kg
(Gould *et al.* 2005)	i.p.	0.2, 2 and 20mg/kg	30 min	Mouse/ C57BL/6J	Pre-pulse Inhibition	0.2, 2 and 20mg/kg
(Davis and Gould 2007)	i.p.	0.2 or 2mg/kg	20 min	Mouse/ C57BL/6	Cognition	2mg/kg
(Tamburella *et al.* 2012)	i.p.	1, 3 and 6mg/kg	?	Rat/ SHR	Cognition & locomotor activity	1, 3 and 6mg/kg
(Janak *et al.* 2012)	i.p.	1mg/kg	45 min	Rat/ Sprague Dawley	Drug seeking (self-administration)	1mg/kg

Date: March 2012. Key words: "atomoxetine" and "rat" or "mouse". Search engine: Pubmed

Appendix 5. Doses of methylphenidate

Reference	Route	Dose	Pre-treatment time	Species/strain	Type of experiment	Effective Doses
(Yan *et al.* 2010)	i.p.	2.5mg/kg	30min	Mouse / NK1R-/-	Locomotor activity	2.5mg/kg
(Kaczmarczyk *et al.* 2013)	i.p.	2.5mg/kg	45min	Mouse /C57BL/6J	Learning/memory	2.5mg/kg
(Yamashita *et al.* 2013)	i.p.	30mg/kg	None	Mouse /C57BL/6J x 129Sv/J	Cliff avoidance reaction (CAR), pre-pulse inhibition	30mg/kg
(Keck *et al.* 2013)	i.p.	0.3, 3, 5, 10, 20 and 30 mg/kg	30min	Mouse / C57BL/6J x 129/Ola	Locomotor activity, novel object recognition	5, 10, 20, 30mg/kg
(Griffin *et al.* 2013)	i.p.	1.25 mg/kg	15min	Mouse /C57BL/6J	Drug-discrimination, locomotor activity	1.25mg/kg
(Zhu *et al.* 2012)	i.p.	0.75, 1.5, 3.5 and 7.5mg/kg	None	Mouse /C57BL/6J	Locomotor activity	3.75 and 7.5mg/kg
(Flood *et al.* 2010)	i.p.	10, 30 and 100mg/kg	30min	Mouse /DBA/2	Pre-pulse inhibition	30 and 100mg/kg
(Rhodes and Garland 2003)	i.p.	15 and 30mg/kg	None	Mouse /Hsd:ICR strain	Locomotor activity	15 and 30mg/kg
(Koike *et al.* 2009)	i.p.	1 and 3mg/kg	30min	Mouse /ICR	Anxiety (EPM)	3mg/kg

Date: January 2014. Key words: "methylphenidate" and "mouse". Search engine: Pubmed

Appendix 6. Weight and food intake of mice in the 5-CSRTT and 5C-CPT

To track animals' weights over the duration of the 5-CSRTT/5C-CPT, mice were weighed every weekday, and fed differing amounts to maintain a constant weight. Only data from mice from homozygous breeding pairs were used in this analysis: mice in one cage were fed together, and cages of mice bred from heterozygous breeding pairs contained both wildtype and NK1R-/- mice, such that the food consumption of the two genotypes could not be separated. Mouse weights and food weights were averaged over each week. These measures were used to calculate how much food was eaten per gram of mouse weight (mouse weight : food weight ratio). The data were analysed with a repeated measures approach, using '*week*' as the within-subjects factor, and '*genotype*' as the between-subjects factor.

Wildtype mice were larger than NK1R-/- mice, and maintained a higher weight throughout the experiment [[LOG10]geno: $F_{(1,46)}$=21.33, P<0.001]. The amount of food consumed decreased over time [[SQRT]week: $F_{(19, 222)}$=95.97, P<0.001], but depended on genotype [[SQRT]geno*week: $F_{(19,222)}$=3.83, P<0.001], such that by the final 5 weeks, NK1R-/- mice consumed more food than wildtypes. When corrected for mouse weight, the genotype-dependent effect on food consumed [[SQRT]geno*week: $F_{(19,222)}$=3.66, P<0.001] was evident with a difference apparent in the latter 6 weeks of the experiments.

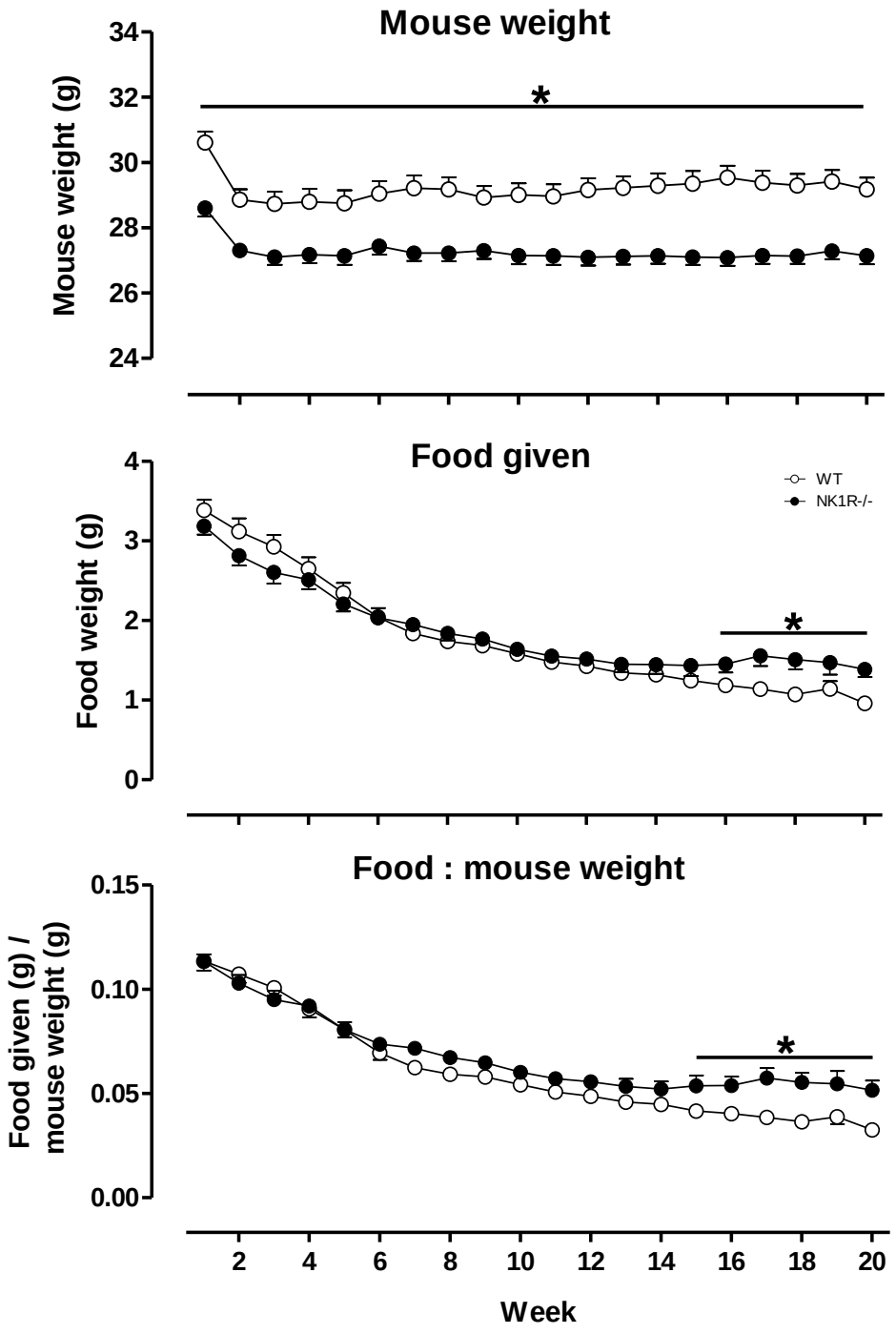

Figure i – The weight (A), food intake (B) and food : weight ratio (C) of wildtype (open circles) and NK1R-/- mice (closed circles) over 20 weeks of the 5-CSRTT and 5C-CPT experiments (presented in Chapters 3, 5 and 6). Data show mean SEM. n= 24. Bars indicate P<0.05.

Appendix 7. Ingredients and calorific value of the normal diet

The diet (2018 Global rodent diet) all mice were fed (apart from where specified) was obtained from Harlan, UK.

	Percentage of weight (%)	kcal (%)
Protein	18.6	24
Carbohydrate	71.7	58
Fat	6.2	18
Fiber	3.5	0
Total	**100**	**100**
kcal/g	**3.1**	

Ingredients
Ground wheat
Ground corn
Wheat midds
Soybean meal
Corn gluten meal
Brewer's yeast

Appendix 8. Ingredients and calorific value of the High Fat Diet

The high fat diet used in Chapter 7 was from Open Source Diets, New Brunswick, NJ, USA.

	Percentage of weight (%)	kcal (%)
Protein	24	20
Carbohydrate	41	35
Fat	24	45
Fiber	11	0
Total	**100**	**100**
kcal/g	**4.73**	

Ingredient	**weight**	**kcal**
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	72.8	291
Maltodextrin 10	100	400
Sucrose	172.8	691
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*		1598
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Red Dye #40	0.05	0
Total	**858.15**	**4057**

*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 167.8
Cholesterol mg/kg = 195.5