The Role of Brain-Derived Neurotrophic Factor in Psychosis-like Behaviour and Addiction

Submitted by

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Abstract

Dopaminergic signalling in the mesocorticolimbic and nigrostriatal networks has been implicated in both the effects of chronic methamphetamine (METH) and in schizophrenia. These networks are also central to addiction development, where dopaminergic sensitization is hypothesized to play a major role in maladaptation to incentive salience. Brain-derived neurotrophic factor (BDNF) is a neurotrophin responsible for the regulation of brain development, plasticity and cell survival and is an important regulator of mesolimbic dopamine D3 receptor (D3R) expression. D3R and attenuated BDNF signalling have been implicated in the pathophysiology of schizophrenia and alcohol use disorders.

This study first aimed to extend previous research on the effects of chronic adolescent METH on schizophrenia endophenotypes, including locomotor hyperactivity and disruption of prepulse inhibition (PPI). The interaction of BDNF and D3R was then studied in double mutant mice with either BDNF heterozygosity, D3 knockout or both. Finally, in operant studies we examined the effects of reduced BDNF and enhanced BDNF receptor signalling on alcohol reinstatement in rats.

BDNF heterozygous (BDNF HET) mice displayed varying and sex-specific responses to chronic METH in locomotor hyperactivity and PPI experiments, and were generally more hyperactive than their wildtype counterparts. Few effects of D3R knockout were detected, suggesting BDNF haploinsufficiency impacts schizophrenia-relevant behaviour via a different mechanism. Sex-specific enhancement of alcohol reinstatement was observed in BDNF HET female rats but not males. Similarly, an increase in TrkB activation with 7,8-DHF also increased reinstatement only in females, highlighting the potential influence of sex hormones on BDNF activity.

These studies provide new insight into the interaction of BDNF and dopamine neurotransmission in psychosis and addiction.

Declaration

I declare that,

i) Except where reference is made in the text of the thesis (Preface), this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

ii) This work was supported by a La Trobe University Postgraduate Research Scholarship and an Australian Government Research Training Program Scholarship.

Signed: 

Samuel John Hogarth August 2020

Preface

All research described in this thesis was conducted at La Trobe University. Mouse and rat breeding colonies were maintained by Emily Jaehne. Experimental data were collected by Samuel Hogarth and Emily Jaehne. The majority of behavioural testing was completed by Samuel Hogarth. In a leave of absence period (3 months) due to injury, Emily Jaehne conducted all experimental procedures. All other work and all data analysis presented in this thesis were conducted by Samuel Hogarth.

Conference abstracts

Hogarth SJ, Jaehne E, van den Buuse M, Djouma E. Investigating the role of Brain-Derived Neurotrophic Factor (BDNF) in alcohol addiction: Studies in BDNF heterozygous mutant rats. Poster presentation. Australasian Neuroscience Society Annual Scientific Meeting 2016, Hobart, Australia, December 2016

Hogarth SJ, Jaehne E, van den Buuse M. Elucidating the interaction between Brain-Derived Neurotrophic Factor (BDNF) and the dopamine D3 receptor in methamphetamine psychosis. Poster presentation. Australasian Neuroscience Society Annual Scientific Meeting, Sydney, Australia, December 2017

Hogarth SJ, Jaehne E, van den Buuse M. Elucidating the interaction between brain-derived neurotrophic factor (BDNF) and the dopamine D3 receptor in methamphetamine psychosis. Poster presentation. The International College of Neuropsychopharmacology (CINP) 31st World Congress, Vienna, Austria, June 2018

Hogarth SJ, Jaehne E, van den Buuse M. Chronic methamphetamine causes disruption of prepulse inhibition in male mice with a combination of reduced brain-derived neurotrophic factor (BDNF) and dopamine D3 receptor levels. Poster

presentation. 11th Federation of Neuroscience Societies (FENS) forum, Berlin, Germany, July 2018

Hogarth SJ, Jaehne E, van den Buuse M. Chronic methamphetamine causes disruption of prepulse inhibition in male mice with a combination of reduced Brain-derived neurotrophic factor (BDNF) and dopamine D3 receptor levels. Poster presentation. Students of Brain Research (SOBR) Symposium, Melbourne, Australia, November 2018

Hogarth SJ, Jaehne E, van den Buuse M. Chronic methamphetamine disrupts prepulse inhibition and locomotor activity in mice with reduced brain-derived neurotrophic factor (BDNF) and dopamine D3 receptor levels. Poster presentation. International Behavioural Neuroscience Society (IBNS) 28th Annual Meeting, Cairns, Australia, 2019

Thesis publications

1. **Hogarth SJ**, Jaehne EJ, van den Buuse M, Djouma E. Brain-derived neurotrophic factor (BDNF) determines a sex difference in cue-conditioned alcohol seeking in rats. *Behav Brain Res*. 2018;339:73-78. doi:10.1016/j.bbr.2017.11.019

2. **Hogarth SJ**, Djouma E, van den Buuse M. 7,8-Dihydroxyflavone Enhances Cue-Conditioned Alcohol Reinstatement in Rats. *Brain Sci*. 2020;10(5):270. doi:10.3390/brainsci10050270

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To my co-supervisor Elly, your input on the alcohol studies was fundamental. Thank you for giving me the chance to work on these operant experiments and other lab assistant duties that provided some much-needed income in my first and second year. Your feedback, along with Maarten's, resulted in my first publication which secured a La Trobe stipend scholarship and made a significant difference to my living circumstances and quality of life.

None of this work would have been possible without the support I received from the other members of the van den Buuse lab, so thank you to everyone who has contributed to my research in some way over the years. I would like to pay special tribute to Emily, who is the glue that holds us together. Even though I relied on your organisational skills for most of my candidature, you taught me the importance of scheduling, planning, and routine, and keeping up to date with the various colony management software (although I did my best to avoid this!). I would have struggled perfusing four mice simultaneously without your help. I also greatly appreciated your useful and well thought out feedback on anything experiment related, and our casual chats in the office about the results of the weekend footy

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Abbreviations

5-HT	5-hydroxytryptamine (Serotonin)
6-OHDA	6-hydroxydopamine
7,8-DHF	7,8-dihydroxyflavone
AC	Adenylyl cyclase
AUD	Alcohol use disorder
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
D1R	Dopamine D1 receptor
D2R	Dopamine D2 receptor
D3R	Dopamine D3 receptor
DA	Dopamine
DAT	Dopamine transporter
DCV	Dense core vesicles
DOPAC	3,4-Dihydroxyphenylacetic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ERK1	Extracellular signal-regulated kinase 1
FEP	First episode of psychosis
GABA	γ-aminobutyric acid
GPCR	G protein-coupled receptor
GWAS	Genome-wide association study

HET	Heterozygote
HIV	Human immunodeficiency virus
ISI	Inter-stimulus interval
IVC	Individually-ventilated cages
JNK	c-Jun N-terminal kinase
L-DOPA	L-3,4-dihydroxyphenylalanine
LHA	Locomotor hyperactivity
LMA	Locomotor activity
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase cascade
mBDNF	Mature brain-derived neurotrophic factor
METH	Methamphetamine
mGluR	Metabotropic glutamate receptor
MK-801	Dizocilpine (aka INN)
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NET	Norepinephrine transporters
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NT3	Neurotrophin-3
NT4	Neurotrophin-4
OT	Olfactory Tubercle

PCP	Phencyclidine
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP	Prepulse
PPI	Prepulse inhibition
PTSD	Post-traumatic stress disorder
SERT	Serotonin transporters
SN	Substantia nigra
SNP	Single nucleotide polymorphism
SUD	Substance use disorder
TH	Tyrosine hydroxylase
Trk	Tyrosine receptor kinase
TAAR1	Trace amino acid receptor 1
TrkB	Tropomyosin-related kinase B
VMAT2	Vesicle monoamine transporter 2
VTA	Ventral Tegmental Area
WT	Wildtype

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Chapter 1 Introduction

1.2 Neurotrophins

Neurotrophins are a collection of structurally and functionally related growth factors that regulate neuronal cell development, function, plasticity, and survival [1]. The study of neurotrophin function within the mammalian brain has gathered substantial traction in recent years, incorporating a comprehensive understanding of the mechanisms through which this class of regulatory proteins modulate neuronal systems.

Neurotrophic factors also play a role in the acute and chronic response to central nervous system (CNS) disruption and disease, which is induced through defined subsets of neuronal network connectivity and plasticity modulation [2]. As central mediators of activity-dependent plasticity, neurotrophins translate environmental stimuli onto neuronal networks via structural and functional adaptation [3]. Through this process, drugs of abuse can trigger maladaptive changes to neuronal networks via neurotrophic response. In addition, most neuroprotective drugs promote neurotrophin synthesis and release within the brain, highlighting a functional role for these proteins in combating CNS dysfunction [2]. Modern research techniques have enhanced the behavioural and molecular interrogation of neurotrophic factor signalling pathways that mediate a host of modulatory effects within the brain, however, the role neurotrophins play in disease is less understood [4].

Neurotrophins play a key role in the developing brain. The neurotrophin or neurotrophic factor hypothesis postulates that developing neurons compete for the limited supply of neurotrophins from innervated tissue, determining synaptic strength and selective neuronal cell death of connections during development [5, 6]. In this system, neuron and target cell populations equilibrate as the survival of neurons is reliant upon feedback from the innervated area of interest, generated via neurotrophin secretion [7]. Neurotrophins are also vital in the maintenance of the mature brain and are key components of long-term potentiation (LTP) and depression (LTD) [8, 9]. In the mammalian system, four neurotrophins have been identified, including nerve growth factor (NGF), neurotrophin-3 (NT-3),

neurotrophin-4 (NT-4), and brain-derived neurotrophic factor (BDNF) [10]. These proteins are synthesized as precursor, or pro-neurotrophins, which are typically cleaved intracellularly into mature forms [10].

All neurotrophins are limited to interacting with two categories of cell surface receptors, tropomyosin-related (or tyrosine) receptor kinases (Trk) and p75 receptors. Trk activation occurs through ligand binding that leads to auto-phosphorylation and ultimately initiates intracellular signalling cascades within the cell, responsible for the majority of survival and growth properties of the neurotrophin [11]. Alternatively, neurotrophic growth factors may trigger dimerization of the p75 receptor, or p75 may act as a co-receptor of Trk, capable of either enhancing or supressing activity. In typical p75 binding the receptor will autonomously promote the induction of apoptosis and cell death, or the advancement of cell survival, and this process is reliant on downstream signalling motifs and targeted feedback mechanisms within the cell [11, 12].

1.3 Neurotrophins in disease: BDNF

Neurotrophins are naturally involved in the aetiology of CNS disease. In most cases, a reduction in these protective proteins within the brain confers a state of vulnerability to the effects of neurodegenerative and neuropsychiatric pathologies, heightened addiction propensity, and an increased risk of developing substance use disorders (SUDs) [13]. Of the four neurotrophins expressed within the mammalian CNS, BDNF is the most widespread and abundant growth factor [14], and changes in BDNF basal activity have been shown to mediate disease-driven dysfunction. The polypeptide has predominantly been implicated in the preclinical setting, playing a substantial role in the development of numerous pathophysiologies in neuronal populations [15]. Despite widespread expression, the vast majority of reported BDNF dysfunction in disease occurs within the mesocorticolimbic pathways, comprised of mesolimbic and mesocortical projections between the midbrain, basal ganglia, and forebrain of the CNS [16]. The mesocorticolimbic projections are associated with processes governing pleasure, reward, and executive function, whose outputs are predominantly moderated by dopaminergic transmission [16]. Taken together, these findings highlight BDNF as a potential mediator of the neurochemical adaptations that occur

within the mesocorticolimbic pathway in response to neuronal disruptions and disease development within the brain.

1.4 BDNF expression and function

BDNF is encoded by the *BDNF* gene that is located on the 11p chromosome, where eight different promotors result in 18 possible gene transcripts. Translation occurs at the endoplasmic reticulum through extracellular protease cleavage of

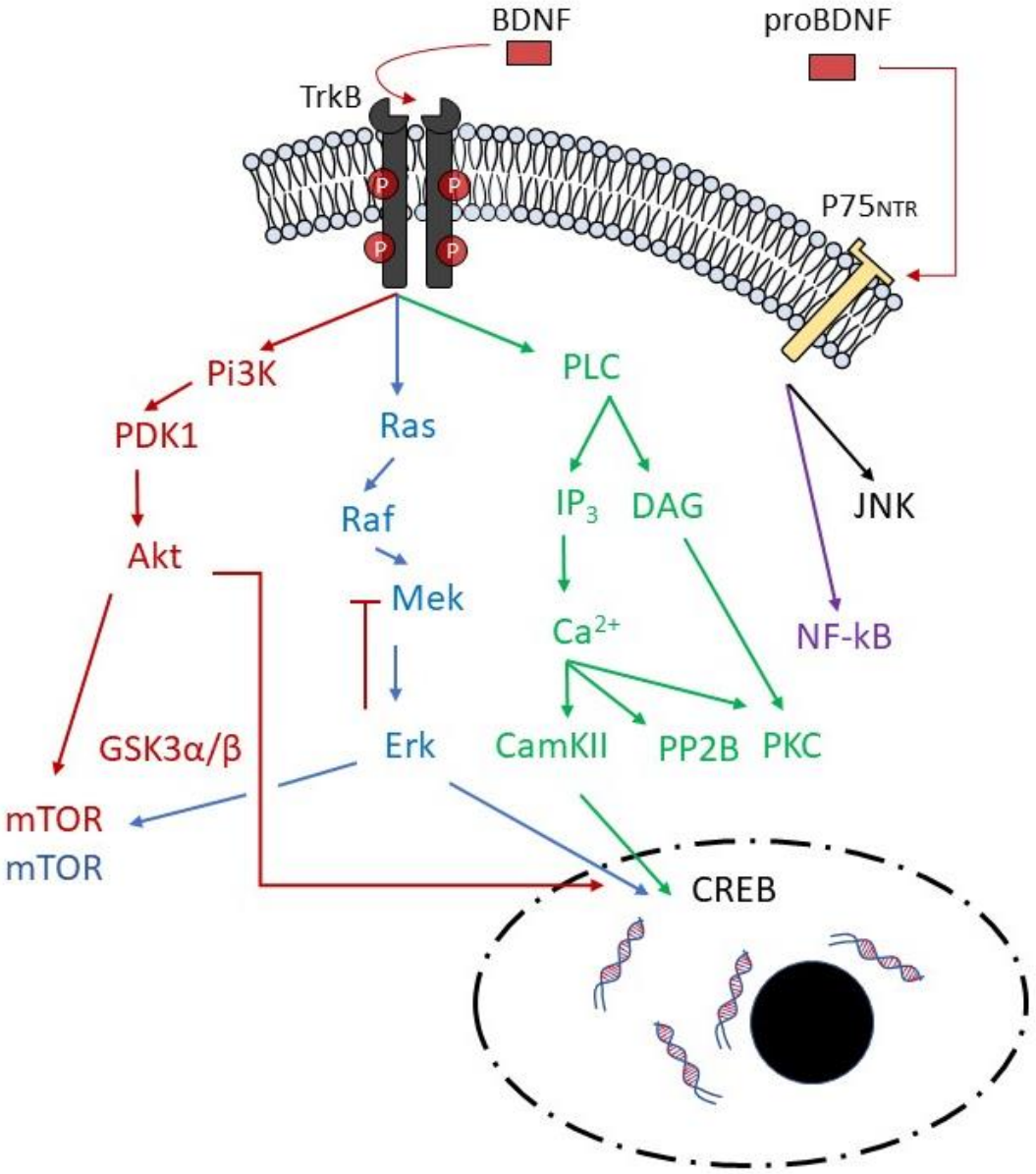

Figure 1.1 BDNF interacts with TrkB to initiate downstream signalling via three main phosphorylation pathways in the CNS. *These are Pi3K (red), MAPK (blue), and PKC (green). Activation of P75$_{NTR}$ by proBDNF triggers JNK and NF-kB activation.*

preproBDNF to proBDNF [17]. ProBDNF is sent to the Golgi apparatus where it is sorted into secretory dense core vesicles (DCV), or cleaved by endoproteases such as FURIN, to mature BDNF (mBDNF) [17]. Within DCVs, protein convertases may also cleave proBDNF into mBDNF prior to exocytosis from the cell [18]. The BDNF gene has four 5' exons and one 3' exon that code for the mature BDNF protein [13, 19]. Combined, this construct is capable of eight different promoter regions that permit up to 18 possible BDNF messenger RNA (mRNA) transcripts to be expressed through alternative sequence splicing. BDNF transcript sequences comprised of exons 1, 2, and 3 are located within the brain. Though it is unknown why BDNF transcription has such innate variability, it is believed this complexity confers varying subcellular locational specificity of the protein and/or mRNA itself, and numerous levels of specialisation through the availability of variable promotor motifs [20].

Mature BDNF interacts with its high-affinity tropomyosin-related kinase B receptor (TrkB) to initiate downstream signalling on several phosphorylation pathways including mitogen-activated protein kinase cascade (MAPK), phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC; Figure 1.1), as well as NMDA receptor activation [10]. This phosphorylation cascade is of great importance during development, where BNDF-mediated signalling can promote neuronal cell growth, differentiation, maturation, and synaptogenesis [21].

ProBDNF that avoids extracellular cleavage binds to the low affinity $p75^{NTR}$, activating apoptosis or cell survival. These pathways are mediated through c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), respectively. Activation of $p75^{NTR}$ stimulates opposing effects to that of TrkB and potentiates long-term depression (LTD), a mechanism to inhibit the growth of redundant neuronal networks through a reduction in trophic signalling [22]. In a study by Nagappan et al., isoforms of BDNF were analysed under low frequency stimulation to model LTD, where they found heightened proBDNF neurotrophin secretion. Unsurprisingly, LTP induced through high-frequency stimulation caused an increased in mBDNF secretion [23]. Based off this work and that of others, it is hypothesized that the amount of proBDNF, relative to mBDNF, creates a dynamic interplay between $p75^{NTR}$ and TrkB activation, which ultimately determines neuronal cell survivability, maintenance, and synaptogenesis in brain.

1.5 BDNF deficiency

BDNF function is also equally important in the mature brain and is a dynamic component of maintaining neurocircuitry pathways through elaboration and refinement of neural network connectivity. As in development, BDNF exerts neurotrophic effects through several processing and enhancement mechanisms, including modulation of synaptic connectomes and their stability, neurogenesis, dendriogenesis, neural organisation, and learning and long-term memory formation [21].

The inherently diverse role of BDNF in the expansion, depression, and maintenance of these systems suggests, somewhat unsurprisingly, that expression and function of the protein can be found to be reduced in a large proportion of cerebral illnesses. These include components of degenerative symptomatology in Parkinson's [24] and Huntington's disease [25], neurochemical changes associated with addiction [25-28], and perturbed functioning in multiple neuropsychiatric conditions [29-32]. What remains unclear is whether alterations in BDNF signalling are primary or secondary in the development of these illnesses affecting the brain. There is evidence to suggest reduced efficiency of BDNF signalling prior to disease onset through variants in the BDNF gene, and these carry an increased risk to the development of related dysfunctions [33]. A single nucleotide polymorphism (SNP) in the pro-region of the BDNF gene in humans reduces secretion of the protein through aberrant mRNA trafficking and disturbed intracellular transport mechanisms [34]. Depending on whether one or both alleles are affected, the amino acid substitution from valine to methionine at codon 66 (val66met) confers an approximate 18% or 29% reduction in activity-dependent secretion of BDNF [33]. This reduction has been associated with the development of neuropsychiatric disorders [30, 32, 33], highlighting the sensitivity of the CNS to changes in neurotrophin expression, and the integral role of BDNF in maintaining regular system function.

1.5.1 The BDNF HET model

Reduced BDNF expression in areas of the CNS relevant to numerous disease pathology has led to the development of heterozygous mutant rodents with a

deletion or null mutation in one BDNF gene allele that confers a 50% reduction in endogenous protein production [35, 36]. As BDNF homozygous knockout rodents do not typically survive beyond the first postnatal month [37], HET animals are the most effective tools in examining a global reduction in endogenous BDNF expression and can aid in determining the extent of neuronal dysfunction instigated by reduced trophic signalling. Animal models are particularly important concerning psychotic disease, where preclinical studies rely on the ability to reproduce aspects of mental illness and complex human psychopathology, a process that is made possible through the careful analysis of behaviours during animal testing. To this end, the effects of genetic modifications such as BDNF heterozygosity can be examined to determine the relative contribution of neurotrophin signalling in pathology development. Initial studies created monoallelic mutations to produce BDNF HET rodents, and these models developed common phenotypic adaptations, including heightened appetite and an increase in weight compared to their wild type counterparts, a small but significant reduction in brain size, and deficits in early neuronal differentiation [38-40]. Adding to this, further work has demonstrated BDNF HET mice have a more aggressive phenotype, coupled with heightened anxiety, reduced fear learning, and impaired long-term potentiation compared to mice with typical BDNF expression [41-43].

Previous studies in these haploinsufficient rodents have also identified several domains affected by altered BDNF-mediated neurotransmission in the adapting brain. As BDNF is known to regulate learning and memory processes, a number of studies have examined the cognitive capabilities of BDNF HET rodents in behavioural tests designed to measure parameters such as cognitive flexibility, defined as the capacity to switch between different mental sets, short term memory, and contextual learning [40, 44, 45]. Significant dysfunction has been observed in each of these fields, highlighting the importance of regulated BDNF expression in cognitive and executive function. In recent years however, the majority of research has shifted focus and now reports primarily on the role of attenuated BDNF signalling in addiction and psychosis symptomatology, and the behaviours that are affected by these domains. BDNF signalling appears crucial to the regulation of seeking addictive substances in reward and relapse behaviours, such as sensitisation to cocaine, and alcohol craving propensity [46, 47]. BDNF HETs also

display increased sensitivity to the hyperlocomotion induced through drugs that increase extracellular neurotransmitter release, such as amphetamine derivatives [48]. These psychostimulants provoke the release of dopamine (DA) and other monoamines from cytosolic and vesicular reservoirs within neurons, thereby increasing local transmission and activating surrounding networks; this effect is acutely prolonged and elevated in BDNF HET mice [49]. Although varied in effect, the result of perturbed neurotrophic signalling in psychosis and addiction coincides with tonic changes in dopaminergic transmission within the brain, which suggests an interaction between BDNF and DA during disease. However, despite growing evidence for a role of BDNF in the development of addiction and psychosis, little progress has been made in determining the mechanisms involved in the contribution of BDNF to these diseases and how attenuated BDNF signalling impacts dopamine-driven behaviours.

1.6 The role of BDNF in disease: Targeting the dopaminergic reward systems

As maladjustments to BDNF expression and secretion during disease occur predominantly in the mesolimbic and mesocortical dopaminergic systems, interrogation of localised dysfunction in these regions can be investigated by examining behavioural domains that converge on these signalling pathways governed by the DA neurotransmitter.

1.6.1 Dopamine

DA is a monoamine synthesized in a two-step process from the amino acid L-tyrosine. Within the brain, L-tyrosine is converted in a rate-limiting reaction by tyrosine hydroxylase (TH) to form L-3,4-dihydroxyphenylalanine (L-DOPA), which is then carboxylated by the enzyme, aromatic acid decarboxylase, to form DA [50]. Physiological functions of the catecholamine within the brain are diverse and include transmission of motivational salience and reward, motor control, arousal, reinforcement, and executive function. The actions of DA cannot be defined simply as either inhibitory or excitatory due to diversity in receptor activation pathways. DA provokes its effects through binding with DA D1, D2, D3, D4, and D5 receptors and trace amino acid receptor 1 (TAAR1) [51]. DA receptors have a preserved

seven-transmembrane structure that can signal through, or independently of G-protein coupled mechanisms within the CNS and can be separated into two groups based on their direct effect on cyclic adenosine monophosphate (cAMP) activation within the neuron. Thus, DA receptors are categorised as either excitatory D1 class (D_1, D_5) or inhibitory D2 class receptors (D_2, D_3, D_4) [51]. The functional property of the D1 class receptors is coupling to $G_{as/olf}$ to activate adenylyl cyclase (AC) that increases production of cAMP and activates PKA causing phosphorylation of nuclear and cytoplasmic proteins. D2R class receptors work in opposition to D1R, inhibiting cAMP production [51]. Ultimately activation of the second messenger cAMP leads to signalling effected through protein kinase A (PKA), which contributes to most downstream events initiated by DA neurotransmission. Following release into the synaptic cleft, excess DA is removed by surrounding glial cells and the DA transporter (DAT), a cell surface protein that reabsorbs the monoamine into the cell, inhibiting further transmission [52].

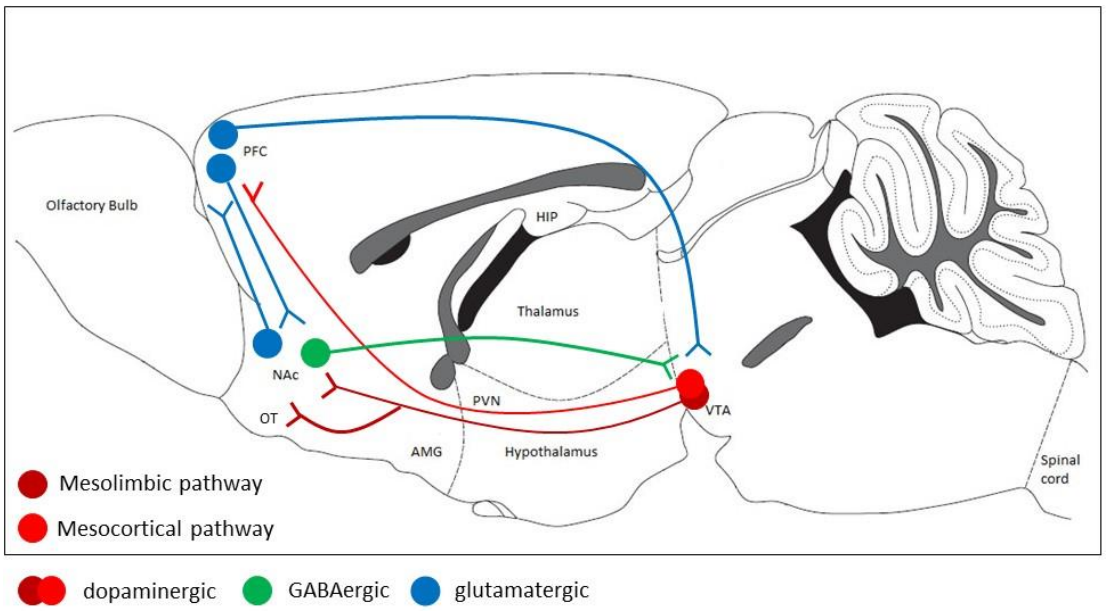

Figure 1.2 The reward neurocircuitry of the mouse brain. *Dopaminergic neurons project from the VTA to the NAc, OT, and mPFC (red). Glutamatergic neurons fire reciprocally between the NAc and mPFC as well as providing feedback mechanisms to the VTA via the mPFC (blue). Inhibitory GABAergic projections signal between the NAc to VTA (green).*

DA synthesis is limited to small clusters of cell bodies located within the substantia nigra (SN), ventral tegmental area (VTA), posterior hypothalamus, periventricular nucleus, arcuate nucleus, and zona incerta [53]. The most prominent source of DA in the brain is the complex network of the basal ganglia, within the VTA and SN at the base of the forebrain [54].

Dopaminergic neurons projecting from the pars compacta of the SN (SNpc) travel to the dorsal striatum caudate nucleus and form the nigrostriatal pathway. Dopaminergic neurons from the VTA fan out and innervate a host of neuronal structures, with prominent branches of cells forming the combined mesocorticolimbic projections. It is within these areas that alterations to dopaminergic signalling become key components of diseases whose symptomatology is defined through aberrant activation of reward and executive function domains. Genetic alterations to BDNF may result in a basal susceptibility to dopaminergic disruptions within these systems, although vulnerability to disease is not typically reliant on genetic factors alone. Temporal and environmental conditions can often contribute to aetiology and should be examined when determining the cause of dysfunction within mesocorticolimbic networks.

1.6.2 Adolescence

Adolescence, defined as the period between child- and adulthood is a stage of development marked by several physical and chemical changes within the mammalian brain. This progression varies in age specificity but can broadly be defined in humans as the maturation stage spanning between 10-22 years. Neurochemical changes include a reduction in grey matter volume that coincides with increased white matter formation and pruning / thinning of weak synapses within the cerebral cortex [55, 56]. Further development is seen in adolescence within the mesocorticolimbic projection, which actively matures through enhanced DA receptor D_1 and D_2 interaction and DA neurotransmitter accessibility [57]. This system is comprised of two dopaminergic pathways connecting the VTA to the nucleus accumbens (NAc) and olfactory tubercle (OT), also known as the ventral striatum (mesolimbic pathway, Figure 1.2), and the second commencing in the VTA and projecting to the prefrontal cortex (mesocortical pathway, Figure 1.2) [58]. Changes to the dopaminergic system are thought to drive adaptive alterations in

this neurocircuitry associated with working memory, cognitive function, and social maturation [59].

Mesolimbic dopaminergic neurons in the VTA that project to the NAc are considered integral in the reward system and the changes leading to reward dysfunction. During adolescence, an imbalance between brain regions occurs, resulting in a reduced rate of development in anterior domains and an enhanced rate in posterior domains [60]. This effects an imbalance between cognitive and control systems regulating the prefrontal cortex and ventral striatum, known as the dual systems model [61, 62]. As a result, dopaminergic corticolimbic pathways mediating reward are poorly regulated by frontal inhibitory circuits, thus enabling increased risk-taking, which is central to the immediate response [63, 64]. During adolescence, the development of abnormal neurocircuitry and brain structure has been proposed to increase the risk of disease development. This can include reduced NAc size [65], a reduction in cerebellar white matter and grey matter volume in the frontal cortex, as well as reduced consistency of white matter in frontal and corticolimbic structures that can forecast future abnormalities and susceptibility to disease [66, 67].

1.6.3 BDNF modulates DA signalling

It is widely accepted that BDNF influences DA signalling within the brain through modulation of various components of dopaminergic neurotransmission. Several studies have examined the relationship between BDNF and striatal DA neurons, and, combined, these findings underpin BDNF as a crucial regulator of dopaminergic tone [68-70]. Preclinical animal studies have reported elevated striatal DA concentrations in BDNF-deficient mice that compromised the dynamic release of DA along the nigrostriatal pathway [71]. Furthermore, quantitative microdialysis investigation has revealed synaptic DA levels in BDNF-deficient mice are increased, and electrically-evoked DA release is attenuated compared to wildtype controls [72]. Reduced release of DA into the synapse in BDNF-deficient rodents is hypothesized to be a homeostatic response to altered DA transporter (DAT) functioning in these mice that inhibits reuptake of the neurotransmitter, resulting in an overall increase in DA neurotransmission. Dampened BDNF signalling can also inhibit vesicular monoamine transporter 2 (VMAT2) and impact

DA storage, resulting in an accumulation of the monoamine at nerve terminals [68]. Within the mesocorticolimbic system BDNF is predominantly synthesized in the VTA, although mRNA from the neurotrophin is also expressed by dopaminergic neurons throughout the midbrain [73], highlighting the widespread importance of intrinsic BDNF expression. Activation of TrkB in the NAc potentiates DA release at nerve terminals within the mesolimbic pathway and modulates NAc neuron output, resulting in functional regulation of the pathway that is dependent on BDNF [47]. Evidence from cell culture experiments also suggests DA neurotransmission can elicit functional changes to the brain through the activation of BDNF-mediated pathways. D_1 and D_2 receptors can transactivate TrkB in neurons [74], as heteromeric complexes of D_1-D_2 receptors are proposed to enhance dimerization and recruitment of the neurotrophin effector to the cell surface [75]. Further interactions have been studied via administration of D_1 receptor agonists in rats, whose effects included upregulation of TrkB, while antagonism of these receptors has the opposite effect, reducing the expression of TrkB within the striatum [76]. Taken together these findings suggest DA and BDNF signalling are inherently linked, and the disruption of this relationship during disease could impact dopaminergic tone, affecting homeostatic trophic balance and potentiating further dysfunction. Combined with the functional relationship between DA and neurotrophin release, there is also strong evidence to suggest BDNF availability can alter DA receptor expression.

1.6.3.1 BDNF mediates expression of the DA D3R

BDNF signalling has been linked to the expression of the D3R following its discovery by the Sokoloff lab in 1990 [77]. Although this expression is lower than D_2 receptor (D2R) expression, immunohistochemical analysis has reported D3R is present on all mesencephalic dopaminergic neurons, where it is hypothesized to act as a negative feedback autoreceptor to dopaminergic signalling. Since its detection, the role of the receptor has been under great scrutiny and has subsequently been examined in numerous investigative domains, ranging from genetic and pharmacological screens, to post-mortem tissue analyses [60, 61]. Combined, these investigations have implicated D3R dysfunction in pathologies of drug addiction, depression, Parkinson's disease, and schizophrenia aetiology [78]. Presynaptic D3R-mediated autoreception has been demonstrated through

dysregulation of dopaminergic tone in D3R-deficient mice. In this study, basal grooming behaviour, which is largely controlled through D_1 receptor (D1R) stimulation, was increased in D3R-deficient mice versus wildtype controls. Mice of either genotype responded similarly to D1R agonism, suggesting increased dopaminergic tone within D3R-naive animals [78]. Pharmacological studies also highlight D3R autoreceptive control of DA release and impulse flow, as well as modulation of the neurotransmitter's direct synthesis within the neuron [79].

Mice deficient in D3Rs have two times the extracellular DA level in comparison to their wildtype counterparts and are more susceptible and have increased response to cues associated with opiates, cocaine, and amphetamines [78]. These effects further highlight a dysregulation of DA signalling that results in a behaviourally hyperactive model through increased basal DA transmission and extracellular monoamine release. Loss of D3Rs results in a minor but significant reduction in the autoregulation of DA presynaptic secretion, and diminished DA reuptake following loss of DAT modulation through downstream G-protein coupled $G_{i/o}$ signalling pathways [80, 81]. The VTA contains D3Rs on both neuronal cell bodies and nerve terminals of neurons reaching from the NAc, suggesting potential mediation of both the production and release of DA into the mesolimbic system [82].

D3R mRNA has been detected in areas relevant to psychosis development including the VTA, SN, NAc, and hippocampus in mice [83], and loss of D3R function in these animals produces hyperactivity that is observed through heightened locomotion [84]. There is evidence to suggest D3R expression is regulated by BDNF [85]. A study by Guillin and colleagues hypothesized anterograde transport of the neurotrophin could promote prolonged neuronal adaptations in target postsynaptic neurons via modulation of DA responsiveness, and that this effect was mediated by D3Rs [85]. To test this theory, DA neurons projecting from the VTA were unilaterally ablated with 6-hydroxydopamine (6-OHDA) within the NAc shell, preventing local innervation. This resulted in a reduction in D3R expression on the denervated side. As depletion of DA itself does not impact D3R expression, it was concluded that a factor released in conjunction with DA may be critical in D3R receptor regulation in postsynaptic neurons. Local infusion of BDNF restored D3R expression in the NAc, suggesting D3R receptor

expression is regulated by BDNF, and this mechanism could also determine behavioural sensitization [81]. D3R expression, which is reduced in BDNF HETs [49], might play a role in neoplastic changes that regulate the mesolimbic dopaminergic signalling system [86]. D3R polymorphisms have also been identified as inherent risk factors for schizophrenia onset. Association studies in FEP schizophrenia patients have identified significant differences in SNPs of the D3R gene, namely the rs6280 gly/gly genotype exhibiting reduced executive functioning compared to ser/ser [32]. Subsequent findings indicate downregulation of the receptor in sensitization models, permitting increased dopaminergic signalling through supressed negative feedback signalling [87, 88]. This process facilitates endogenous sensitization, and combined these theories suggest the D3R may be integral in DA dysfunction. This is supported by evidence of altered D3R activity and gene polymorphism interactions in schizophrenia pathophysiology [89-92]. Consequently, the relationship between D3R and BDNF requires further investigation and interrogation of combinatory effects on the mesolimbic DA system during psychosis.

1.7 BDNF and psychosis

Psychosis is a disruptive symptom of several neurodevelopmental, drug-induced, and psychiatric conditions, broadly defined by the World Health Organisation and Diagnostic and Statistical Manual of Mental Disorders (DSM-5) through the presence of hallucinations (sensory perceptions lacking equivalent stimuli) and delusions (firm beliefs formed through the false interpretation of reality) [93]. Psychosis development can be primary and defining in disease, or occur as a result of environmental conditions, such as substance abuse and psychoactive drug-taking. Regardless of origin, psychotic symptoms are thought to arise from dysfunctional signalling in perception and information processes within the brain. Moreover, pathological convergence has been shown in specific regions of the mammalian brain and these findings have established a neural framework in which shared dysfunction can be examined in different psychosis models. As a result, psychosis development can be interrogated through differing aetiology and levels of comparison can be drawn between different developmental routes, and via analysis of the common substrates and pathways involved. One of the

neurochemical adaptations that has been identified in emerging psychoses is the dysregulation of BDNF and its mediation of subcortical dopaminergic reward networks and associated pathways innervating the frontal cortex. Thus, reduced BDNF has been identified as a hallmark symptom in various psychosis-presenting illnesses. In particular, the disruption of neurotrophic signalling has been implicated in schizophrenia and in drug-induced psychosis following substance abuse. However, the exact role of BDNF in disease progression and the mechanisms through which perturbed signalling occurs, remains largely unknown. Due to recent evidence of neurotrophin-modulating capacity within the dopaminergic system, the most promising avenue of understanding psychosis development in the context of BDNF-mediated reward circuitry, is through the examination of the effects of psychotropic drugs that are known to promote hyperdopaminergia neuropathy. Methamphetamine (METH; N-methyl-alpha-methylphenethylamine) is a widely abused hyperdopaminergic drug in today's society that can induce a psychosis with significantly detrimental neurotoxic effects and has demonstrated to be associated with alterations in BDNF function and signalling.

1.7.1 Methamphetamine

METH is a synthetic drug originally designed by the Japanese military in 1919 to stimulate troops and maintain alertness during battle. In World War 2 high doses were also administered to kamikaze pilots prior to suicide missions as a means to override the fear of death. Post war, METH was made available to the public and this stimulated severe overuse and dependence on the euphoric effects of smoking or injecting the drug acutely. During the 1950s, the amphetamine derivative was prescribed as a dietary supplement, and later as a stimulant often used by college students and long-haul truck drivers to maintain alertness. Unfortunately, due to the substantial addictive profile and ready availability of METH, drug abuse shortly ensued, provoking a ban on the narcotic by the US government in 1970.

METH, also known as ice, crank, speed, or crystal, is a highly addictive psychostimulant with significant and long-lasting neurotoxic and neuroinflammatory effects on the mammalian brain. METH is categorised as an amphetamine-type synthetic drug and has pharmacodynamic properties similar to that of amphetamine, with the additional capability of rapid blood brain barrier

(BBB) diffusion and prolonged effect duration [94, 95]. As a result, users of METH have a higher propensity to develop substance addiction compared to amphetamine users, and chronic METH exposure has been shown to result in severe mental and physical impairment [96]. Pathologies can include increased risk of infection with HIV and hepatitis, stroke, kidney failure, periodontal disease, hypertension [97, 98], and an increased risk of development of diseases such as Parkinson's, schizophrenia, and psychosis [99, 100]. The majority of detrimental effects observed in METH users are a result of neurotoxicity, defined as the reversible or permanent physical damage to neuronal structures and their associated function, which evoke changes in behaviour and driving network degeneration [101].

1.7.1.1 METH abuse prevalence

METH use has increasingly become the drug of choice in a large proportion of substance abusers globally. Within Australia, during the beginning of the 1990s, legislation was introduced to control the precursor chemicals required for amphetamine sulphate [102], resulting in a steady increase in the production of METH over amphetamine, accounting for up to 91% of criminal seizures in 2000-2001 [103]. In addition, stronger forms of METH are now more readily available as the pure crystal form is in greater circulation and is predominantly made domestically [103]. In Australia a reported 1 in 70 people, or 1.4%, recorded having used METH in the past year [104]. The National Drug Household Survey has also recently stated 6.3% of Australians over the age of 14 have tried METH in the past and the use of pure METH substrates such as ice has increased in injecting drug users from 68% in 2017 to 75% in 2018 [104, 105]. Additionally, as of 2017 the percentage of new Australian users constituted the highest rate of METH addiction development globally. These data highlight the significance of providing therapeutic intervention to combat the social and economic burden of METH use in Australia and around the world.

1.7.1.2 Acute METH neurobiology

Due to its high lipid solubility, METH rapidly diffuses into the brain across the blood brain barrier (BBB) and into the CNS [95]. As METH shares structural homogeneity with DA, it is withdrawn from the synaptic cleft into the neuron through either DAT,

creating competitive uptake inhibition with DA, or through passive diffusion [101, 106]. Like DA, METH is an agonist of TAAR1, an intracellular G protein-coupled receptor (GPCR) which regulates catecholamines in the brain [107]. Activation of TAAR1 increases cAMP and phosphorylates DAT, norepinephrine transporters (NET), and serotonin transporters (SERT).This increases synaptic monoamine levels by acting on synaptic and vesicular monoamine transporters, the majority of which is via DAT regulation [108]. Upon binding to TAAR1, METH triggers phosphorylation of PKA and PKC, causing a potential reverse function of these monoamine transporters. This in turn increases intracellular calcium and creates DA efflux, as METH also impairs vesicle monoamine transporter 2 (VMAT2) and facilitates DA release into the synaptic cleft (Figure 1.3) [109]. This mechanism is proposed to underlie the main action of neurotoxicity [110]. Neurotoxic effects include neuronal apoptosis, immune response and neuroinflammation through astrocyte and microglia activation, and impairment to dopaminergic and serotonergic neuron terminals [111].

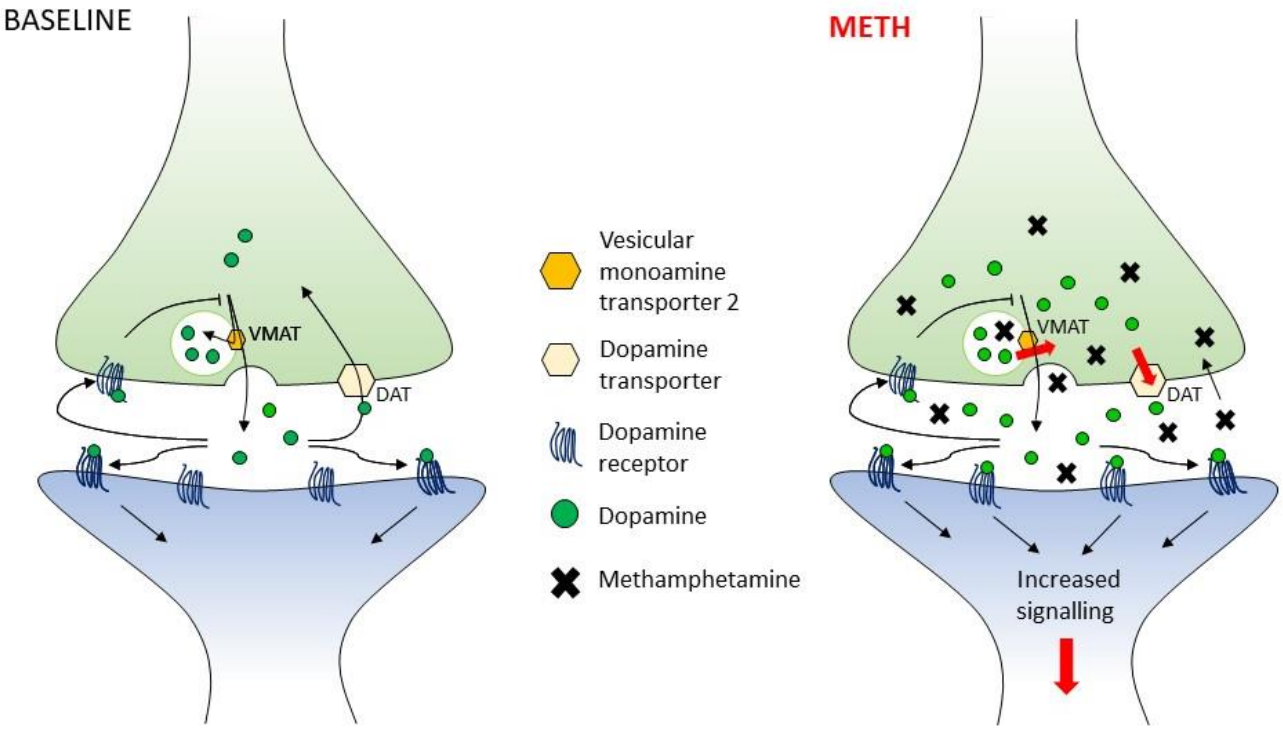

Figure 1.3 Neurochemical adaptations occur at the synaptic cleft. *Response to acute METH intake, resulting in increased post-synaptic signalling.*

1.7.1.3 METH-induced excitotoxicity

Research on the excitotoxicity of METH has recently highlighted the glutamatergic system as an important component of its effects [112]. The concentration of glutamate, the foremost excitatory neurotransmitter within the CNS, increases extracellularly in response to METH [98]. The accumulation of glutamate in the synapse increases calcium ion flow within the neuron intracellularly [113], and activates NMDA and metabotropic glutamate receptors leading to protein kinase C (PKC) activation which secondarily upregulates NMDARs [114] and increases Ca^{2+} influx. Increased cellular Ca^{2+} activates kinases, nitric oxide synthase, and phosphatases which ultimately results in endoplasmic reticulum stress and the initiation of apoptosis, reactive oxygen species, and neurotoxic substrates [98]. Endoplasmic reticulum stress has also been associated with METH-induced dopaminergic toxicity and DA D_1 receptor activation at nerve terminals [115].

1.7.1.4 Neurobiology of chronic METH

Long-term METH abuse has the potential to cause prolonged striatal serotonergic and dopaminergic deficits. In heavy METH abusers the level of dopaminergic markers is reduced in the striatum months or years after abstinence. These alterations over chronic intake have been proposed to lead to one of the most detrimental consequences of METH abuse; the development of a drug-induced psychosis [31, 116, 117]. A study in 2010 found recreational METH use conferred a 2- to 3-fold increase in the likelihood of psychotic symptom development [116]. Symptoms include delusions, delirium, hallucinations, and paranoia [118]. Antipsychotic medications are used to resolve psychosis, although 5-15% of abusers fail to fully recover [119]. Typically, these symptoms are transient and subside 1-2 weeks after METH administration, with gradual reduction of heightened subcortical DA within individuals [109]. However, a proportion of METH consumers do not recover, and their symptoms persist, resulting in psychotic indications that have been compared to paranoid schizophrenia [120].

Chronic METH use causes behavioural sensitization, which is the progressive enhancement of a reaction induced by a stimulus during repeated exposure. Sensitization can remain for weeks following withdrawal and can often be produced through contingent or non-contingent administration, and increased exposure to

METH can result in enhanced sensitivity to the behavioural effects of the drug [121]. Sensitization is the proposed mechanism by which subcortical hyperdopaminergia could arise and present in psychosis within other illnesses, such as the shared symptoms between schizophrenia and METH users. This is supported by studies showing increased DA release in schizophrenia patients to an amphetamine challenge dose, suggesting inherent sensitization within the dopaminergic neurocircuitry of the brain [122].

1.7.1.5 BDNF and METH

Despite BDNF being repeatedly implicated in the neurochemical response to METH abuse [31, 49, 123-125], there are marked inconsistencies in experimental results. It has recently been reported that repeated exposure to METH results in significantly higher serum levels of BDNF during withdrawal, and these serum levels decrease following an extended 30-day abstinence [124]. Meanwhile, high plasma levels of BDNF have also been described that persist 1 month post METH withdrawal [126]. Upregulation of rodent BDNF/TrkB signalling in the NAc shell suggests the signalling pathway is a mediator of the depressive state observed in withdrawal [125], while increased expression has also been described in both the dorsal and ventral rat hippocampus [127]. It was hypothesized that alterations to BDNF availability within the hippocampus infer modification of local synaptic transmission, consistent with previous studies underlining similar BDNF upregulation [128, 129]. These findings correlate with human studies where serum BDNF is elevated in METH-dependent users compared to healthy controls [124]. However, in a separate experiment Chen and colleagues presented opposing evidence; they collected serum from human METH abusers and showed a reduction in BDNF during the first 3 weeks of withdrawal [130]. They demonstrated reduced BDNF in extinction may impair neuroprotective function following repetitive METH exposure [130]. Taken together, these findings could suggest psychostimulants such as METH reduce neuroprotective function through a reduction in BDNF, causing maladaptive neural responses that disrupt homeostatic neurotrophin balance and ultimately lead to psychosis.

BDNF also acts to mediate the neurotoxic effects of METH and is known to prevent nitrous oxide-induced cell death in the hippocampus via the ERK1 and PI3K

pathways [127]. These results support the theory that enhanced acute activation of BDNF in the hippocampus is a protective mechanism intended to defend neurons and maintain plasticity. We have previously demonstrated through endogenous manipulation that perturbed BDNF signalling attenuates changes in behaviour associated with chronic METH-induced psychosis [31]. These findings require further investigation and may suggest dynamic changes in BDNF signalling are involved in a state of persistent METH psychosis and sensitization.

1.7.1.6 METH and Schizophrenia

A large body of evidence has emerged underlining the symptomatic resemblance of METH-induced psychosis to the positive symptoms of paranoid schizophrenia. These theories stem from comparable altered functioning within the mesolimbic dopaminergic system, shared prefrontal cortical dysfunction [131], and evidence of genetic risk factors overlapping both states of psychosis [132]. Consequently, recent preclinical studies have highlighted METH as a risk factor in schizophrenia development, and this theory has led to further investigation. Neuroimaging studies examining METH users have shown correlations between DAT binding levels in the prefrontal cortex and positive symptom severity, as well as a host of other dopaminergic signalling alterations [108, 120]. Genome-wide association studies (GWAS) have identified increased risk factor genes for METH psychosis in schizophrenia patients and these were not present in non-dependent METH users [132]. Genetic associations have been shown in Taiwanese populations that identified familial risk to schizophrenia increased the likelihood of psychosis development during METH use [133]. Schizophrenia and METH psychosis have similarities in neurobiological and cellular changes, including a reduction in DAT binding in the striatum [134, 135], and decreased hippocampal and amygdala volume [136, 137].

Longitudinal studies have reported METH psychosis hospitalization often results in the transition of an acute drug-induced psychotic state to a primary psychosis such as schizophrenia [138, 139]. These observations highlight METH as an inducer of an underlying predisposition to primary psychosis development in genetically-vulnerable individuals, and recently use of the psychostimulant has been deemed a significant factor in schizophrenia aetiology [140, 141].

1.7.2 Schizophrenia

Schizophrenia is a multi-genetic syndrome affecting an approximate 24 million people worldwide, and ranks in the top 10 for disabling conditions in young adults [142]. The disease encompasses a broad spectrum of psychiatric disorders clustered into positive, negative, and cognitive domains, and is estimated within the general population to have a prevalence of 0.4%; 4 in every 1000 persons will develop the condition across their lifetime [143, 144]. Australia has an approximate prevalence of 1%, although this higher, and likely more accurate reflection, is largely due to more sophisticated classification systems and ease of access to mental health diagnosis within developed nations. Although prevalence of schizophrenia is lower than that of other psychiatric illnesses, such as bipolar [145], major depressive, and post-traumatic stress disorders (PTSD), this illness places a comparably large burden on the healthcare system due to its debilitating effects on social behaviour and cognition. People are most affected during their early to mid-working life, from 15-44, where the overall burden comes in at 4th highest for men and within the top 5 for women [146].

Despite the significant detriment worldwide, very few advancements have been made in schizophrenia patient treatment outcomes since the discovery of first-generation antipsychotics in the 1950s, and second generation in the 1980-90s [147]. While these treatments are effective, and their efficacy has improved over time, they are targeted almost solely to the positive symptoms of schizophrenia [148], and do not inhibit cognitive and negative deficits, with 10-40 percent of patients non-responsive to first- and second-class antipsychotics [149]. This is not surprising given the markedly diverse symptom profile between patients, which highlights the need for further treatment development.

There is currently no cure for schizophrenia, and this is due in part to a poor understanding of the complex neurophysiological pathways leading to disease onset [120]. However, with new techniques and technology, it is possible the underlying multimodal aetiology can be unravelled [150]. Schizophrenia research has entered a new era of sophistication, in which preclinical models can aid in the investigation of aspects of schizophrenia previously left untouched [151]. We are fortunate to live in an age where robust genetic tools can be implemented to

examine the effects of protein manipulation and malfunction, and their consequent augmentation of the underlying neurocircuitry caused by disease. Using these tools, studies have been conducted on genetically-modified animals that mimic aspects of the schizophrenia phenotype and permit partial disassembly of the mechanisms involved in schizophrenia pathophysiology.

1.7.2.1 Behavioural phenotype

Schizophrenia has a broad symptomatic profile that affects several domains of cognition and behaviour. Identification of the disease is obtained through analysis of clusters of symptoms broadly grouped together and defined as either positive or negative symptoms, or social, or cognitive deficits [152]. In its infancy, schizophrenia research proposed two principal symptom profiles, these were the negative-positive dichotomy, involving a weakening of emotional, activity driven volition, and a loss of unity within intellectual and emotional activities [153]; a loss of normal function and a gain in aberrant function, respectively. Since then our understanding of the biological basis to schizophrenia development has progressed somewhat, driven through improvements to molecular and behavioural tools used to decipher more complex and subtle abnormalities. This has led to the development of a schizophrenia symptom profile encompassing an array of clusters that are dispersed across disease time points. When active, schizophrenia can induce a range of disorganised and impaired conscious states. The DSM-V diagnosis requires two of five symptomatic criteria are met to qualify disease presence/onset [154]. These include positive symptoms such as delusions and hallucinations, cognitive deficits such as disorganized thinking and behaviour, and negative symptoms. To effectively study schizophrenia and attempt to understand the dysfunctions occurring within the brain, it is imperative that the spectrum of behavioural phenotypes is understood.

1.7.2.2 Negative and cognitive symptoms

Negative symptoms refer to a subset, or cluster, of dysfunctional behaviours observed in schizophrenia symptomology that result in a loss of normal function. These are typically founded in the emotional state and include alogia, avolition, marked apathy, and blunting of expressive responses [155]. Negative symptoms of schizophrenia are considered the most detrimental to an individual of all

clustered phenotypes and have been correlated with decreased functional outcome in patient populations. Difficulties can include social withdrawal, lowered motivation, speech difficulties, movement issues, and diminished responsiveness and communication, which contribute to reduced quality of life and poor socio-occupational functioning.

Cognitive functioning can be moderately to severely diminished in schizophrenia and is a core feature of the disease. Deficits are observed in the domains of working memory, verbal learning, attention, and executive function and typically predate the onset of psychosis [156]. Cognitive deficits are the major contributor to disability, social, and economic burden, making the amelioration of these symptoms a treatment priority. Deterioration of the pathways between temporal and frontal regions of the brain are central to the development of cognitive deficits although the mechanism by which this occurs is still unknown. Antipsychotics have little effect on cognition following first-episode psychosis and effective pharmacological treatments targeting these processes are yet to be implemented.

1.7.2.3 Positive symptoms

The positive symptoms of schizophrenia are classified through a ‘gain of function’ in normal neuronal activity and a loss of touch with reality [157]. One such alteration in schizophrenia is the onset of delusions. These can include thoughts and feelings of being controlled by an external force, thought broadcasting, whereby the individual projects their thoughts vocally into the external world, and thought insertion, in which others perceive beliefs are held within the schizophrenic mind [158]. Hallucinations are a second form of gain of function behaviour that defines schizophrenia diagnosis. Types of hallucinations include auditory inputs that encompass the perception of internal voices that can maintain a commentary or engage with the auditory voice [159]. Other positive modalities include disorganized behaviour and thought disorders, whereby patients have illogical conversational sequences and disconnected content patterns [160]. Overall, these gain of function behaviours that are not typically seen in healthy individuals, cause significant distress to schizophrenia patients and contribute to a state of psychosis.

While current pharmaceutical interventions into the positive symptoms of schizophrenia are broadening [161], the lifelong persistence of the disease is such

that relapse is not uncommon [162, 163]. Downstream indicators of disabilities related to positive symptoms, such as reduced social functioning, deteriorating physical health, and unemployment, often diverge between patients and can have severely detrimental impacts if left untreated [164]. A loss of touch with reality is a common positive symptom, which is associated with significant adverse patient outcomes and contributes to increased risk of suicide mortality [164, 165]. Epidemiology studies show schizophrenia patients are 2-3 times more at risk of disease-related death compared to the general population [164]. Unfortunately, this gap is widening with divergence in morbidity rates on the rise for the past few decades. Despite this, the schizophrenia fatality rate remains unchanged [166], suggesting the gap is a depiction of reduced mortality in the general population, signifying a lack of improved treatment options and disease management strategies for current schizophrenia patients. Side effects of second-generation antipsychotics include weight gain and metabolic syndrome, and these medications have been hypothesized to increase relative morbidity [167], although more recent evidence highlights a reduction in long-term mortality risk in patients who do not use antipsychotic medication during follow-up [168]. Taken together, this evidence demonstrates the heterogeneity of treatment outcomes and underlines a need for an updated pathophysiological model of psychosis prevention in patients.

1.7.2.4 Pathophysiology of schizophrenia

The difficulty in understanding and treating schizophrenia stems from a lack of knowledge of the pathophysiological mechanisms causing the illness. As schizophrenia encompasses a wide range of heterogeneous deficits, there is no one pathological abnormality accountable for every behavioural and neurochemical alteration observed. However, overarching hypotheses exist regarding clusters of behaviours that are affected by components of the general dysfunction. While treatment remains incomplete, the most efficacious antipsychotics are targeted toward DA receptor blockade, and consequent modulation of the dopaminergic signalling system [169].

1.7.2.5 Hyperdopaminergic signalling

Hyperdopaminergia as a pathophysiological trait of schizophrenia has long been accepted due to repeated behavioural and pharmacological observations. A review in the 1980s by Lieberman et al., discussed studies showing amphetamine-fuelled increases in DA could induce psychotic symptoms with marked resemblance to those of schizophrenia phenotypes [170]. Pharmacological studies supported this finding with drug interventions designed to reduce DA levels having ameliorating effects on psychosis [171], and consequent evidence revealing the efficacy of antipsychotics correlated strongly to their DA receptor affinity for antagonism [172]. The DA pathway is considered the primary target for both typical and atypical antipsychotic drugs, which act as D2-preferring DA receptor antagonists, suggesting an integral role for the neurotransmitter in psychosis development [173]. Consequently, DA signalling disruption has long been a focus of schizophrenia research, with studies dating back to the 1970s suggesting abnormal DA receptor density is capable of causing further pathophysiology [174]. However, early findings were limited in functional relevance due to a lack of knowledge regarding dopaminergic loci in the perturbed brain and limited understanding of induced monoamine activation. Post-mortem analysis has since correlated increased D2 receptor density and striatal DA concentration in schizophrenia patients, with unchanged DA transporter (DAT) density [175].

In recent times the DA hypothesis has developed further, with studies investigating pre- and post-synaptic alterations with greater sophistication. Howes et al. recently compared unaffected individuals to schizophrenia patients and found increased TH in the substantia nigra of schizophrenics [176], suggesting increased signalling potential in dopaminergic neurons and their termination in the striatum of the schizophrenic brain. While the analysis of chronic schizophrenia patients has failed to show significantly increased dopaminergic signalling compared to regular state, the acute phase of psychosis has clear upregulation of presynaptic DA synthesis capacity and release, suggesting presynaptic malfunction [177, 178]. Whether this pathophysiology is primary or secondary in nature is still under contention. The effects of hyperdopaminergia have clear implications for the development of positive symptoms but there is less evidence for their involvement in negative and cognitive deficits observed in schizophrenia.

Arguably, the most compelling evidence for dopamine-induced negative and cognitive symptomology is prefrontal cortex hypofunction. The medial prefrontal cortex receives afferent dopaminergic input from the VTA and reduced function in this area is associated with reduced executive function [179]. The prefrontal cortex is known to have regulatory control over subcortical DA systems and a reduction in regular neural patterning within the frontal cortex correlates with hyperdopaminergia in these regions within schizophrenia sufferers [177]. More recent research has associated deficits in cognitive tasks during the prodromal phase of patients with increased striatal DA synthesis [180], which is further correlated to deficiencies in cortical processing during cognitive tasks prior to schizophrenia onset, suggesting potential interplay between both neuropathologies. What remains unclear is the driving force mediating changes in dopaminergic signalling and the order in which cortical and subcortical dysregulation occurs.

1.7.2.6 Hypoglutamatergic signalling

Hypoglutamatergic signalling is another mechanism by which the symptomatology of schizophrenia may be derived. Signalling via glutamate is the primary excitatory form of neurotransmission within the human CNS, and accounts for over 60 per cent of metabolic activity [181]. Signal transmission transpires through activation of ionotropic or metabotropic glutamate receptors. There have been theories regarding altered glutamate levels in schizophrenia patients from as far back as the 1950s when glutamic acid was implemented as a treatment strategy [182]. Over time the idea of a general deficit in glutamatergic neurotransmission was narrowed down and now focuses primarily on the N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor.

The NMDA receptor hypofunction hypothesis was established after administration of ketamine and phencyclidine (PCP), two non-competitive NMDA receptor antagonists, was shown to elicit behavioural effects virtually indistinguishable from aspects of both negative and positive schizophrenia symptom domains [183, 184]. These dissociative drugs provoked delusions, thought disorder, hallucinations and negative symptoms [184], and have resulted in schizophrenia relapse in patients following exposure. Additionally, post-mortem tissue analysis in schizophrenia

studies display reduced subunit density of NMDAR1 in the frontal cortex, highlighting receptor hypofunction [185], although these results are inconsistent with some other reports and require additional investigation.

Supporting the theory of NMDA receptor dysfunction, genetic studies have identified associations between schizophrenia development and NMDA receptor subunit gene variants, as well as genes related to NMDA receptor activation [186]. The hypoglutamatergic theory can also partially explain the delayed onset of schizophrenia, as peak inception in adolescence coincides with NMDA receptor signalling maturation within the dorsolateral prefrontal cortex [187]. While NMDA receptor-mediated reduction in signalling can explain a range of schizophrenia pathologies, the DA hypothesis has superior construct validity due to the greater correlative power of DA-stimulating drugs and their associated risk. In addition, due to the heterogeneous assortment of behavioural characteristics within the disease, it is unlikely a single neuropathology can be held solely accountable. For example, hypoglutamatergic function cannot attest to the externally observable symptoms, or phenomenology, of psychosis observed in schizophrenia patients.

1.7.2.7 Combining pathophysiology

The dysfunction of presynaptic DA transmission can describe the development of positive symptom profiles of schizophrenia but does not support cognitive deficits and negative symptoms common to the disease. Hypoglutamatergic models can account for these deficits, although they hold poor construct validity, in that the glutamate antagonism in animal models does not correlate to human schizophrenia aetiology. Therefore, it is possible a combinatory effect of these neuropathologies could be responsible for eliciting the neurochemical changes necessary for the disrupted behaviours observed. Clinical aspects of schizophrenia may be explained by hypoglutamatergic NMDA receptor dysfunction present on neurons connecting the mesolimbic dopaminergic system. DA neurons in the midbrain are under regulatory control from glutamatergic neurons projecting from the frontal cortex (see Figure 1.2). Functional neuroimaging has proposed hypoglutamatergic signalling may therefore precede, and be primary to, dopaminergic alterations [188]. Furthermore, changes to DA firing patterns in neurons mediated through the

NMDA receptor antagonists, ketamine and PCP, upregulate DA release in response to an amphetamine challenge [189].

1.7.2.8 Schizophrenia chronology and aetiology

According to epidemiological studies, schizophrenia onset peaks in early 20s for men, and mid to late 20s for women, who also exhibit a second vulnerable period during menopause. However, as mentioned previously, patients often display deleterious cognitive functioning well before the first episode of psychosis (FEP), suggesting a sustained period of neurological dysfunction priming prior to classification. Longitudinal studies in the 1960s by Gerd Huber identified subtle cognitive and behavioural deficits, often only self-perceivable and present at pre-psychotic time points [190, 191]. We now know these signs of illness preceding acute and prolonged psychosis occur in the 'prodromal' phase, a term derived from the Greek *prodromos*, meaning forerunner [192]. The prodromal phase of schizophrenia is a retrospectively identified set of initial symptoms temporally related to the onset of psychosis, with high negative predictive validity to patient outcome [191]. These symptoms can be vague and are often misinterpreted as other mental illness-related qualities, preventing the use of prodromal deficits as a reliable identifying marker of schizophrenia onset.

The second disease state is the active or acute phase, whereby patients exhibit a combination of positive, negative, and cognitive symptoms that can appear at heterogeneous progressions in varying intensities, but characteristically result in the first psychotic episode [193]. Typically, diagnosis occurs within the active phase and is present for a period of 4-8 weeks, by which time antipsychotics and remediation therapy are implemented (given patients submit for diagnosis and treatment) [194]. Successful treatment leads to remission, the stage in which affected persons can live a relatively unaffected life, until a further psychotic episode manifests and additional management is required to return to remission once more. The cyclic interchange between remission and psychosis can continue throughout the patient's life and correlates to environmental stimuli driving supplementary neurochemical imbalances [195].

Despite having diminutive predictive power, the progression of the prodromal phase to psychosis during cortical development and remodelling suggests a

complex aetiology leading to disease onset. While the underlying adaptations that cause altered pathophysiology of standard neuronal networks remain unknown, there are identified risk factors for schizophrenia in broad domains of genetic susceptibility, neurodevelopment, and environmental stimuli, which are often sex-specific. The finding, that disease onset peaks between 3 to 4 years later for women than for men [143], with a second vulnerability period for females around the age of menopause, suggests that increased susceptibility in females at this age and highlights a potential role for sex hormones, namely progesterone and estrogen, whose ovarian production fluctuates and begins to taper off at approximately 50 years of age [196]. In addition, schizophrenia development tends to be more severe in males, and has an increased incidence ratio of 1.4 compared to females [197]. Taken together, these findings suggest a potential neuroprotective role for female hormones in schizophrenia development.

1.7.2.9 BDNF and schizophrenia

BDNF dysfunction has been implicated in the pathophysiology of a number of neuropsychiatric disorders including depression, bipolar disease, anxiety-related disorders, and schizophrenia [198]. This is due to the involvement of the neurotrophin in synaptic transmission, development, and reactivity to environmental stimuli including those associated with schizophrenia aetiology. Alterations in BDNF signalling and expression, in combination with environmental stimuli and genetic susceptibility, may combine to produce dysfunctional neuronal signalling and behaviours associated with schizophrenia [29, 199]. In post-mortem schizophrenia tissue, varying and contrasting observations have been made regarding BDNF activity. Some studies report region-specific alterations in BDNF, such as increased frontal cortex expression [200]. Meanwhile, more recent studies have highlighted a reduction in expression of the neurotrophin and its receptor, TrkB, in the cortices [29, 199]. A similar dichotomy can be seen in hippocampal regions with enhanced [201] and reduced [201] reports. Furthermore, TrkB expression appears to be downregulated in the hippocampus of schizophrenia patients [200]. A convergent finding within schizophrenia research is the enlargement of ventricles and reduction in grey matter, primarily in the cortical and hippocampal regions of the brain [202-204]. Collectively, these neuroanatomical

changes could be driven in part by altered BDNF function during development and may contribute to the progression of schizophrenia pathophysiology [198].

1.7.2.10 The two-hit hypothesis

The two-hit hypothesis combines multiple hypotheses on the aetiology of schizophrenia through an amalgamation of genetic and environmental risk factors acting within vulnerability periods during neural development. The theory proposes disease acquisition occurs through offences to the brain that have an accumulative or combinatory effect, whereby the interaction of these insults initiates disease onset [205-207]. Offences (or hits) can be genetic or environmental factors and may lay dormant, having no observable effect on the psychotic state, until a further hit during neurodevelopment leads to the onset of psychoses and eventually schizophrenia [205]. This theory of disease progression can be used to explain the prodromal phase of schizophrenia as deficits are present from an early age, but psychosis remains absent up until secondary insult. Hits occurring *in utero* and during adolescence, when the brain is most at risk, have the strongest correlation to schizophrenia development [208]. Genetic mutations or related issues during pregnancy most likely act as a first hit and result in a vulnerable neurodevelopment trajectory. Second hits could include environmental factors such as drugs and other stressors, and these would have amplified effects in 'schizophrenia-primed' neurocircuitry. A number of animal model studies have begun to examine gene + environment interactions [209, 210], but further work is required to untangle these connections and drive the two-hit hypothesis forward into further clinical examination.

1.7.3 Animal models of psychosis

Animal models of schizophrenia and other mental disorders are important preclinical tools for understanding the molecular and neurochemical changes preceding psychosis. They provide a platform to trial pharmacological intervention and are the first step in preventing disease and improving patient outcome. Unfortunately, this method is not as straightforward in schizophrenia and related psychoses due to the complicated interactive aetiology and developmental course of the disease that has proved difficult to replicate thus far. Owing to this complexity, the most popular method of studying schizophrenia neurobiology is

through behavioural endophenotypes that can be used to measure dysfunction and symptoms [32, 211]. Endophenotypes represent subsets of symptoms that occur in disease and are often derived from fewer neurocellular perturbations, with clear genetic founding, that can be analysed and assessed more easily than the symptoms as a whole. Through this method the positive symptoms of schizophrenia can be examined in animal models via endophenotype analysis. Schizophrenia endophenotypes model specific dysfunctions observed in the disease and are also important determinants of the neural substrates governing drug-induced psychosis. In addition, two-hit animal models can be used to measure the combination of 'environment + gene' interactions on endophenotypes capable of measuring specific symptomatology [212]. Schizophrenia two-hit animal models are in their infancy but already show a more realistic representation than previous models of the aetiology of the syndrome and better represent the conditions under which psychosis may develop through combinatory processes [213].

1.7.3.1 Face validity of schizophrenia mouse models

In preclinical research, face validity can be defined as the degree to which a behaviour exhibited by an animal shares similarity with the specific symptoms of a human condition. However, when studying mouse models of schizophrenia, it is unrealistic to expect similar behaviours between humans and rodents. To this end, preclinical studies instead focus on relevant parallels, such as brain areas involved, and neurotransmitter systems affected by the disease [214].

Furthermore, it is important to note that individual rodent behaviours should not be used as animal models of schizophrenia. Due to the heterogeneity of the disease, no single behavioural test can represent its complete symptomology. Instead, different behavioural mouse models represent different aspects of the disease, together covering the broader schizophrenia pathophysiology. Paradigms may target negative symptoms, for example by examining social withdrawal, reduced nesting behaviour, decreased preference for social novelty, and altered aggression [215]. Cognitive symptom analyses of decreased working memory are modelled through deficits in the T-maze and 8-arm radial maze, and general cognitive deficits include decreased spatial learning in Morris water maze and decreased spatial

learning in 8-arm radial maze [215]. The combination of investigating direct and indirect models will ultimately lead to a more sophisticated framework for testing the pathological and molecular traits of schizophrenia symptomatology [214, 215].

1.7.3.2 Disrupted prepulse inhibition as an endophenotype

Sensorimotor gating is a form of CNS inhibition that filters out irrelevant data and permits the focused processing of more salient information. Prepulse inhibition (PPI) is an operational measure of sensorimotor gating and is a reflex-like behaviour, activated in response to external stimuli to prevent information overload and cognitive fragmentation [216]. Given that the reduced ability to filter sensory material is considered a core psychopathological construct of schizophrenia, altered PPI has been labelled as an endophenotype of the disease and used to measure disruptions in gating that occur prior to cognitive feedback. It is important to note that some other psychiatric and neurodegenerative diseases are accompanied by disruption of PPI, including obsessive compulsive disorder, Huntington's disease, Parkinson's disease, Alzheimer's, and autism spectrum disorders [217-220]. PPI of acoustic startle can be measured through a reduction in the evoked startle response [221]. Startle inhibition occurs when a weaker pre-stimulus, or prepulse, is applied prior to a more intense startling stimulus, or pulse, priming the system and attenuating the reaction that would otherwise occur. A number of investigations examining schizophrenia symptomatology have reported altered sensorimotor gating, including greater startle propensity and reduced PPI, in schizophrenia patients versus healthy matched controls [222-224]. Disruption of sensorimotor gating is also evident in schizophrenia familial studies, where the heritability of PPI deficits is 47% higher compared to unaffected families, and positively correlated with the vulnerability of psychosis development [225]. In addition, a reduction in PPI is displayed by first-degree asymptomatic relatives of schizophrenia patients [226], and by subjects who score highly on clinical tests designed to measure psychosis susceptibility [227]. Combined, these results suggest commonality of genes governing PPI and psychotic disease and that disrupted PPI can be used as a biomarker and as an endophenotype of schizophrenia and psychosis development.

1.7.3.3 PPI animal models

As the neurochemical pathways governing gating mechanics are relatively well conserved between rodents and humans, PPI is considered a cross-species measure of sensory gating, with good test re-test reliability and strong face-, and construct validity [228, 229]. Reduced PPI in human schizophrenia patients has prompted preclinical studies in rodents examining gating deficits observed through the acoustic startle response [230]. This is measured by recording whole-body startle while the animal is subjected to a range of auditory pulse and prepulse combinations that vary in intensity and interstimulus interval (ISI; refer Figure 1.4).

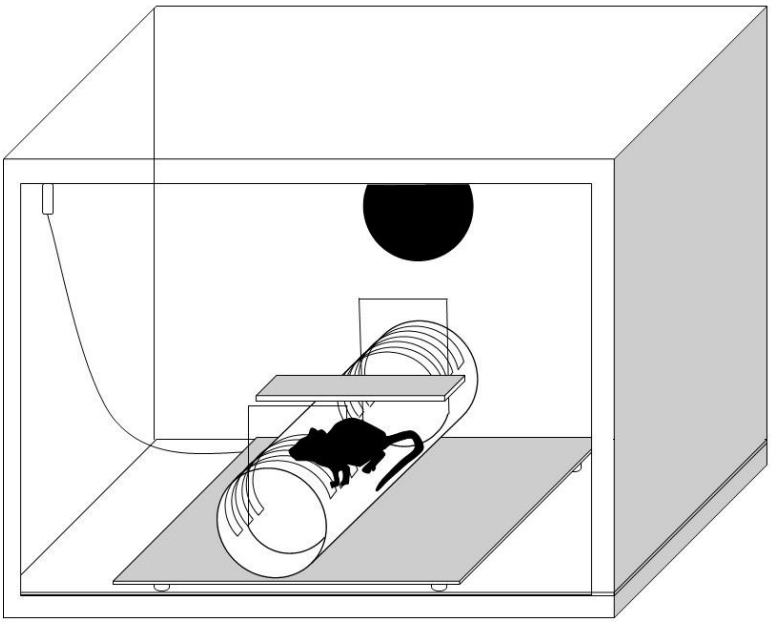

Figure 1.4 Illustration of the mouse prepulse inhibition chamber*. Mice are held in a Perspex cylinder within the box while the acoustic startle response is measured over repeated and varied prepulse intensities and interstimulus intervals.*

Humans and rodents display parallel response patterns of PPI, and neurochemical studies have replicated schizophrenia gating deficiencies in rats and mice through pharmacological and developmental manipulations [231], although rarely in combination. Importantly, the evoked disruption of rodent PPI is often reversible through the administration of antipsychotic drugs, suggesting strong predictive validity in animal models comparing cognitive and psychotic deficits with targeted drug therapies [229]. Preclinical testing has focussed on pharmacological screening, as well as providing a platform for genetic studies, aimed at unravelling the correlation between attenuated PPI and psychosis development [230].

1.7.3.3.1 Disrupting PPI

It is well documented that PPI is affected by alterations to the serotonergic, glutamatergic, and dopaminergic systems. In rodents, disrupted startle inhibition occurs following administration of drugs targeting receptors associated with all of these neurotransmitter systems. Dopaminergic drugs shown to attenuate PPI typically stimulate the D2R and include both direct and indirect modes of receptor stimulation, such as the non-selective DA receptor agonist – apomorphine (APO), and amphetamine respectively [231]. Selective D2R agonists can also disrupt PPI, with a reduction in PPI first demonstrated in a rat study examining the D2R agonist, quinpirole [232]. Studies on the role of DA receptor mediation of PPI has since expanded to include the majority of D2R agonists and, combined with the fact that a reduction in PPI is blocked via antipsychotic medications, suggests sensorimotor gating is disrupted primarily through DA D_2 receptor family signalling. Thus, PPI regulation can be examined and used to study dopaminergic hyperactivity in preclinical animal models and is applicable to the examination of schizophrenia aetiology.

Glutamatergic signalling, particularly via the NMDA receptor, has similarly been demonstrated as a component of PPI regulation. NMDA receptor antagonists, such as phencyclidine (PCP) and MK-801, have psychotomimetic effects in humans, and studies have likened symptoms obtained during their administration in humans and rodents to features of the cognitive deficits present in schizophrenia [233-235]. Furthermore, atypical antipsychotics can reduce the effect of NMDA receptor antagonists on PPI, although with varied efficacy, suggesting substantial

complexity within the circuitry governing gating disruption [236]. Despite limited understanding of the neurochemical pathways surrounding NMDA receptor mediation of sensorimotor gating, the disruption of PPI by antagonists of this glutamate receptor, combined with the efficacy of antipsychotic drugs in restoring these deficits, highlight that PPI can be used as an endophenotype of hypoglutamatergic function. Thus, PPI can be used as a measure of both hypoglutamatergic and hyperdopaminergic signalling within the CNS to model the positive symptoms, and to a lesser extent, the cognitive deficits of schizophrenia.

1.7.3.4 Locomotor hyperactivity (LHA) as an endophenotype

Like PPI, LHA is an established behavioural representation of brain mechanisms involved in ADHD [237], Huntington's Disease [238], dementia [239], and the positive symptoms of schizophrenia. As the definition of psychosis in schizophrenia is inherently human, animal studies are unable to model the complex behavioural outputs of hallucinations and delusions in the rodent brain. Therefore, preclinical research must rely on aspects of rodent behaviour capable of representing schizophrenic endophenotypes and their associated psychoses that can be quantifiably measured through an experimental paradigm. At a simplistic level, enhanced dopaminergic activity increases LMA through heightened stimulation of the motor cortex, executed through alterations in nigrostriatal and mesolimbic DA pathways via the two key mechanisms implicated in schizophrenia development, hyperdopaminergic and hypoglutamatergic neurotransmission [216].

1.7.3.4.1 LMA animal models

Despite high construct validity, LMA holds poor face validity when comparing human and rodent systems as the behavioural output of DA stimulation differs between species [214]. Despite this caveat, LMA analysis can be employed in behavioural design with relative ease, has high throughput, and is easily implemented in studies targeting dopaminergic neurocircuitry during disease [214]. Rodent LMA analysis involves testing in an open field, in which the animal is free to move around and explore the cell. This platform can be used to examine a variety of quantifiable motifs including horizontal locomotion, stereotypies, and vertical locomotion, or rearing, which is recorded within the field by tracking movement through the disruption of photoelectric beams (Figure 1.5). Results from

photocell analysis can then be compared between genotypes or drug treatment groups to determine DA-driven endophenotype effects on animal activity [240].

To date, the majority of research focussing on LHA as an endophenotype of schizophrenia has involved the use of psychotropic drugs that act via stimulation of monoamine release, ultimately resulting in elevated extracellular DA [214]. DA signalling was first associated with rodent hyperactivity when experiments in rats used 6-OHDA to partially deplete the neurotransmitter from the NAc, leading to attenuation of amphetamine-induced hyperlocomotion. This result suggested subcortical DA was an important mediator of hyperactivity [241], and further highlighted enhanced LMA as a measurable output of subcortical DA activity. More recent analysis of LMA in mice treated with acute amphetamine has described similar effects of the psychostimulant on behavioural hyperactivity [31, 215], and these findings have clinical relevance to schizophrenia, where increases in subcortical DA are associated with positive symptom development.

Modification of NMDA receptor signalling can also affect LMA. Like PPI, LMA is sensitive to hypoglutamatergic transmission and studies implementing NMDA receptor antagonism in animal models result in a dose-specific response. Low to moderate doses of MK-801 and PCP induce hyperlocomotion in mice and rats [242, 243], and in humans these drugs at similar concentrations are known to incite aspects of psychosis that are comparable to schizophrenia positive symptoms.

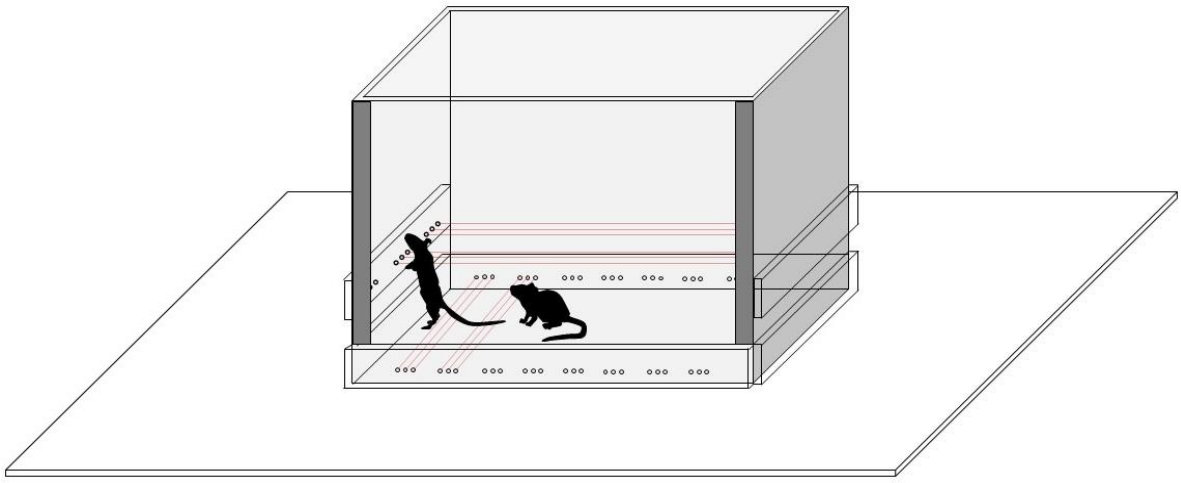

***Figure 1.5 Illustration of the mouse locomotor photocell arena**. Mice are held in a clear box while locomotion is measured before and following acute treatments. Photoelectric beam disruption records movement including vertical (left mouse) , and horizontal (right mouse) planes. Note: Only one mouse is present within a chamber during testing.*

Therefore, analysis of the hypoglutamatergic-induced LMA response in rodents may be relevant to psychosis research and observations made in this system may be relevant to further understanding schizophrenia development.

1.8 BDNF and addiction

Over the years BDNF has been highlighted not only as a mediator of psychosis symptomatology, but also as a partial regulator of addiction. Alcohol use and abuse are reported to significantly affect BDNF signalling, preventing neuronal maintenance of the mesocorticolimbic DA system, and enhancing an addictive phenotype following excessive alcohol consumption.

1.8.1 Alcohol / ethanol

Chronic intake of alcohol, also known as ethanol, is an addictive substance use disorder that often precedes long-term chronic disease. Excessive alcohol consumption causes a variety of primary injuries through liver impairment and neurodegeneration, frequently resulting in public and domestic violence and a broad spectrum of social dysfunction disorders [47]. While acute alcohol intake can lead to behaviour-related injury, long-lasting dependence can alter blood pressure, cardiovascular health, mental stability, and is increasingly correlated with liver cancer progression [244]. Alcohol correlates with diabetes onset in a curvilinear pattern, as lower rates of consumption have a protective effect while heavy drinking is associated with increased diabetes development [245]. Alcohol consumption can also impact infectious disease progression and has been identified to interact with human immunodeficiency virus (HIV) [246], pneumonia [247], and tuberculosis [248]. These interactions are largely driven by compromised immune response in heavy drinking individuals with established alcohol dependence [249]. Alcohol use disorder (AUD) development, a form of SUD, is also associated with concurrent substance use, in which the risk of the development increases with concurrent use of other drugs such as marijuana and/or cigarette smoking [250].

The prevalence of alcohol abuse is high, with 5.3% of all deaths and 5.1% of the global burden of disease and injury attributed to alcohol in 2018 [251]. Alcohol is consistently reported as the most commonly abused legal psychoactive substance in adolescents, and given its significant economic and social burden,

understanding the neurochemical adaptations that lead to alcoholism is of great importance. While the aetiology of AUD has not yet been recognised, amelioration of alcohol addiction may be achieved through a better understanding of the excitatory vs. inhibitory pathway imbalance facilitating ethanol craving.

1.8.2 Alcohol addiction neurobiology on DA system

Ethanol acts as an indirect facilitator of DAergic activity, and DA release from presynaptic terminals within the mesocorticolimbic dopaminergic system of the brain. As it does not selectively bind to DA receptors, the underlying mechanism of ethanol-induced DA release has been shown, predominantly in animal models, to be indirect DAergic modulation [252]. Studies highlight increased release of the neurotransmitter at synaptic terminals [253], and an increase of extracellular DA through modification of γ-aminobutyric acid (GABA; GABAergic) interneuron opioid receptors in the NAc [254].

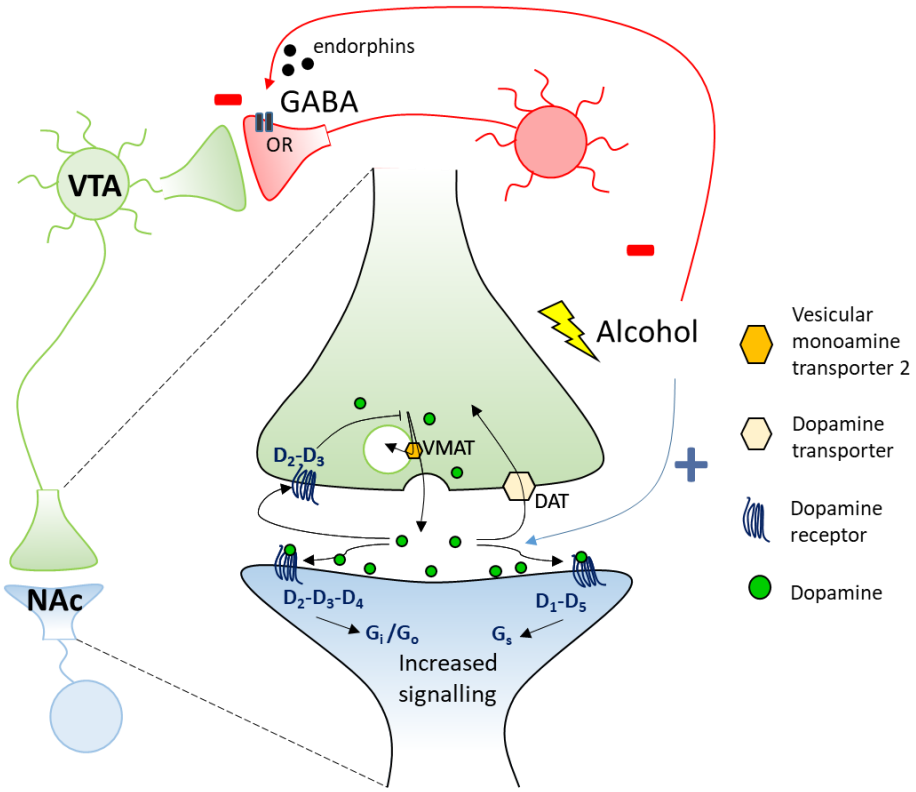

***Figure 1.6 A proposed mechanism of alcohol-induced hyperdopaminergia**. Ethanol interacts with signalling systems within GABAergic neurons in the VTA and reduces their inhibitory effect on DA neurons innervating the NAc. Ethanol increases opioidergic activity, disinhibiting DAergic neurons through opioid receptors in the VTA.*

Theoretically, the inhibition of GABAergic neurons in the VTA, that would otherwise dampen DA release through tonic modulation, would result in enhanced presynaptic DA release (Figure 1.6) [255]. It is important to note that ethanol also impacts other neurotransmitter systems through modulation of synthesis and functional capacity, for example the serotonergic (5-HT) and glutamatergic systems [256], however, this thesis will focus primarily on DA signalling.

Similar to that of other addictive substances, alcohol stimulates DA efflux from cells within the VTA that promote behaviourally-driven motivation and reward [257]. Downstream stimulation of the NAc and prefrontal cortex via dopaminergic innervation is postulated to reinforce drinking and elicit salience to alcohol consumption. Consequently, the mesolimbic DA system has been proposed to mediate a neurobiological addiction to alcohol through reward-based learning [258].

In the acute setting, ethanol exposure modulates activity of GABA receptors and reduces ion flow through N-methyl-D-aspartate (NMDA) glutamate receptors, resulting in neuron inhibition [259]. Chronically, repeated ethanol intake promotes upregulation of NMDA receptor trafficking, increasing excitability potential [260]. This process can lead to an amplified reaction to cues associated with drinking and establishes a state of hyperexcitability upon cessation of alcohol intake. It is hypothesized glutamatergic plasticity at the synapse of DA neurons may regulate aspects of the vulnerability to developing substance addictions, such as AUD [258].

1.8.3 BDNF and alcohol addiction

BDNF has been implicated in alcohol use disorders and the underlying mechanisms enabling addiction. Alcohol intake has been shown to reduce BDNF expression over intermittent, repeated, moderate, and chronic exposure in rodents [261, 262]. Neuronal networks adapt to recurrent ethanol presence, altering a homeostatic balance that leads to craving in ethanol absence and reducing BDNF expression [263].

BDNF can also regulate drug sensitization and drug self-administration, although this regulation appears to be regionally specific, and differs between addictive drugs. For example, cocaine craving in rats is increased following infusion of BDNF into the NAc [46], while a reduction of BDNF in the same region is correlated with heightened alcohol preference [264]. BDNF in the medial prefrontal cortex (mPFC) has a similar role with elevated endogenous activity within the mPFC reducing cocaine self-administration [265], while a reduction of levels of the neurotrophin escalates alcohol drinking in rats [266-268]. BDNF in the dorsal striatum may act as a negative regulator for alcohol intake [27] and amygdaloidal BDNF represses anxiety-like behaviour and ethanol intake through increased dendritic spine density [269]. These similarities and differences between brain regions and between addictive substances, infer local specificity of the role of BDNF in reward pathways.

BDNF HET models have been used primarily in the two-bottle choice paradigm where mice develop an increased sensitivity to ethanol exposure [270, 271] and a preference for alcohol consumption [270]. Rat models are favourable due to their enhanced learning capabilities and comparable neurocircuitry to humans [272]. More specifically, similar to humans, rats show a strong alcohol deprivation effect which is not as pronounced in mouse models [273]. The role of BDNF within the dorsal striatum has been studied in rats using operant, restricted, and *ad libitum* ethanol access [274, 275]. However, no analyses have been done in BDNF haploinsufficient rats. Furthermore, studies on the role of BDNF in alcoholism have yet to compare and acknowledge potential sex-specific interactions, with previous studies primarily testing male and rarely female animals, but never in parallel. This is despite the suggestion that female rodents have increased sensitivity to BDNF attenuation [270] and accelerated alcohol dependence [276], reflecting potential sex-specific mechanisms in alcohol addiction.

1.9 Schizophrenia and AUD Comorbidity

Substance use disorders (SUDs) are the most common psychiatric comorbidity in patients with schizophrenia. Individuals with the disease are six times more likely to develop a drug use disorder compared to the general population [277] and between 20-70% of surveyed patients report comorbid substance abuse. AUD is the most common co-occurring disorder [278], with an estimated 20% of

schizophrenia patients presenting with a lifetime AUD diagnosis. SUD comorbidity is thought to be derived from a combination of psychological and biological factors.

It was first hypothesized that alcohol and other drugs were used as a form of self-medication [279], acting to alleviate aversive positive and negative symptoms and antipsychotic side effects. However, AUD often precedes schizophrenia and there is evidence to suggest schizotypal individuals, who are at higher risk of psychosis development and exhibit characteristics of schizophrenia, have an increased propensity to consume alcohol prior to psychosis onset [280]. Furthermore, neuropathological changes associated with schizophrenia are also thought to promote the positive reinforcement of dopamine-driven behaviour, contributing to the overall vulnerability to addiction [281]. This is based on alterations to neural circuitry regulating positive reinforcement in schizophrenia that overlay on substrates governing addiction, primarily the mesocorticolimbic dopamine system. The theory is supported by experimental interventions in schizophrenia animal models, that display accelerated positive reinforcement and motivational effects of reward networks that drive addictive behaviour [278].

1.10 Thesis outline

The overarching aim of this project is to determine the role of BDNF in the mesocorticolimbic DA system, in mediating aspects of METH psychosis and alcohol use disorders, that occur following prolonged intermittent exposure. Three studies are included.

1.10.1 Study 1: METH in BDNF HET mice

We have shown previously that BDNF HET mice become sensitized to METH following an escalating dose regime in which the amount of METH received is incrementally increased over a 3-week period during adolescence [31]. Sensitization is observed weeks after the chronic injection phase, similar to previous escalation protocols that have been implemented to replicate real life scenarios leading to psychosis. This method has superior construct validity compared to acute METH administration, as the frequency of drug taking better represents the escalation seen in human users at similar time points. Acute high doses of METH without prior escalation can also induce excitotoxicity and

confound sensitization results. Previous work from our laboratory identified that BDNF HET mice display enhanced hyperlocomotion to acute amphetamine treatment as well as a relative lack of effect of chronic METH pretreatment on the locomotor response to an acute amphetamine challenge compared to saline-pretreated BDNF HET mice. This led to the proposal of endogenous sensitization in BDNF HET mice [31]. These findings require replication and expansion to a range of chronic doses to determine the strength and temporal relationship of the disruption to METH sensitization in BDNF HETs. Firstly, it was considered important to replicate previous work and confirm the endogenous sensitization observed in HET mice. Furthermore, while it is still unknown how much METH exposure is required for the development of these sensitisation effects, this thesis will use protocols with shortened METH exposure or reduced drug dosage that reflect diverse METH use in human users. This study, designated 'Low-dose study', aimed to provide detailed dose/effect information on the interaction of BDNF deficiency and METH sensitisation, and could shed light on the importance of early intervention in METH users and prodromal schizophrenia.

1.10.2 Study 2: METH in BDNF HET D3KO mice - D3R-mediated sensitization hypothesis

METH-dependent subjects develop sensitization to the effects of DA-releasing psychostimulant drugs. Importantly, DA receptor affinity varies between D1- and D2-like receptor families and the response to stimulation therefore differs between individual DA receptor subtypes depending on rates of DA occupancy [282]. Compared to the D1R and D2R, D3R have markedly higher affinity for DA [283], which means a small change in D3R expression or function may confer dramatic effects on synaptic DA regulation. DA receptor affinity is measured by the constant, Ki, defined as the substrate concentration needed to reach 50% receptor occupancy. D3R have a Ki of 30nM, compared to 2300nM for D1R and 2000nM for D2R [284]. This is relevant when considering DA release measurements that confirm prolonged and up to 14-fold elevated DA extracellular concentrations within the striatum following administration of D-amphetamine in rodents [285]. Extracellular DA concentration typically sits at 50nM. The D-amphetamine dose used in these studies (2mg/kg) increased DA concentration to approximately 700nM [286]. At this higher concentration D3R occupancy has been calculated at

95+%, compared to D2R 27% and D1R 25% [87]. Given the prolonged increase in striatal DA tone it can be theorized that some form of receptor tolerance may occur in response to repeated amphetamine exposure. Due to the varied affinity of the DA receptors, tolerance would occur at differential rates, first at the D3R. As D3R predominantly play an inhibitory role in DA neurotransmission, loss of postsynaptic and autoreceptor mediation would facilitate D1R and D2R postsynaptic signalling and contribute to sensitization development through loss of negative feedback mechanisms that would usually promote DA reuptake and inhibit further release [87, 287].

This is supported by PET displacement/competitive binding studies demonstrating a reduction in D3R binding by the D3R-preferring radioligand, ^{11}C-[PHNO], in the dorsal striatum of DA sensitized human amphetamine- [288] and METH-dependent users [285]. Similar reductions in D3R binding have been observed in the NAc of rodents, demonstrating increased vulnerability to cocaine intake and seeking behaviours in rats [289]. Although it should be noted that these findings cannot be attributed solely to D3R activation, due to poor D2-like receptor ligand specificity, a genetic approach using D3KO mice also demonstrated an increase in vulnerability to cocaine-taking and seeking behaviours [289], further highlighting a role for these receptors in sensitization. Adaptive downregulation of D3R has also been implicated in the development of amphetamine sensitization in rodents [290, 291], where D3R mRNA and protein levels are reduced in limbic forebrain regions following chronic amphetamine treatment. Taken together, these findings suggest downregulation of D3R in DAergic systems in response to sensitizing psychostimulant drugs.

1.10.3 Study 2: METH in BDNF HET D3KO mice

D3R expression, which is reduced in BDNF HET systems, may play a role in DA sensitization within mesocorticolimbic networks. To examine this relationship, a genetically-modified double-mutant mouse was developed for use in this project. This thesis is the first to combine genetic alteration in two systems: BDNF HET and D3R knockout. This new preclinical BDNF HET / D3KO double mutant model provides a method to study the role of the D3R in the involvement of BDNF in psychoactive disease development and allows further interrogation of the

relationship between BDNF and the DA system, specifically the D3R, in psychosis endophenotype pathophysiology. These mice were tested for changes in PPI and LMA to assess the development of neuroadaptations associated with the positive symptoms of schizophrenia.

1.10.4 Study 3: Alcohol studies in BDNF HET rats

Finally, in rats the studies in this thesis aimed to first examine how BDNF heterozygosity contributes to aspects of alcohol addiction, such as the persistence to acquire alcohol and the craving propensity following withdrawal. Secondly, studies in this thesis will examine this relationship in reverse through BDNF receptor agonism by 7,8-DHF that may provide additional evidence of the role of BDNF in alcohol use disorders and addiction.

Chapter 2 General methodology

2.1 Animals

2.1.1 Mice

Male and female mice were bred and housed at the La Trobe Animal Research and Teaching Facility (LARTF; La Trobe University, Melbourne). Experiments conducted in mice incorporated four distinct genotypes. These included wildtypes (WT) with no genetic manipulation, BDNF heterozygotes (BDNF HET) possessing only one functional copy of the BDNF gene, DA D3R knockouts (D3KO) that lacked D3R, and double mutants, which combined D3R loss and BDNF haploinsufficiency within the same animal. BDNF HETs used in these studies were first established through targeted deletion of the BDNF gene via disruption of exon 5, resulting in complete removal of BDNF mRNA expression in the homozygote mutant mouse [37]. These mice were backcrossed onto the C57BL/6 background strain to obtain heterozygote mice that have an approximate 50% reduction in BDNF protein [35]. D3KO mice were a generous gift of Dr John Drago, University of Melbourne, Australia, and were originally established by targeted deletion of the mouse D3 gene [84, 292] in the 129/sv strain prior to backcross onto C57BL/6. C57BL/6 are among the most active mice and are a common strain for backcrossing a new mutation onto a standard background. This is predominantly because C57BL/6 mice breed well and are widely used in different laboratories for preclinical research. Furthermore, PPI and LHA experiments appear largely replicable across laboratories and few strain differences have been identified [293].

Mice used in the D3R double-mutant study were bred via a two-step breeding strategy (Figure 2.1):

- Step 1: BDNF HET / D3R double mutants were generated by first crossing BDNF HET mice with D3R knockouts, generating 50% BDNF HET / D3R HET and 50% BDNF wildtype / D3R HET. These animals were used as founding breeders of the double-mutant line as well as the single-mutant controls and wildtype controls, all with the same genetic background. This also ensured equal maternal behaviour irrespective of the genotype of the offspring.

- Step 2: Mating BDNF HET / D3R HET mice with BDNF wildtype / D3R HET generated eight possible genetic combinations. To account for maternal behavioural effects, genotype/sex combinations were randomised. The experimental groups that were tested are highlighted in Figure 2.1.

Total number of mice used across METH studies:

- Low-dose project n= 192.
- BDNF/D3KO double mutant project n= 193.

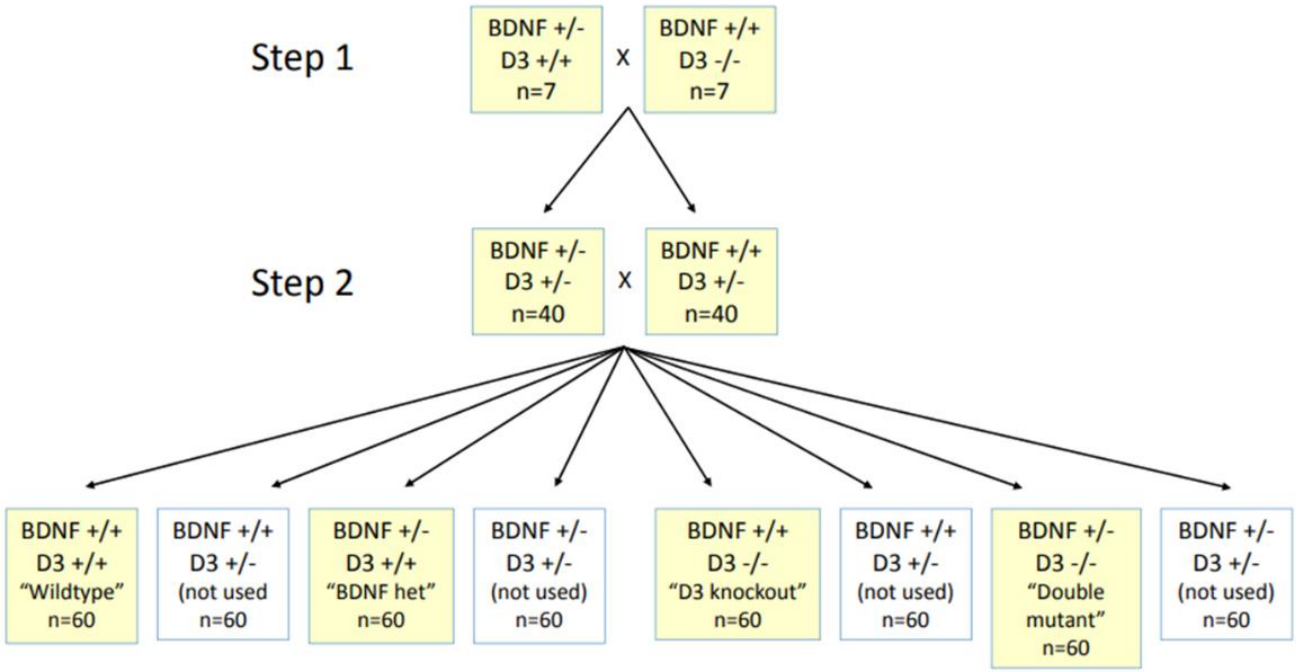

Figure 2.1 Two-step breeding protocol. *Diagram depicts the strategy used to obtain four genetically distinct groups of mice for psychosis-like behavioural analysis. Note n numbers in the figure relate to numbers used for animal ethics application.*

2.1.1.1 Housing

Mice were housed in individually-ventilated cages (IVC; Tecniplast, Buguggiate, Italy) in same-sex groups of 2-5 and were provided with a stage and 'fun tunnel' (cardboard roll) as standard enrichment material. To account for confounding littermate effects, the number of mice per litter per treatment combination did not exceed two mice per experimental group. Mice had *ad libitum* access to standard rodent chow and drinking water and were maintained on a 12-hour light/dark cycle,

8am on, 8pm off (varied ±1 hour depending on daylight savings but remained constant for mice throughout experiments). Cages were cleaned every fortnight and all experiments were conducted during the light period prior to any cage cleaning to avoid possible behavioural disturbance. BDNF HET and WT mice from study one, and BDNF HET, D3KO, double mutant and WT mice from study two were genotyped via PCR through analysis of samples sent to Transnetyx (Cordova, Tennessee, USA).

2.1.2 Rats

Male and female BDNF HET and WT rats were bred and housed in LARTF, while wildtype rats used in a follow-up study were purchased from the Animal Resource Centre (ARC, Canning Vale, Australia) at 6 weeks of age and housed in LARTF for the remainder of the experiment. The BDNF HET rat model was on a Sprague-Dawley genetic background and originally established by Zinc Finger Nuclease (ZNF)-induced knockout (SD-BDNF^{tm1sage}, SAGE Labs). Rats from both cohorts were kept in same-sex groups of 1-4 in reverse light cycle conditions (12 hour: on 8pm, off 8am ±1 hour depending on daylight savings) with *ad libitum* access to standard rat chow and drinking water. Cages were cleaned once per week outside of experimental testing hours. The litter effect was accounted for by implementing the same rule established in the mice, having no more than 2 littermates per experimental group for analysis. BDNF HET and WT rats from cohort one were genotyped via PCR through analysis of samples sent to Transnetyx (Cordova, Tennessee, USA).

2.1.3 Ethics approvals

All experiments conducted in mice and rats were approved by the La Trobe University Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals enacted by the National Health and Medical Research Council of Australia (NHMRC). Ethics numbers approved and completed during these projects include: 16-05, 16-76, 15-64, and 18073.

2.2 Species selection

This study incorporated both mice and rats in behavioural paradigms, based on practicality and differences between species in testing domains. Compared to rats, mice are a more economical model for conducting psychosis research incorporating substantial drug dosing regimens. This project would have required significantly more funding to replicate escalating METH use in rats based on the significant weight differences and dosage requirements. Furthermore, the majority of genetically-modified models, such as D3KO, are created in mice and are therefore more readily accessible in this species.

That being said, there are notable differences in behaviour between rat and mouse species regarding behavioural tests conducted in this thesis. Pharmacological differences in regulation of PPI across species include reduced sensitivity to PPI disruption via D2R agonism in mice compared to in rats [294]. Furthermore, it has been shown that D1Rs play a more important role in modulation of PPI in mice compared to rats, where direct DA agonism with APO disrupts PPI via the D1R [230]. Interestingly, in rats PPI is not disrupted substantially by D1 agonists [295], suggesting there are differences in the regulation of PPI across rodent species regarding contribution of D1 and D2 receptor-mediated modulation of sensorimotor gating. Unlike PPI, rats and mice both respond to METH challenge with comparable locomotor hyperactivity [296-298]. To our knowledge the neural circuitry governing LHA is preserved across rodent species and therefore did not play a role in selection of species. Finally, previous studies have investigated the aptitude of mice and rats in acquiring, extinguishing, and reinstating operant self-administration of alcohol [299-301]. Rats were chosen for addiction studies in this thesis based on their superior learning capabilities, strong alcohol- deprivation effect, and compulsive drinking patterns following extinction, which is yet to be demonstrated in mice [302].

2.3 METH treatments in mice

During adolescence at 6±0.5 weeks of age, mice were randomly assigned to one of three dose regimens of METH ((±)-Methamphetamine-HCl, National Measurement Institute, Pymble, NSW, Australia), or alternatively, administered a volume-matched saline vehicle solution (0.9% sodium chloride) to act as control. METH and saline solutions were administered via intraperitoneal (IP) injection at a volume of 5mL/kg. Animal weights were measured twice weekly (Monday and Wednesday) to ensure accurate dosage across the five-day injection week. The escalating dose design in these studies covers a 3-week window of adolescence in mice that overlaps with adolescence/young adulthood in humans, a period associated with enhanced vulnerability to the effects of psychotropic drugs and psychosis development (see [303] and General Introduction).

Previous work from the laboratory on the effects of METH pretreatment in mice has demonstrated a dopaminergic sensitizing effect following a 3-week escalating dose regimen [31]. To further interrogate this sensitization model, a low-dose protocol was developed with reduced METH exposure over two alternative 3-week schedules, as summarized in Table 2.1. Briefly, mice in the low-dose protocol that receive the least exposure (designated 100) were given daily injections of 1mg/kg METH Mon-Fri for 1 week prior to 2 weeks of 2x saline injections Mon-Fri. The second group were administered 1mg/kg METH once daily Mon-Fri for 3 weeks, and an additional injection of saline in the 2nd and 3rd weeks (designated 111). The final METH treatment group received the original escalating protocol described below (designated 124). Mouse in the BDNF/D3KO double mutant project were all administered 1mg/kg METH Mon-Fri in the first week, 2x 2mg/kg METH in the second, and 2x 4mg/kg in the third week. The twice daily injection of METH is required to escalate dosage over time, acting to mimic how a drug user may escalate their METH use over time. Two injections per day maintains a relatively uniform concentration of METH over the 24-hour period and prevents overdose in the third week when daily concentrations are too high for a single injection. Saline-matched controls received the same frequency of injections.

Table 2.1 METH administration protocols used in mice in this thesis.

IP injection protocol	Week 6 Mo-Fr 9am	Week 7 Mo-Fri 9am +4pm	Week 8 Mo-Fri 9am + 4pm
Saline (SAL)	SAL	SAL + SAL	SAL + SAL
Low-dose 1 week (100)	1 mg/kg METH	SAL + SAL	SAL + SAL
Low-dose 3 weeks (111)	1 mg/kg METH	1 mg/kg METH + SAL	1 mg/kg METH + SAL
Escalating dose (124)	1 mg/kg METH	2 mg/kg METH + 2 mg/kg METH	4 mg/kg METH + 4 mg/kg METH

2.4 Behavioural Analysis

2.4.1 Mice

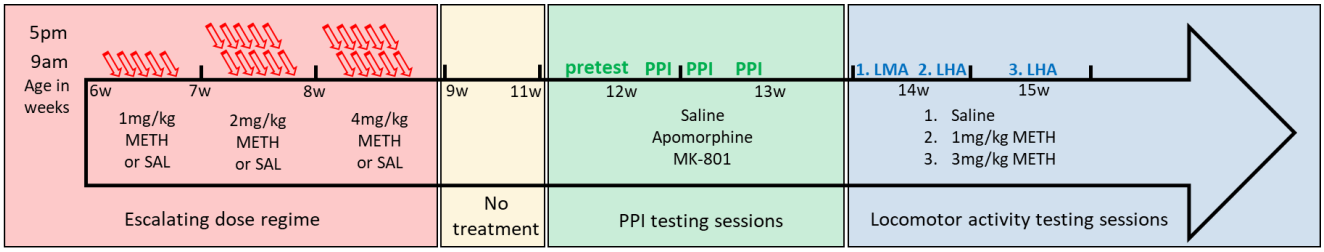

Figure 2.2 Timeline of mouse studies described in this thesis

Following one of three chronic METH injection phases or a saline equivalent (highlighted in red in Figure 2.2) mice were left untreated for two weeks to enable METH washout and for long-lasting sensitization to occur (highlighted in yellow). Mice were subsequently tested in 4 prepulse inhibition experiments (highlighted in green), 1 locomotor activity session (LMA) and 2 METH-induced hyperactivity sessions (LHA; blue).

2.4.2 Prepulse Inhibition

Following the two-week 'resting' phase after 3 weeks of chronic METH or saline injections, mice were tested for acoustic startle responses and PPI to determine baseline and psychostimulant-driven responses. Mice were placed in Perspex

cylinders within San Diego Instruments SR-Lab automated startle boxes (San Diego, CA, USA; Figure 2.3). A protocol of 104 trials was used over 40-45 minute per session. Every session began with a 3-minute 65 decibel (dB) background noise phase, followed by 8x 115dB pulse-alone startle stimuli. This block of pulse-alone stimuli was repeated at the end of the PPI session. Within each session, 16 additional pulse-alone trials were pseudorandomly inserted throughout the experiment and startle responses for each mouse were calculated over four blocks of these pulse-alone stimuli to establish habituation. Analysis of PPI was conducted on the average of the 32 pulse-alone trials.

A prepulse trial was defined as a prepulse stimulus of 2, 4, 8, 16dB above background that preceded the 115dB pulse at an inter-stimulus interval (ISI) of 30ms or 100ms prior to the startle noise. In total, 8 trials were pseudorandomly conducted for each PP intensity at both ISIs. A further 8 no-stimulus trials were pseudorandomized within each session to determine any non-specific motor effects or background vibration.

Startle responses were measured through vibrations detected by the PPI chamber and relayed to the SR Lab software for analysis. PPI is a percentage value calculated by the difference between median responses to the eight pulse-alone startle stimuli compared to the median responses to the pulses preceded by one of the four prepulses.

Four PPI sessions were conducted in each mouse with a 2-3 day washout between experiments:

1. Pre-test with no injection. Acclimatization to the PPI chamber.
2. Saline IP injection directly prior to PPI session.
3. 3mg/kg APO (R-(-)-Apomorphine, Sigma Aldrich, Castle Hill, NSW) IP injection directly prior to PPI session (mice weighed same day).
4. 0.2mg/kg MK-801 ((+)-MK-801 hydrogen maleate, Sigma Aldrich, Castle Hill, NSW) IP injection 15 minutes prior to PPI session (mice weighed same day).

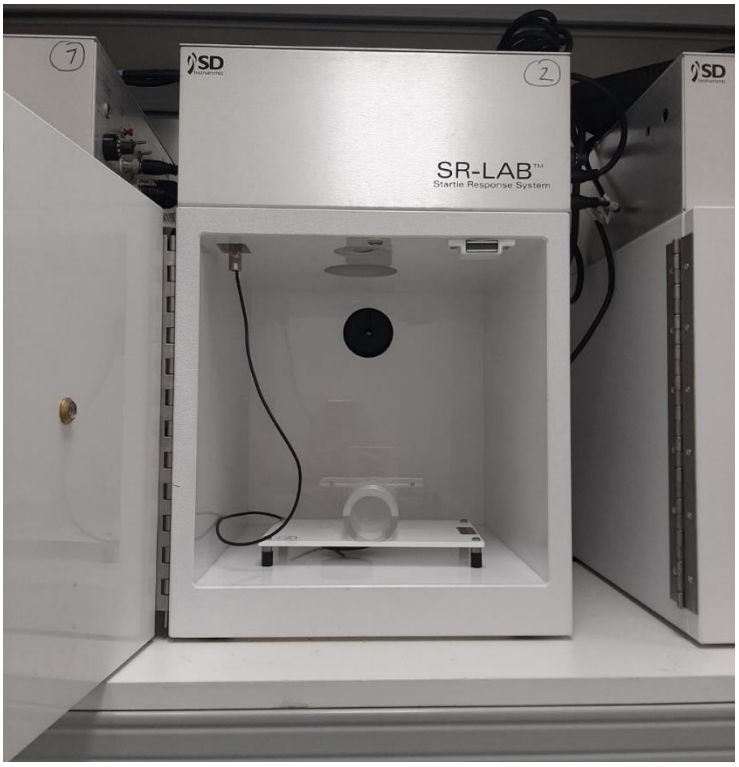

Figure 2.3 Mouse prepulse inhibition chamber*. Mice are held in Perspex cylinders while the acoustic startle response is measured over repeated and varied prepulse intensity and interstimulus intervals.*

These drugs were selected due to their capacity to disrupt PPI via different neurochemical pathways, shown previously by the van den Buuse lab [304] and others. The order of saline, APO, and MK-801 injection trials was pseudorandomised to ensure each cohort of mice was tested in a different rotational order from the previous group. All mice were analysed for differences in startle and PPI, with protocols that have been previously defined [240]. As expected, PPI response increased with PP dB levels. As we did not observe any major interactions on individual PP data, average PPI at 30- and 100ms ISI, and average startle value were compared between experimental groups within each PPI chapter. Average PPI for each ISI was examined based on limited interaction between PPs and experimental variables. Raw PP data separated by prepulse intensity is available in the appendix for both PPI studies.

There is some debate within the literature whether habituation of PPI occurs across trials [305, 306], which is hypothesized to be a result of habituation to startle stimulus alone trials [307]. PPI has also been reported to increase with repeated testing [308]. However, findings are inconsistent, with some studies citing no

habituation or extinction effects over multiple trials [305]. To account for potential behavioural adaptation, PPI sessions incorporate pulse-alone trials at the beginning of the test to prompt rapid startle habituation function and stabilise startle magnitude. In addition, consecutive PPI session drug treatments were pseudorandomised in the current study, across the three PPI trials (SAL, MK-801, APO), to prevent potential confounding effects of habituation.

2.4.3 Locomotor activity

Locomotor activity of up to sixteen mice simultaneously was measured in a well-lit room in LARTF. Five-seven days after PPI experiment completion, mice were tested in locomotor photocells (Seamless Open Field Arena, Med Associates, Inc. Fairfax, VT, USA) over three 180-minute sessions (Figure 2.4). Mice were placed individually in these photocell chambers and habituated for the first 60 minutes of the session. At 55-60 minutes, mice received an acute IP injection (29G needle) before being returned to the photocell where activity was recorded over the following 120 minutes. Mice received METH in an escalating administration protocol to attempt to prevent variable sensitization that may have occurred if doses were pseudorandomly ordered:

1. Saline vehicle in the first session
2. 1mg/kg meth in the second session
3. 3mg/kg meth in the final session.

Two-four days was left between sessions to allow drug wash-out. Photocell chambers recorded locomotion via interruption of photoelectric beams positioned along the walls of the chamber. Mouse activity was recorded and analysed by the Activity Monitor software (Med Associates Inc.) and outputs for statistical analysis included cumulative horizontal distance moved every 5 minutes.

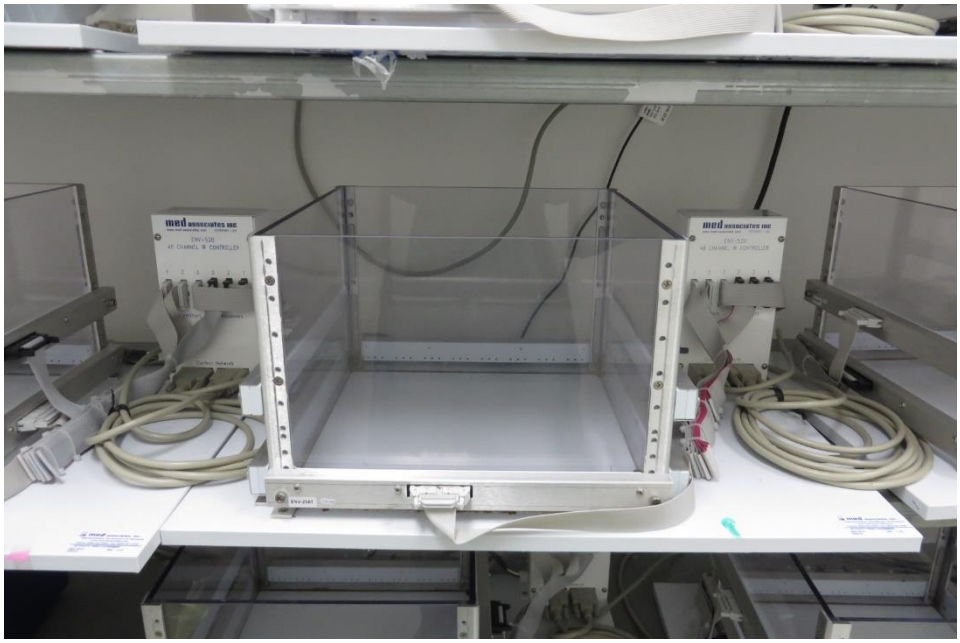

Figure 2.4 Mouse locomotor photocell arena. *Mice are held in a clear box while locomotion is measured following acute injection. Photoelectric beam disruption records horizontal movement.*

2.4.4 Rats

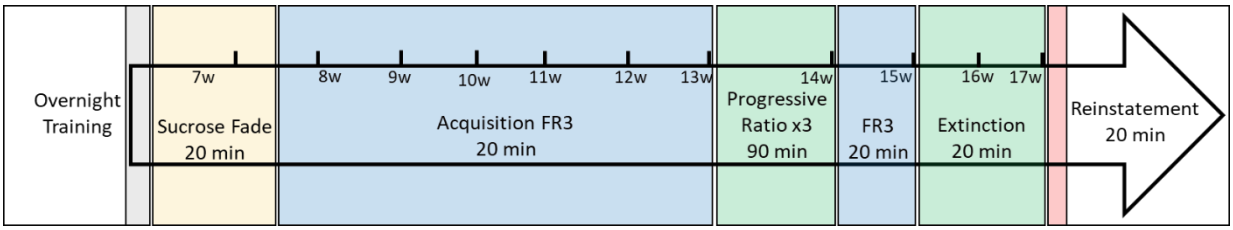

Figure 2.5 Timeline of rat operant alcohol self-administration study in BDNF HET rats

2.4.4.1 Alcohol operant self-administration

BDNF HET rats and WT controls were trained to press an active lever in operant chambers (Med Associates, St Albans, VT, USA). Briefly, each chamber was held in a sound-proof cubicle with a fan to provide airflow and to mask external noise (Figure 2.6). Two retractable levers (visible during operant sessions) were placed below a stimulus light and adjacent to a fluid receptacle. Levers were positioned at opposite corners of the chamber. A single drop of vanilla essence was placed onto

a plastic dish under the active lever to act as an olfactory cue and the stimulus light was set to illuminate upon completion of the required number of presses of the active lever only. A controlled liquid dispenser fed each receptacle. Initially, each animal was introduced to the operant chamber for an overnight training session which ran for approximately 16 h. During this session, rats could explore the chamber and learn to lever press for rewards of a solution containing 5% v/v ethanol and 5% w/v sucrose. Pressing the opposite lever resulted in water being dispensed. The levers were set to dispense fluid on a fixed ratio of FR2; 2 lever presses = 100 microliter reward. Rats were provided with food pellets within the chamber to ensure they had adequate access to food overnight.

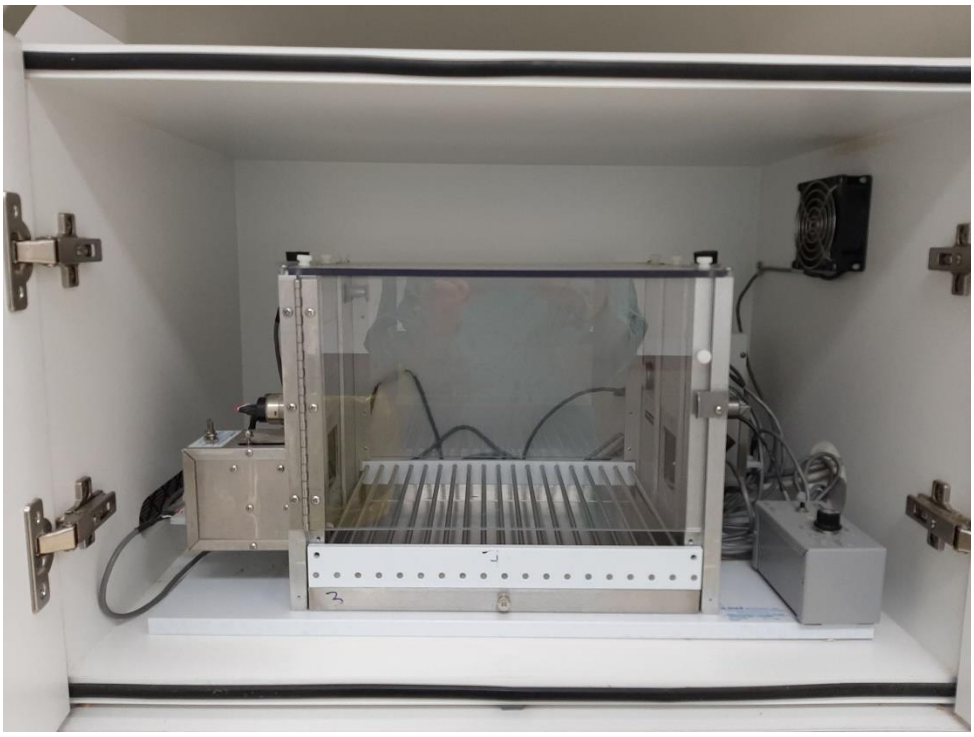

Figure 2.6 Rat operant self-administration chamber*. Rats were held within the operant chamber and trained to discriminate between the active and inactive lever for an ethanol reward. The number of lever presses was recorded.*

Following overnight training, rats were placed into the operant chambers for daily 20 min sessions, five times a week (Monday–Friday). Ethanol and water response levers were alternated between sessions to avoid place preference, with a small drop of solution (either water or the ethanol-containing solution) left in each receptacle to indicate the current orientation. Similar to the overnight training, both

vanilla essence and a stimulus light acted as cues to signal reward availability. A standard ‘sucrose fading' protocol was administered (5% sucrose, 5% ethanol 1-5 days, 2.5% sucrose, 10% ethanol from 6 to 8 days). This allowed the aversive taste of ethanol to be overcome through gradual reduction of the concentration of sucrose over a 9-day period. Once sucrose was withdrawn, a 10% ethanol solution was applied to all future sessions.

Once 9 days of sucrose fade were complete, rats successfully responded to a 10% ethanol solution under a 3:1 fixed ratio requirement (FR3, 3 lever presses equal one reward) within a 20-min daily exposure. Rats that did not successfully learn to lever press were removed from further analysis (n = 6). For each session, the total number of ethanol and water responses were recorded. Ethanol availability was again paired with a stimulus light and vanilla essence olfactory cue. Operant responding sessions for ethanol continued up to day 37 until a consistent baseline response was obtained.

Once a baseline level of responding was achieved, a progressive ratio (PR) reward system was used as previously described [27] to investigate the role of BDNF on “breakpoint”. During this phase, the press requirement is progressively increased for each consecutive reward. For example, the first reward is delivered after one press, second reward delivered after three presses, and the third reward delivered after six presses. The water lever followed the same progressive ratio. Breakpoint represents the point during the PR protocol at which the animal ceases to press the active lever the sufficient number of times for a drug reward to be administered. This protocol occurred every second day for a period of one week for a total number of three 90-min PR sessions (Monday, Wednesday, and Friday). The 20-min FR3 condition was conducted in-between PR sessions (Tuesday and Thursday) and resumed after the last PR session for one week prior to commencing extinction testing.

Following breakpoint analysis and a further 7 days of FR3 responding to 10% ethanol, extinction training began on day 49 on 10% ethanol. During this phase, both the ethanol solution and water, as well as the conditioned cues (vanilla essence and stimulus light) were withheld from the chamber. Sessions continued daily for 20 min. During the extinction phase lever pressing no longer resulted in a

reward, rapidly causing a diminished propensity to lever press. Extinction sessions continued until lever pressing activity reached a low baseline, or equal to the water lever (14 days).

Following extinction, the olfactory cue (vanilla essence) and stimulus light were re-introduced in conjunction with a single drop of ethanol (100 µl) dispensed from the receptacle to act as a primer, however, no alcohol reward was dispensed following lever pressing. Rats typically re-instated and returned to pressing the active lever containing the ethanol solution. Lever pressing activity was recorded for the full 20 min reinstatement session. Reinstatement lever pressing was compared to the average lever presses taken over the last 5 days of extinction, prior to reinstatement.

2.4.4.2 Alcohol operant self-administration - 7,8-DHF

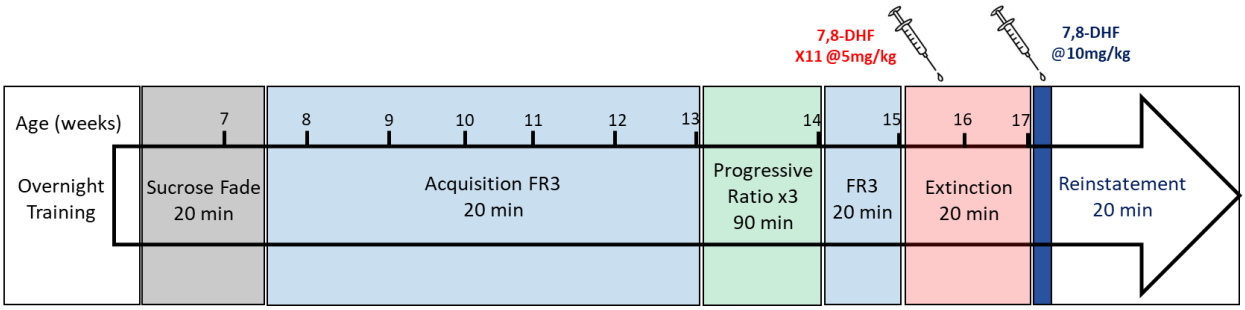

Figure 2.7 Timeline of operant alcohol self-administration study in Sprague Dawley rats with subchronic 7,8-DHF.

Following the observation of significant sex differences in the effect of BDNF deficiency in cue-induced reinstatement, a second cohort of rats was analysed in the alcohol self-administration protocol. The follow-up study consisted of 24 male and 24 female wildtype Sprague Dawley rats tested in an experimental design that closely resembled the previous study, with similar overnight training, sucrose fade, acquisition, and progressive ratio phases. Extinction and reinstatement were similar to the original protocol but included an 11-day subchronic IP injection drug regime. Rats were given 5mg/kg 7,8-dihydroxyflavone (7,8-DHF; Tokyo chemical Industry, Nihonbashihoncho, Chuo-ku, Tokyo) or a saline vehicle during the last two weeks, directly following the end of testing on each day. On the final day, the rats receiving 7,8-DHF were given an acute 10mg/kg dose of the drug approximately 30 minutes prior to the reinstatement session (Figure 2.7).

2.5 Statistical Analysis

All statistical analysis was conducted using IBM SPSS (Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp). Analysis of variance (ANOVA) statistical models were used to examine effects of between-subject variables such as genotype, sex, and pretreatment. When significant differences were observed in genotype (4 groups: WT, D3KO, BDNF HET, DM), genotype was split into D3R and BDNF subgroups (2x2 design: BDNF HET vs. BDNF WT, D3KO vs. D3R WT) to determine potential genotype interactions and relative contributions to behaviour.

A repeated-measures ANOVA was used to analyse within-subject factors in mice' behavioural data. These included change in mouse bodyweight over the experiment, prepulse intensity, interstimulus interval, and drug effects in PPI, and time bin analyses and drug effects in LHA experiments. In rats, operant data were analysed with repeated measures ANOVA to determine differences in active and non-active lever activity during acquisition, progressive ratio, extinction, and reinstatement.

ANOVA were considered statistically significant when $p < 0.05$. All graphs are represented as the mean ± SEM unless stated otherwise. In cases where data were not normally distributed and sphericity could not be assumed, as demonstrated by a significant Mauchly's test, Greenhouse-Geisser correction was used to interpret results.

Chapter 3 Evaluation of METH-pretreatment regimens on prepulse inhibition in WT and BDNF HET mice

3.1 Introduction

3.1.1 PPI is a measure of sensorimotor gating and a behavioural endophenotype of schizophrenia

Clinical observations which have consistently shown inability of schizophrenia patients to optimally filter or ‘gate’ incoming stimuli have led to a large literature on animal models of sensorimotor gating deficits [309, 310]. As outlined in Chapter 1, PPI is an operational measure of sensorimotor gating and has historically been implemented in clinical and preclinical schizophrenia research in bottom-up, phenotype-driven investigations. Weak prepulses prime the brain for subsequent sensory stimuli and reduce further information processing through the prevention, or dampening, of motor outputs. The theory that PPI deficits model aspects of positive symptoms of schizophrenia is largely derived from observations that sensorimotor gating deficits can be induced by psychostimulant drugs, and the reversal of these PPI deficiencies by first-generation antipsychotics. A multitude of studies have used PPI in evaluating rodent models of schizophrenia to elucidate underlying neurocellular perturbations [230, 311-314]. PPI of startle is demonstrated to have a genetic component in mice [315] and shares common neurocircuitry with psychosis-like behaviours. Thus, investigation of PPI can be used to review potential schizophrenia neurocircuitry and act as a pharmacological screen for novel therapeutic substrates [313]. The neuropharmacology of PPI is well defined in human and animal representations, predominantly implicating glutamatergic, DAergic and serotonergic signalling systems and networks [40, 214, 231, 304, 311, 316, 317].

3.1.2 PPI is attenuated in hyperdopaminergic and hypoglutamatergic systems

Early studies on disrupted PPI focused on enhanced DAergic neurotransmission, modelling DAergic hyperactivity in schizophrenia [231]. First demonstrated by Swerdlow and colleagues, rats treated with either amphetamine or APO displayed reduced PPI [318]. More recent investigations have replicated attenuated sensorimotor gating following direct and indirect DA receptor activation, demonstrating dose-dependent sensorimotor gating deficits [230, 304, 319-321].

Comparable PPI disruption is observed with attenuated glutamatergic signalling through NMDA receptor blockade by phencyclidine (PCP) and MK-801 [234]. Due to their psychotogenic properties, PCP and MK-801 elicit substantial behavioural effects in humans that have been compared to the positive symptoms of schizophrenia [322, 323]. Subsequently, NMDA receptor antagonism was used to develop early models of psychosis and test the effects of antipsychotic drugs [231, 324]. Glutamatergic signalling has also been further implicated in PPI regulation through evidence of L-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)- and metabotropic (mGluR)-receptor influence. For example, mice lacking the AMPA GluR1 receptor subunit displayed reduced PPI and striatal hyperdopaminergia [325], and studies in mice without mGluR1 report similar findings of diminished PPI [326]. These studies highlight the involvement of glutamatergic and dopaminergic signalling in PPI regulation and highlight regions of overlapping neurocircuitry between psychosis, behavioural sensitization reward pathways, and modulation of the networks governing precognitive reflex response.

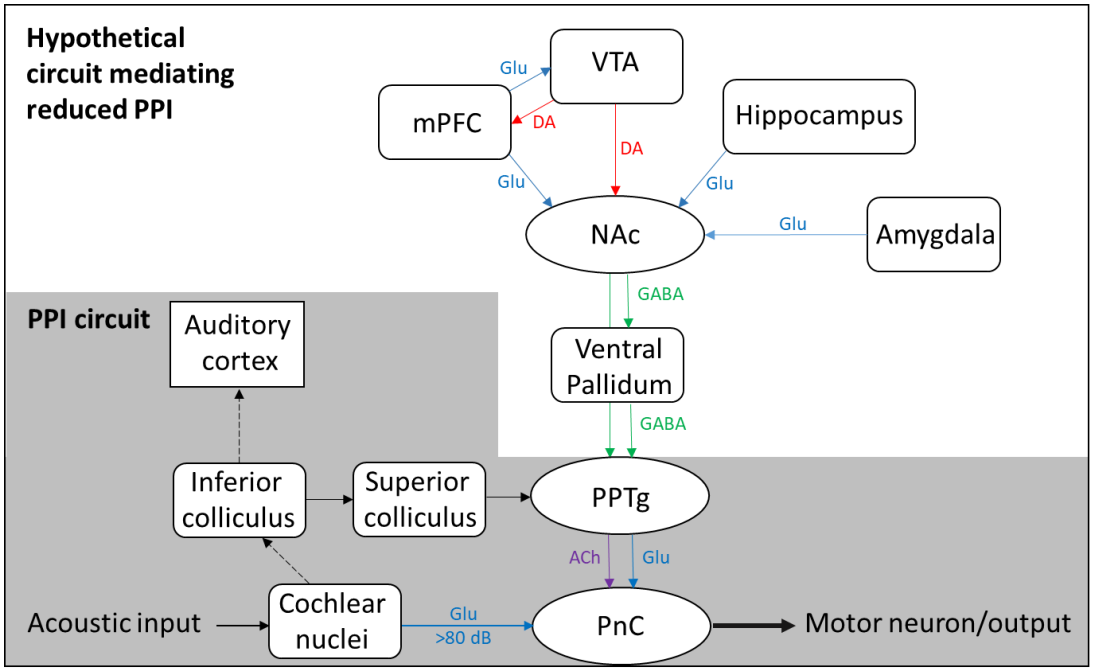

Figure 3.1 An adapted hypothetical circuit of proposed corticolimbic-striatal circuitry governing prepulse inhibition*. In this system, feedback from cortical and subcortical regions converge on the pedunculopontine nucleus (PPTg) which projects to the caudal pontine reticular nucleus (PnC), a structure associated with PPI regulation. Ventral tegmental nucleus (VTA), medial prefrontal cortex (mPFC), nucleus accumbens (NAc), acetylcholine (Ach), dopamine (DA), g-aminobutyric acid (GABA), glutamate (Glu).*

3.1.3 PPI networks are modulated by neurocircuitry associated with psychosis

PPI is mediated by brainstem nuclei located between the midbrain and the medulla (Figure 3.1). Studies have demonstrated the role of the inferior and superior colliculi, reticular formation (pedunculopontine (PPTg) and to a lesser extent, laterodorsal tegmental nuclei), and caudal pontine reticular nucleus (PnC) in the reflex pathway governing complete expression of PPI [327]. The PnC is central to this model, acting as the sensorimotor interface where excitatory and inhibitory afferent signals combine to determine whether sensory stimuli are relayed to motor outputs [328]. Given these observations, it is likely that disruption to PPI occurs when maladaptive signalling impacts the PnC.

The NAc receives input from the VTA and limbic areas, including the hippocampus, cingulate gyrus, and amygdala. Increased DAergic signalling in the NAc has consistently been implicated in attenuated PPI [329] and is involved in mediating GABAergic neurotransmission to and from the ventral palladium, innervating the PPTg which in turn modulates the PnC. Therefore, PPI is sensitive to subcortical DA neurotransmission, which is also implicated with psychosis symptomatology and reward-related behaviours.

The majority of sensory cortical areas within the CNS are interconnected by the PFC and discrete subcortical domains [330] that are hypothesized to impact brain networks and guide neuronal signalling of motor outputs. Although the precise mechanisms have not been fully elucidated, PPI networks are regulated by forebrain circuitry in the cortico-striato-pallido-pontine (CSPP) and cortico-striato-pallido-thalamic (CSPT) networks [331]. The PFC is comprised of organised layers of glutamatergic pyramidal neurons (70-80% of non-glial cells) and GABAergic interneurons, which largely function as modulators of incoming synaptic signalling [332]. Therefore, disruption of NMDA receptor signalling within the PFC could impact PPI through PFC disruption, or reduced efficacy of associated cortical inhibition of mesocorticolimbic DA [331, 333].

These models of neurotransmitter perturbations in schizophrenia will be used in this chapter, in combination with attenuated BDNF protein expression. Furthermore, to examine the effect of sensitized DAergic neurocircuitry, mice

described in this chapter were given chronic METH in late adolescence, providing a putative environmental x genetic interaction of disrupted PPI.

3.2 Aims

The first aim of this experiment was to examine potential dose-dependent effects of METH pretreatment on PPI regulation, including hyperdopaminergia and hypoglutamatergic functioning. This was achieved through acute administration of APO and MK-801, respectively. Previous results by Manning et al. described reduced baseline PPI following an escalating METH-pretreatment regimen in BDNF-deficient mice, and no significant interaction with genotype or METH-pretreatment on APO- or MK-801-induced PPI disruption [304]. Therefore, as the second aim of this study, we predicted METH-pretreatment would impact PPI in a dose-dependent fashion and that BDNF HETs would respond differently to METH-evoked PPI disruption than WT control animals.

3.3 Methods

3.3.1 Animals and statistical analyses

A total of 169 mice was used in experiments described in this chapter (Table 3.1). Statistical analysis was conducted on groups separated by genotype, sex, and METH pretreatment resulting in 16 experimental groups for comparison. Two weeks after pretreatment, mice were tested in San Diego Instruments SR-Lab automated startle boxes (San Diego, CA, USA; Figure 2.3) over 3x 45-minute experimental sessions, as outlined in the General Methods (Chapter 2.3.2).

Table 3.1 Number of mice used in PPI experiments outlined in this chapter

	WT				**BDNF HET**			
	SAL	**100**	**111**	**124**	**SAL**	**100**	**111**	**124**
Male	12	12	11	10	11	10	10	10
Female	10	10	10	9	10	10	11	8

Statistical analysis was conducted on PPI percentage at 30ms and 100ms ISI, averaged across the four prepulse intensities. Startle amplitude was analysed as the average startle reactivity over the four startle blocks, as described in Chapter 2. Outliers were detected using Z scores calculated for the saline, APO, and MK-801 average PPI values. Five mice were removed from analysis (1 male WT 124, 1 female HET saline, 1 female HET 100, 2 female HET 124) based on average baseline PPI values positioned outside 3 standard deviations of the mean. A repeated measures ANOVA was used to compare differences in PPI between sex, genotype, and pretreatment following separate IP injection of saline, 3mg/kg APO, and 0.2mg/kg MK-801 prior to session commencement.

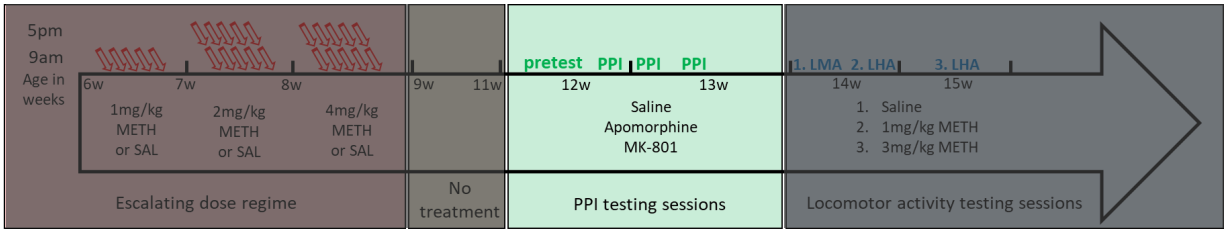

Figure 3.2 A timeline of METH pretreatments and PPI experiments outlined in this chapter. *Mice underwent 3 PPI sessions pseudorandomly ordered across a 2-week testing phase (highlighted in green). The three injections included: saline, 3mg/kg APO, and 0.2mg/kg MK-801.*

3.4 Results

3.4.1 Bodyweight of mice

Mice were weighed twice per week during METH or saline pretreatment and once before every acute drug IP injection. Analyses of bodyweight included 4 timepoints: Initial weight, end of pretreatment, end of no-treatment (NT) phase, and end of behavioural analyses (Figure 3.3). Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated for bodyweight change over the 4 timepoints ($\chi^2(5) = 154.78$, $p < 0.001$), so the Greenhouse-Geisser correction was used to adjust results. Adolescent mice' bodyweight increased significantly in all groups across the experiment timeline (main effect of weight change, $F(1.95, 293.78) = 124.50$; $p<0.001$). There was no difference in bodyweight between any of the pretreatment groups, although male mice were significantly heavier than female mice at each timepoint and BDNF HET gained significantly more weight over the experiment timecourse than WT mice (weight change x sex, $F(1.95, 293.78) =$

34.25; p< 0.001; weight change x genotype, F(1.95, 293.78)= 124.50; p<0.001; Figure 3.3).

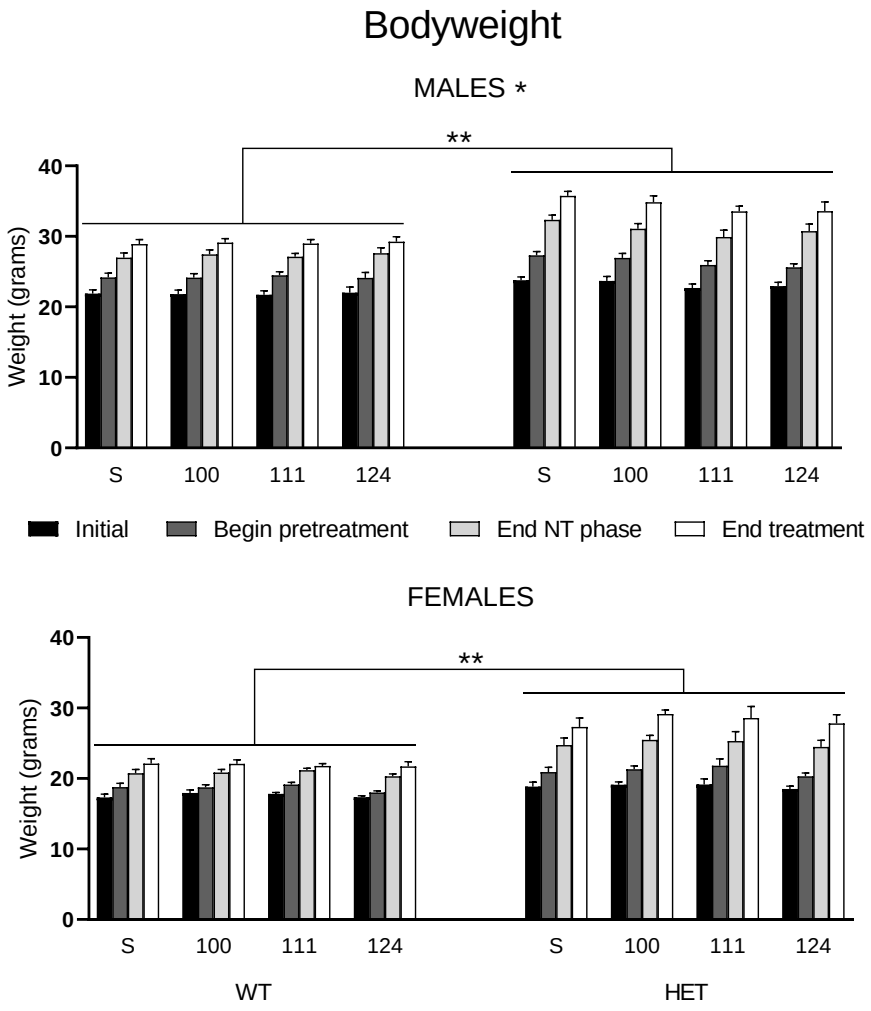

Figure 3.3 Bodyweight of mice across the BDNF HET low-dose study. *Males were significantly heavier than females and BDNF HET mice were significantly heavier than WT mice. No difference in weight was observed between METH and saline pretreatment groups. *= p < 0.05 for difference between males and females, ** = p< 0.05 for difference between BDNF HET and BDNF WT mice. 100 = 1 week of low-dose METH-pretreatment, 111= 3 weeks of low-dose METH pretreatment, 124= 3 weeks of escalating dose METH-pretreatment.*

3.4.2 Baseline PPI

Baseline PPI was recorded following acute IP administration of saline. At 30ms ISI pretreatment groups differed significantly in baseline PPI (pretreatment main effect, $F(3,148)= 4.72$, $p= 0.004$), and significant interactions were observed between pretreatment and sex (interaction, $F(3,148)= 3.98$, $p= 0.009$) and pretreatment and genotype (interaction, $F(3,148)= 4.23$, $p= 0.007$). Based on the study aims and these initial results, data were split by genotype and re-analysed. This secondary ANOVA revealed PPI in BDNF HET mice was equivalent across sex and pretreatment groups. In contrast, WT groups displayed significant differences in average PPI (pretreatment effect, $F(3,76)= 8.87$, $p< 0.001$). Tukey's post hoc analysis revealed significantly lower PPI in 111- and 100-pretreated mice compared to SAL WT controls ($P< 0.05$; Figure 3.4). There was no significant difference between SAL and 124 mice, although the escalating METH protocol group had significantly higher PPI than 111-pretreated animals ($p< 0.05$; Figure 3.4).

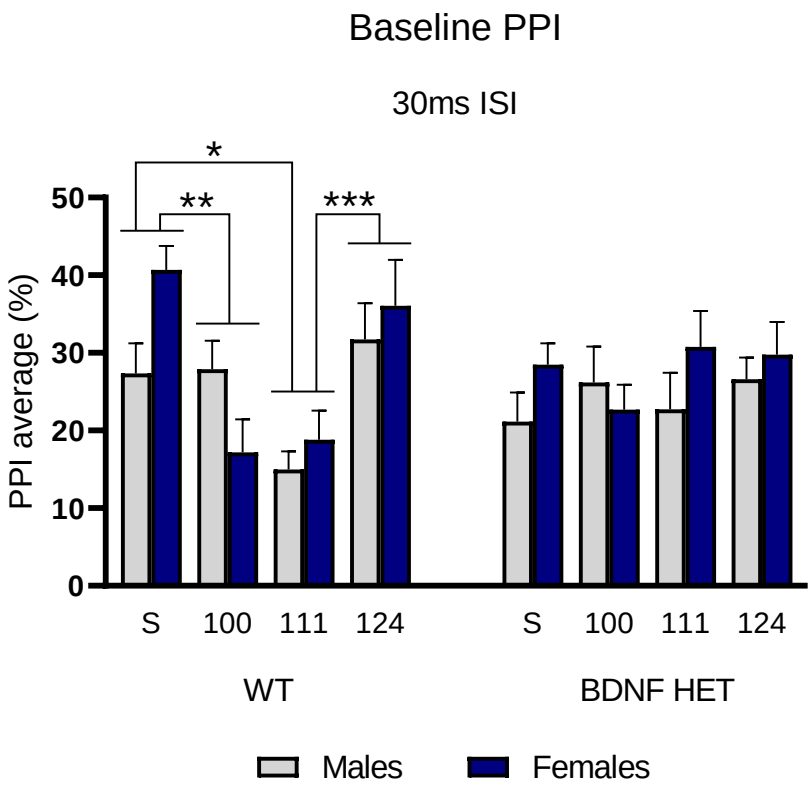

Figure 3.4 Average baseline PPI at the 30ms ISI following acute saline injection. ** = p< 0.05 difference SAL- and 111-pretreated mice, **= p< 0.05 between SAL- and 100-pretreated mice. *** = p< 0.05 difference between 111- and 124-pretreated mice. Data are mean ± SEM.*

At 100ms ISI, no significant sex interactions were observed, so data from males and females were combined. A significant pretreatment x genotype interaction ($F(3,148)= 3.23$, $p= 0.024$) again prompted further examination of the data split into WT and HET groups. Tukey's post-hoc and pairwise comparison of pretreatment groups revealed that in BDNF HET PPI did not differ significantly between the type of pretreatment regimen. In contrast, WT mice which were pretreated following the 111-protocol displayed significantly lower baseline PPI compared to SAL and 124-pretreament groups ($p< 0.05$; Figure 3.5).

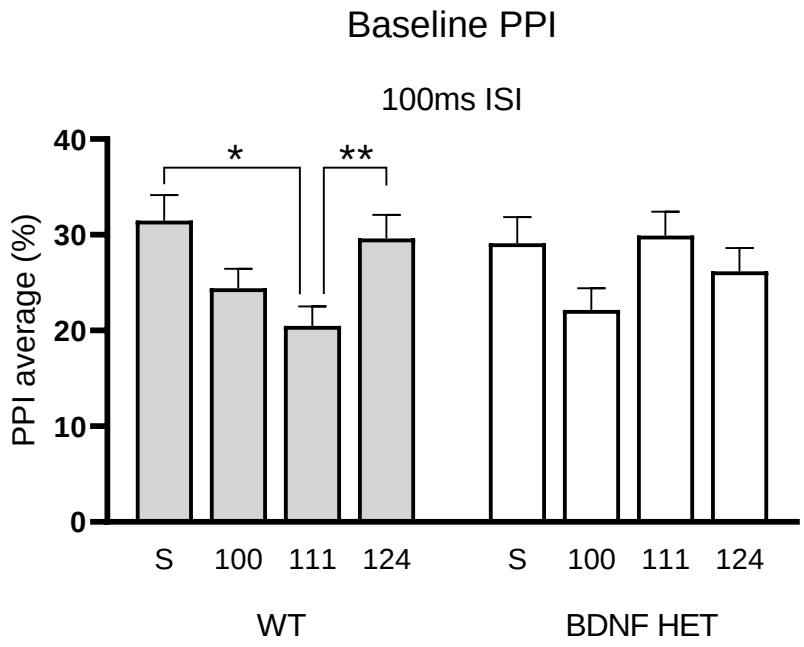

Figure 3.5 Average baseline PPI at the 100ms ISI following acute saline injection. ** = p< 0.05 difference between SAL- and 111-pretreated WT mice, **= p< 0.05 between 111- and 124-pretreated WT mice. Data are mean ± SEM.*

3.4.3 Baseline startle

Univariate analysis of baseline startle was conducted on the average startle amplitude recorded during assessment of baseline PPI. Male mice had a significantly larger startle reactivity compared to female mice (main effect of sex, $F(1,148)= 55.65$, $p<0.001$; Figure 3.6) and BDNF HET mice had significantly higher startle compared to WT controls (main effect of genotype, $F(1,148)= 11.87$, $p= 0.001$; Figure 3.6). There was no effect of pretreatment on baseline startle, and no significant interactions between variables were observed (Figure 3.6).

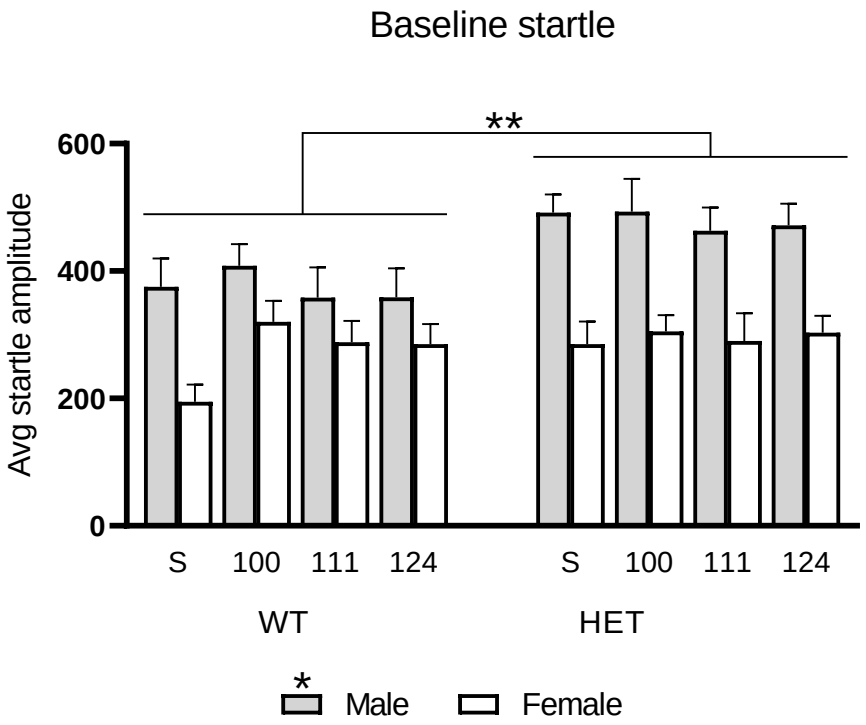

Figure 3.6 Startle amplitude of mice treated with acute saline. ** = p< 0.05 difference between male female mice, **= p< 0.05 between BDNF HET and WT. Data are mean ± SEM.*

3.4.4 APO

At an ISI of 30ms, acute APO significantly attenuated average PPI compared to baseline (main effect of APO, $F(1,148)= 135.68$, $p<0.001$). Genotype and sex did not affect change in average PPI. Pretreatment groups differed significantly in their response to APO treatment (interaction, $F(3,148)= 3.65$, $p= 0.014$), but post hoc analysis revealed no significant difference between any of the METH treatment regimens compared to chronic saline (Figure 3.7 A). At the 100ms ISI APO significantly reduced baseline PPI in all groups (main effect of APO, $F(1,148)= 86.19$, $p<0.001$), and a strong trend was detected for a greater APO-induced reduction in PPI in females compared to males (interaction, $F(1,148)= 3.82$, $p= 0.053$). No significant differences in the effect of APO were detected between genotype or pretreatment groups (Figure 3.7 B-C).

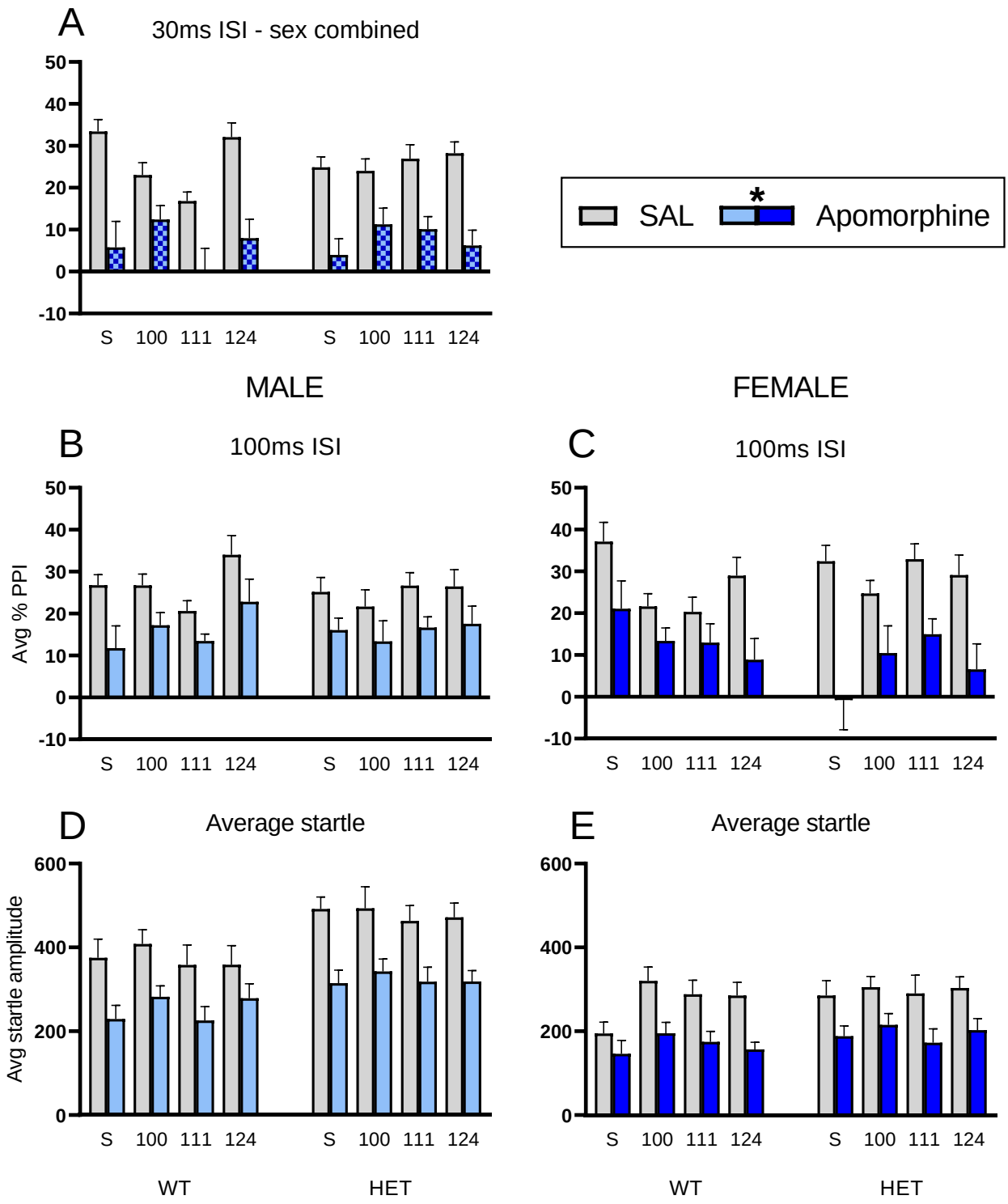

Figure 3.7 Average PPI and startle response across acute saline and APO sessions. (A) *Sex-combined 30ms ISI PPI.* ***(B)*** *Male 100ms ISI PPI.* ***(C)*** *Female 100ms ISI PPI.* ***(D)*** *Male average startle amplitude.* ***(E)*** *Female average startle amplitude. *= p< 0.05 represents significant difference between saline and APO-induced PPI and startle. Data are mean ± SEM.*

3.4.5 APO startle

Compared to baseline startle, the average startle reactivity in the APO session was significantly reduced (main effect of APO startle, $F(1,148)= 235.26$, $p<0.001$; Figure 3.7 D-E). In line with differences in bodyweight between sexes, these analyses also revealed significantly reduced startle amplitude in female mice compared to males (main effect of sex, $F(1,148)= 6.11$, $p= 0.015$). No further interactions were detected.

3.4.6 MK-801

At 30ms ISI, mice receiving MK-801 had a significant reduction in PPI compared to baseline values (main effect of MK-801; $F(1,147)= 70.4$, $p<0.001$). A 3-way interaction between genotype, pretreatment, and MK-801 (interaction, $F(3,147)= 2.69$, $p= 0.048$) led to analysis split by genotype, exposing a pretreatment-specific difference in PPI. In 111-pretreated WT mice the effect of MK-801 was attenuated compared to saline-pretreatment (WT mice, MK x pretreatment interaction, $F(3,75)= 3.08$, $p= 0.032$; Tukey's multiple comparison $P< 0.05$). In contrast, in BDNF HET mice, MK-801 significantly reduced PPI in all pretreatment groups. Consequently, BDNF HET mice that were pretreated with the 111 regimen displayed a greater reduction of PPI following MK-801 administration compared to 111 WT mice (MK-801 x genotype, $F(1,37)= 6.30$, $p= 0.017$; Figure 3.8 A; Tukey's multiple comparison $p<0.05$).

As in 30ms ISI, average PPI at the 100ms ISI was significantly reduced across all groups by acute MK-801 treatment (main effect of MK-801, $F(1,147)= 150.78$, $p< 0.001$; Figure 3.8 B). A significant interaction (MK-801 x genotype x pretreatment, $F(3,147)= 3.37$, $p= 0.02$) prompted separation of the groups by genotype, which showed no significant differences in BDNF HET mice. WT mice however, displayed a pretreatment interaction (WT mice, MK-801 x pretreatment, $F(3,72)= 3.00$, $p= 0.036$) but post hoc testing confirmed no significant difference between genotypes.

3.4.7 MK-801 startle reactivity

Startle reactivity following acute MK-801 did not significantly differ from baseline startle, although a strong trend was detected for a reduction in amplitude (main effect of startle, $F(1,147)= 3.80$, $p= 0.053$; Figure 3.8 C-D). No significant

interactions were detected among any within- or between-subject variables for average startle reactivity.

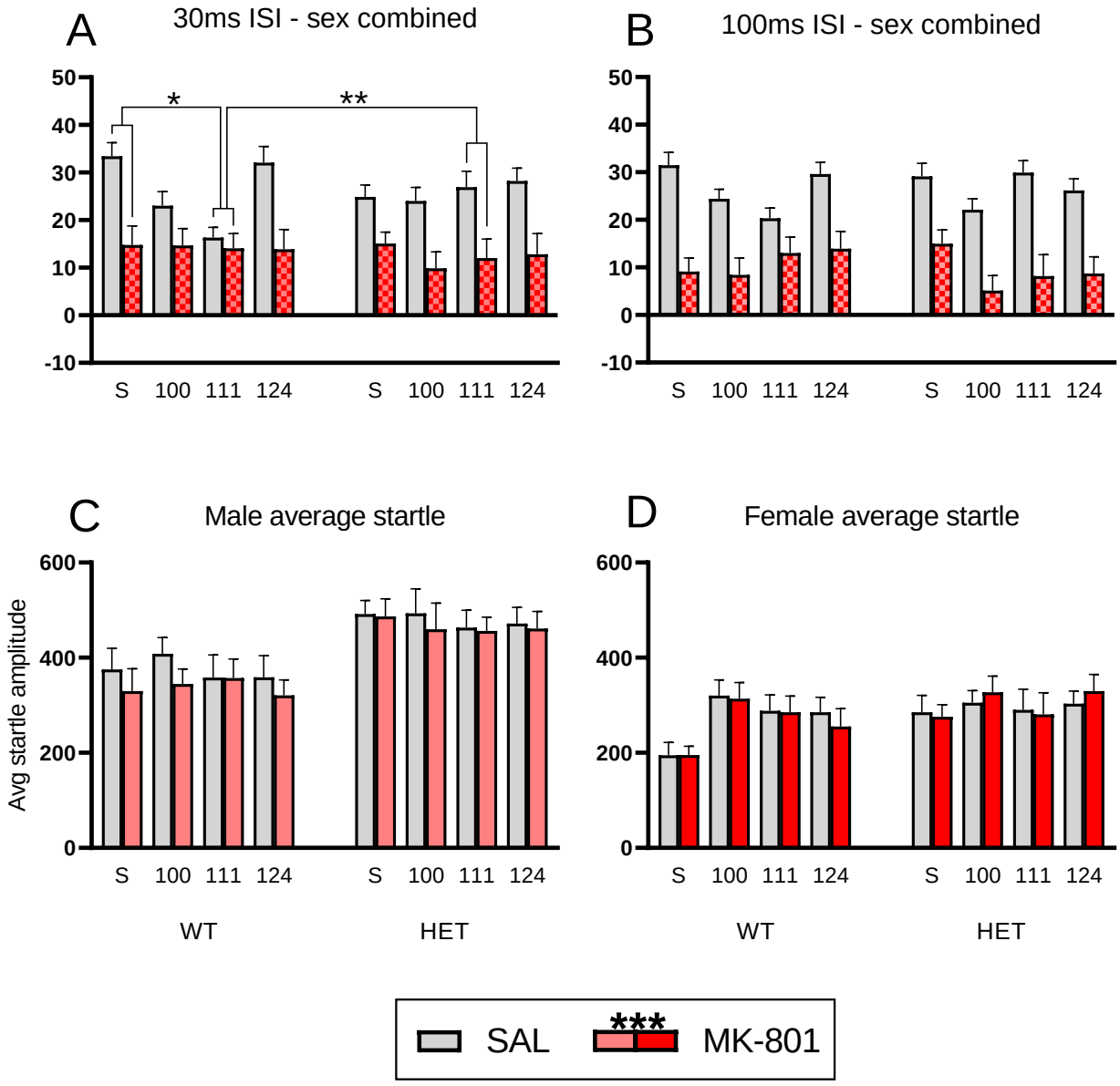

Figure 3.8 Average PPI and startle response across acute saline and MK-801 sessions. (A) *Sex-combined 30ms ISI PPI *= p< 0.05 for difference between saline- and 111-pretreated WT mice. **= p<0.05 for difference between 111-pretreated WT and BDNF HET.* ***(B)*** *Sex-combined 100ms ISI PPI.* ***(C)*** *Male average startle amplitude.* ***(D)*** *Female average startle amplitude. ***= p< 0.05 difference between saline and MK PPI sessions. Data are mean ± SEM.*

3.5 Discussion

The primary aim of this experiment was to examine potential dose-dependent METH-pretreatment effects on PPI disruption. These analyses included comparison between baseline PPI and PPI deficiency in states of hyperdopaminergia and hypoglutamatergic signalling. Furthermore, this study sought to replicate previous work in the BDNF HET model and confirm involvement of BDNF in mediating PPI.

3.5.1 Bodyweight and baseline startle

Results on mouse bodyweight support previous findings that BDNF HET mice are heavier than WT and predisposed to enhanced weight gain [334]. No pretreatment effects were detected for weight or startle amplitude, suggesting potential neurobiological adaptations to METH exposure do not impact mouse weight. The null effect of pretreatment on mice' bodyweight is favourable to the analysis of pretreatment on PPI regulation, as differences in PPI should reflect changes in sensitization of neuronal networks as opposed to other, potentially non-specific METH-induced effects (potentially reflected by bodyweight changes). It is also important to note that variation in baseline startle reactivity has been shown to be a potential confound in assessing PPI levels in mice and humans [335, 336]. This is relevant when small variations in startle reactivity between experimental groups impact relative PPI. For example, a group of mice with lower acoustic startle response may have a lower PPI response to a low intensity acoustic startle stimulus, compared to a group of mice with higher basal startle amplitudes [311, 336]. This may have had some effect on data obtained in this study, based on a small increase in ASR in BDNF HET mice (Figure 3.6), although this is likely due to enhanced weight of these animals and not inherent differences in startle amplitude.

3.5.2 Baseline PPI reduction by METH pretreatment is dose-dependent

Baseline PPI was recorded following acute saline injection. Results revealed that compared to saline-pretreated controls, PPI was reduced in 111-pretreated mice (30- and 100ms ISI) and in 100-pretreated at 30ms ISI. Surprisingly, these deficits were not evident in 124-pretreated mice, who displayed similar startle inhibition to

control animals. Combined, these effects may provide evidence for altered PPI regulation at lower METH pretreatment doses, although the reason for this differential effect remains unclear. It is possible that our findings demonstrate potential dose-dependent compensatory mechanisms. Administration of higher doses of METH, such as in the 124-regimen, may trigger compensation in the mouse brain that preserves PPI regulation. Compensatory mechanisms such as changes in receptor availability and altered neurotransmitter signalling systems are hypothesized to occur in response to CNS disruption to prevent maladaptive phenotypes [216]. In contrast, it is possible that the low-dose METH-pretreatments provoking attenuated baseline PPI may not trigger these protective mechanisms within the brain, due to their lower doses, and therefore these mice show deficits in some behaviours.

These results are in line with earlier studies showing a lack of METH-pretreatment effect on baseline PPI observed in the 124 escalation model [304]. A discrepancy between previous and current experiments is the lack of baseline genotype effect in the current study. Unlike previous results in this model, BDNF HET and WT mice had comparable baseline PPI at 30- and 100ms ISI. Earlier studies have shown varying results. Most recently in our lab, a genotype effect of attenuated PPI in BDNF haploinsufficient mice compared to WT controls was described [304]. However, the present result is similar to an earlier study where we did not find a difference between baseline PPI in BDNF HET mice [337], although it should be noted that these mice did not follow a METH-pretreatment regimen prior to sensorimotor gating analysis and were not subjected to repeated IP injection. Therefore, the saline injection received prior to baseline PPI analysis may have become a mild but significant stressor and could have impacted sensorimotor gating differently between studies [337]. These disparities are also reflected in BDNF HET rat models that have displayed equivalent [32] and unequal PPI regulation [40] between genotypes, and studies in mice which report no difference in baseline PPI between BDNF HET and WT [338] or selective BDNF deficiency in forebrain-restricted mutant mice and control animals [339]. In summary, it remains unclear whether BDNF heterozygosity impinges on baseline PPI regulatory networks. Further studies will need to address these discrepancies between findings.

3.5.3 APO-induced PPI deficits are not impacted by METH-pretreatment or BDNF heterozygosity

Results obtained during APO challenge were largely unchanged between pretreatment, sex, and genotype. As expected, APO attenuated PPI and average startle reactivity, demonstrating a role for D1/D2 receptor activation in mediating PPI. Based on the findings presented here we do not provide evidence for BDNF HET influence over D1/D2 receptor-mediated PPI deficits. Furthermore, no variance in either startle or PPI was detected following METH-pretreatment regimens, signifying DAergic regulation of PPI is not altered following METH sensitization. These results confirm previous observations in the BDNF HET model, where Manning described significant APO-induced disruption of PPI, but no effect of METH-pretreatment or genotype on sensorimotor gating deficits [304].

3.5.4 MK-801 administration reduces PPI independent of METH-pretreatment and BDNF depletion

Similar to results obtained in APO challenge, administration of MK-801 disrupted PPI largely independent of BDNF and METH-pretreatment. However, 111-pretreated mice displayed significant variance. While 111-pretreatment attenuated the effect of MK-801 in WT mice, the same wasn't observed in BDNF HET mice, demonstrating a greater effect of MK-801 in 111-treated HET compared to 111-treated WT. Combined with baseline PPI analyses, these results could infer some form of PPI network interference that is specific to a low repetitive METH dose, although the reason for this dose-specific effect remains unclear. Startle was comparable between all groups, indicating that NMDA receptor blockade had little effect on the acoustic startle of these mice. Once again these results reflect previous observations in the BDNF HET model of non-significant differences on MK-801-induced PPI deficits between genotype and pretreatment [304].

3.6 Conclusion

Few effects of BDNF heterozygosity were observed in mice on PPI. These results provide limited evidence for a role of BDNF in mediating inhibition of acoustic startle at baseline, or in states of hyperdopaminergia or hypoglutamatergic function. Furthermore, some subtle differences were observed between ISIs. This may be

due to differences between neural circuitry determining short and long ISI response that could contribute to differences in sensorimotor gating, although this remains to be defined. Recent studies have identified short ISI response pathways that may bypass some components of the PPI neural network to expediate fast sensorimotor gating reactions, indicating that several midbrain pathways may act separately and in parallel to mediate PPI [340]. Thus, separate response networks may have contributed to ISI variances observed in this chapter.

Findings from this study are in line with our earlier research on the effects of APO and MK-801 in METH-pretreated BDNF HET mice, where we reported few interactions. However, there are also contrasts to previous BDNF HET experiments, such as equivalent baseline PPI between genotypes described in this study. The dose and frequency of METH administered during pretreatment had few effects on PPI other than reduced baseline and MK-801 effect in 111-pretreated WT mice. The lack of effect to METH highlights potential compensatory mechanisms that may prevent disruption of baseline PPI at higher METH doses.

Chapter 4 Comparison of METH-pretreatment regimens on LHA in WT and BDNF HET mice

4.1 Introduction

Stimulant drugs that recreate behaviourally distinct phenotypes of psychosis disorders can be used to elucidate the underlying neurocircuitry that contributes to the aetiology of these diseases. Locomotor hyperactivity (LHA) is an established behavioural representation of amplified DA signalling within the mesocorticolimbic network and can be used as a potential endophenotype of the positive symptoms of schizophrenia, to model changes in DA signalling along this common final pathway [216]. As discussed previously, enhanced signalling in subcortical dopaminergic neurons has been identified as a core pathophysiological trait of psychosis. Typical antipsychotics that ameliorate psychosis symptoms in schizophrenia are largely DA receptor antagonists [341]. These drugs prevent heightened DA transmission via blockade of the D2 receptor. Thus, increased DA signalling has been widely implicated in psychosis aetiology and is measurable in animal models through analysis of psychostimulant-induced LHA [216].

It is important to note that although the underlying neurobiology may be shared between species, the consequence of enhanced DA signalling on behaviour varies between humans and rodents. Therefore, LHA holds relatively poor face validity for positive symptoms of schizophrenia. On the other hand, because hyperlocomotion, or LHA, represents dysfunctional DAergic signalling, parallels can be drawn between LHA in rodents and aspects of human psychosis-like behaviour that may occur as a result of heightened DA signalling. Thus, DA-sensitive behaviours converge on dysfunctional signalling within subcortical regions of the brain and, in the case of rodent hyperactivity, have good construct validity due to their mediation by drugs that target similar neurochemical structures in schizophrenia patients.

LHA can be measured in rodent models with relative ease and is used extensively to study changes in the underlying dopaminergic neurocircuitry of mice. METH is a drug that indirectly increases dopaminergic neurotransmission in the mouse brain, and this can be quantitatively assessed through locomotor analysis of mice following administration of the drug.

4.2 Aims for low-dose BDNF HET

The van den Buuse lab has previously examined the effect of an escalating METH pretreatment regimen on LHA [31]. This treatment protocol was designed by Manning and van den Buuse to replicate METH abuse in human users who escalate their drug intake over time. Manning et al. (2016) observed dose-dependent changes in sensitization to psychostimulant-induced LHA in mice following an escalating METH pretreatment protocol [31]. This sensitization was disrupted in BDNF HET mice, highlighting that a form of endogenous sensitization may be occurring in the absence of complete BDNF expression. However, these experiments were conducted over multiple facilities with different apparatus and require replication to confirm LHA findings. Therefore, this chapter aimed to replicate previous results and confirm if endogenous sensitization to DA was occurring in BDNF HET mice following an escalating METH pretreatment protocol. Secondly, we aimed to expand insight into the sensitizing effects of METH on LHA by exploring different METH pretreatment regimens. As in the previous chapter, this was achieved by reducing the total amount of METH given to the mice during pretreatment. These studies included the introduction of a standard METH dose that remained constant across the three-week injection phase (111-pretreated) and a subchronic dosing strategy that was administered for one week only (100-pretreated). These studies bear potential clinical significance for less frequent METH users who may develop psychosis-like symptoms with only limited exposure to the drug.

4.3 Methods

4.3.1 Mice and statistical analysis:

Locomotor activity was measured at the La Trobe Animal and Teaching Research Facility. Mice used in this study were the same as those used for PPI studies (see Chapter 3). Approximately 1 week after PPI experiment completion, the mice were tested in Med Associates locomotor photocells over 3x 180-minute sessions, as outlined in the General Methods (Chapter 2.3.3; Figure 4.1). Mice received:

1. Saline vehicle in the first session.

2. 1mg/kg meth in the second session.

3. 3mg/kg meth in the final session.

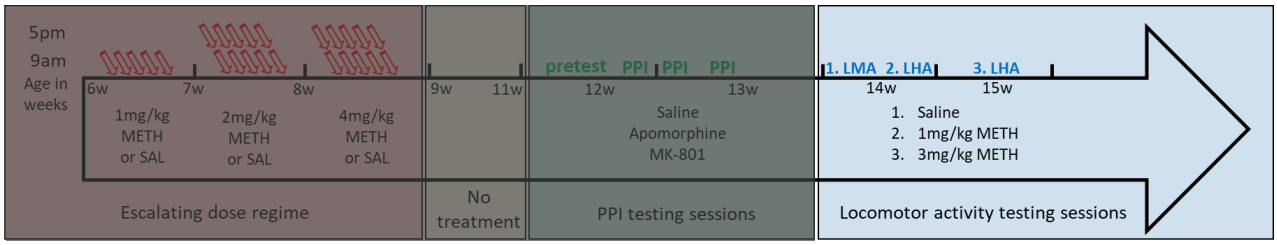

Figure 4.1 Timeline of METH experiments described in this chapter*. Baseline locomotor activity and hyperactivity were tested in weeks 14 and 15 of the low-dose METH experiment, approximately 1 week after PPI studies. Three separate locomotor trials were conducted starting with a baseline session. Three to five days later hyperactivity was measured following an acute 1mg/kg METH challenge dose, and finally after a similar wash-out period, a third trial was conducted with an acute 3mg/kg METH injection in week 15. Approximately 1-week later mice were humanely killed, and brains were dissected and fresh-frozen.*

A total of 168 mice were used in experiments described in this chapter (one mouse was humanely killed due to sickness between PPI and LMA studies). Statistical analyses were conducted on 16 experimental groups, separated by sex, genotype, and drug pretreatment. Distance moved over the 120 minutes post-injection was analysed in 5-minute bins or as the total following acute injection of vehicle or METH.

Total distance over 120 minutes was used to determine outliers across saline, 1mg/kg-, and 3mg/kg METH injection activity. As a result, 4 mice were excluded from further analysis based on having values outside 3 standard deviations of the mean (saline pretreated: 2 female WT; METH pretreated: 1 female HET 100, 1 male HET 124), resulting in n = 164 for locomotor activity investigation (Table 4.1). A repeated-measures ANOVA was used to compare vehicle vs. METH-induced hyperlocomotion between genotype, sex, and pretreatment.

Table 4.1 Number of mice per experimental group tested in low-dose BDNF HET locomotor study

	WT				BDNF HET			
	Saline	**100**	**111**	**124**	**Saline**	**100**	**111**	**124**
Male	12	12	11	11	11	10	10	9
Female	8	10	9	9	11	10	11	10

4.4 Results

4.4.1 Bodyweight

No significant changes in bodyweight were observed other than high bodyweight in male mice and greater weight gain in BDNF HET mice compared to WT mice over the duration of the experiment. METH-pretreatment did not affect mouse bodyweight. For details, see BDNF HET PPI Chapter 3.4.1.

4.4.2 Baseline locomotor activity

Baseline locomotion was measured in the first LMA session following acute saline IP injection. Univariate analysis of the total distance moved in the 2-hour period post-injection phase revealed no significant differences between any of the pretreatment or genotype groups. A strong trend was detected for higher baseline locomotor activity in females compared to males (main effect of sex, $F(1,148)= 3.83$, $p= 0.052$), but no significant difference in baseline activity was recorded across any between-subject variable (Figure 4.2).

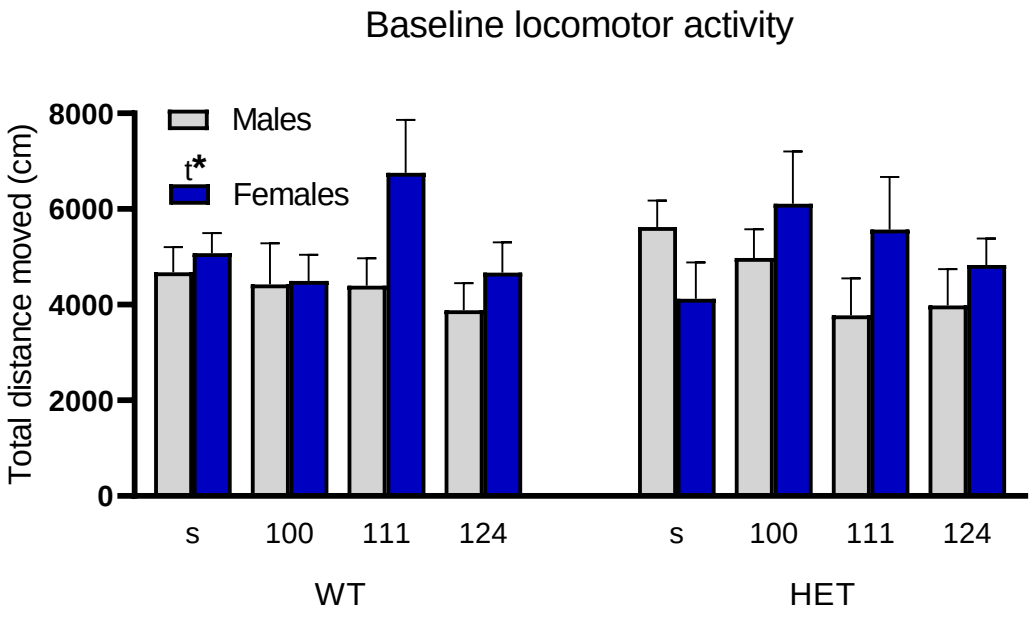

Figure 4.2 Total baseline locomotor activity post-saline injection. *Saline and METH pretreated male and female BDNF HET and WT mice injected with saline were analysed for baseline locomotor activity. The total distance moved over 2 hours post injection did not differ significantly between sex, pretreatment, or genotype groups. A trend for increased baseline locomotion was detected in females: t* = 0.052. In all cases data are mean ± SEM.*

4.4.3 LHA to 1mg/kg METH challenge

Variability in baseline LMA between individual mice was accounted for by combining the saline challenge session with the acute 1mg/kg METH session in a repeated-measures ANOVA design. Acute 1mg/kg METH significantly enhanced baseline locomotor activity vs. acute saline (main effect of METH, $F(1,148)= 119.57$, $p<0.001$; Figure 4.3). The locomotor response to the low METH dose did not differ between the sexes or genotypes so data were grouped. A pretreatment effect prompted post-hoc analyses (interaction, METH x pretreatment, $F(3,148)= 5.58$, $p= 0.001$), although no significant variance between pretreatments were identified, a trend for increased LHA was detected for all METH groups compared to saline (Tukey's: SAL:100 $p= 0.107$, SAL:111 $p= 0.068$, SAL:124 $p= 0.112$; Figure 4.3).

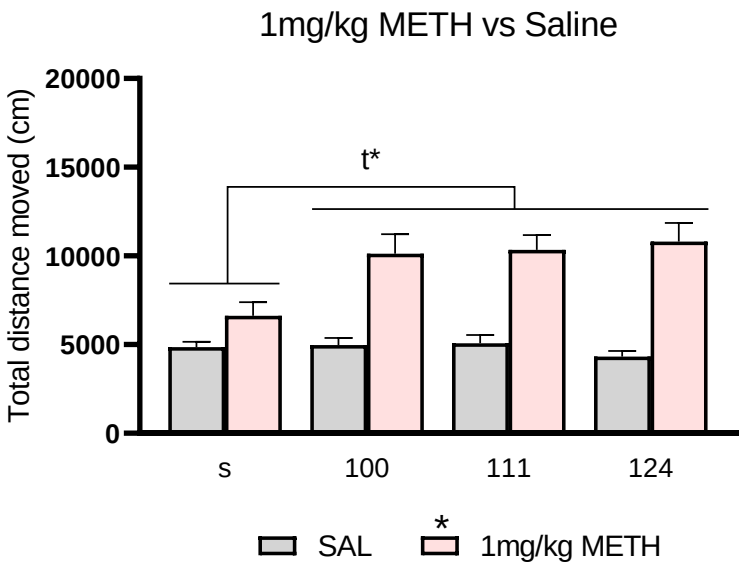

Figure 4.3 Total distance baseline vs 1mg/kg METH (sexes combined). *Hyperactivity to 1mg/kg METH was compared to LMA obtained 2-hours post-injection of the saline challenge. The total distance moved increased significantly following acute METH (*P<0.001). There was no main effect of sex or genotype on LHA, but a trend was detected for increased LHA in METH-pretreated mice (t*= 0.067-0.112). In all cases data are mean ± SEM.*

4.4.3.1 Saline vs. 1mg/kg METH: first hour

Mice were most active during the first hour post-injection. Following a peak in activity between 0-60 mins post-injection (Figure 4.4), the hyperlocomotion response to 1m/kg METH decreased and plateaued over the second hour of activity. Based on these observations, LHA between 0-60 mins post-injection was analysed separately to compare differences between experiment groups during the phase of greatest change in activity in response to METH. No significant differences in LHA were detected between male and female mice, so sexes were grouped. During the first hour HET mice responded to 1mg/kg METH with significantly higher LHA compared to WTs (METH x genotype, $F(1,148)= 4.99$, $p= 0.027$; Figure 4.5). A significant pretreatment effect was detected (METH x pretreatment, $F(3,148)= 8.06$, $p< 0.001$) leading to post-hoc analysis showing LHA in 100- and 124-preteated mice compared to saline-pretreated mice ($p< 0.05$), and a strong trend for higher activity in 111-pretreated mice compared to saline-pretreated mice ($p< 0.056$; Figure 4.5).

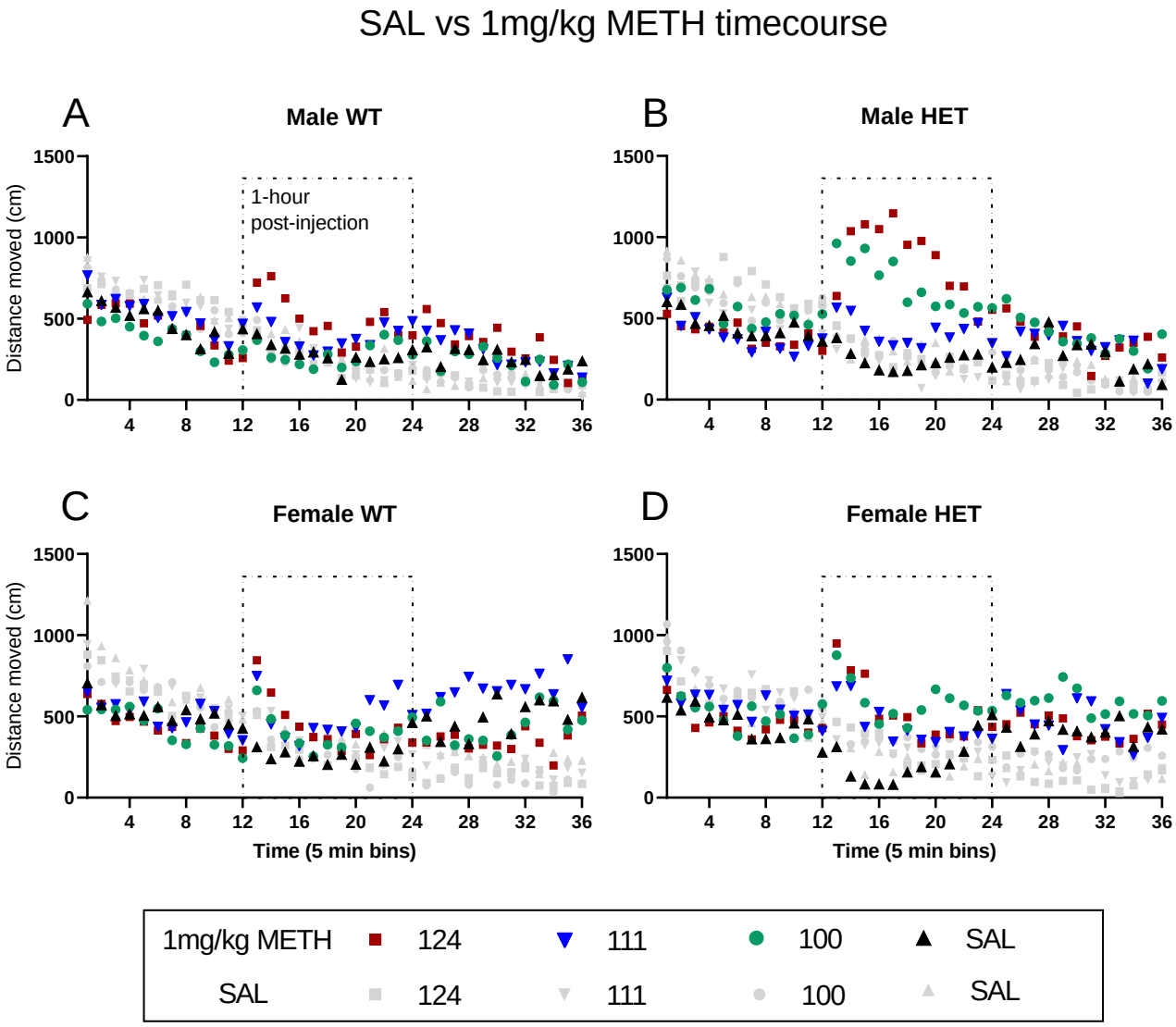

Figure 4.4 Saline and 1mg/kg METH timecourse. *Distance moved over the 2-hour timecourse was compared between acute saline and 1mg/kg METH over 1-hour post-injection. Graphs are split by sex and genotype groups. Saline or METH was injected at time bin 12 (60 mins) following 1 hour of habituation to the photocell cage. In all cases data are represented as the mean.*

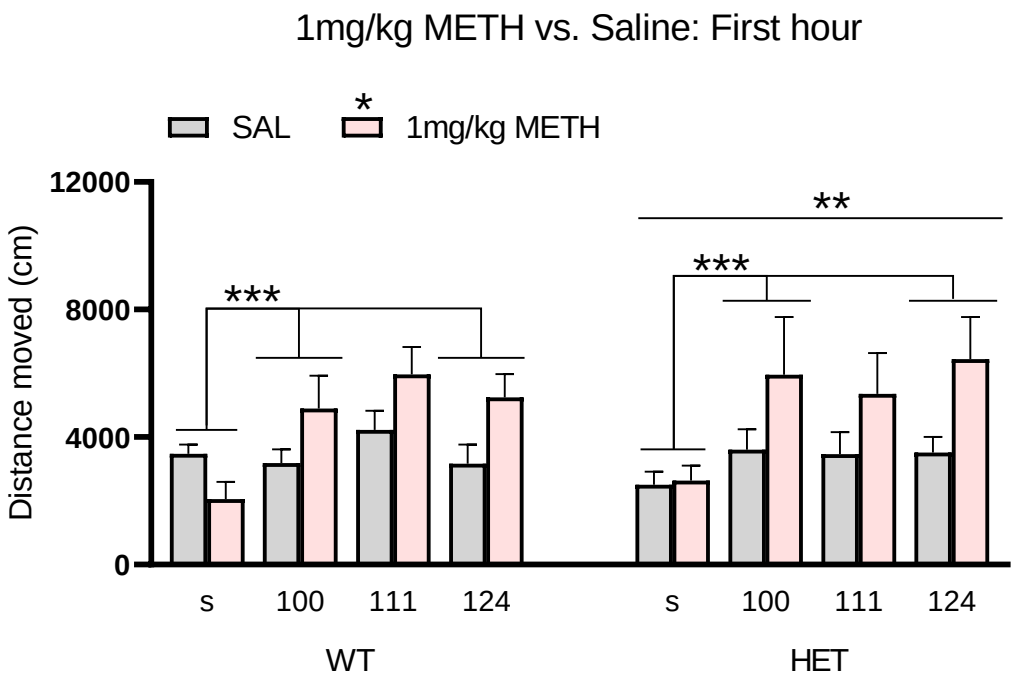

Figure 4.5 First hour total distance: Baseline vs 1mg/kg METH (sexes combined). *Hyperactivity to 1mg/kg METH was compared to LMA after saline injection over the first hour. There was no main effect of sex on hyperactivity, so male and female data were combined. The total distance moved increased significantly following acute METH (*P<0.001) and LHA was increased in BDNF HET mice (**P= 0.027). Tukey's post hoc comparison revealed a significant increase in hyperactivity in 100- and 124-pretreated vs. saline-pretreated mice (***P< 0.05)*

4.4.4 LHA to 3mg/kg METH challenge

To compare LMA between acute saline and 3mg/kg METH trials, data were analysed in a repeated-measures ANOVA. 3mg/kg METH significantly increased locomotor activity above saline session activity (main effect of METH, $F(1,148)= 925.81$, $p<0.001$). Hyperactivity was affected by genotype (interaction of METH x genotype, $F(1,148)= 16.58$, $p=<0.001$) and pretreatment (interaction of METH x pretreatment, $F(3,148)= 7.67$, $p<0.001$), and a strong trend was detected for a three way interaction between METH, genotype, and sex (interaction, $F(3,148)= 3.80$, $p= 0.053$). Given the strong interaction trend and previously observed sex-specific variance in locomotor activity, the data were split into male and female groups for subsequent analyses. Male BDNF HET mice displayed increased locomotor activity compared to male WT mice following acute 3m/kg METH (interaction of METH x genotype, $F(1,78)= 18.33$, $p<0.001$; Figure 4.6). In contrast, total distance moved in male mice over the 2-hour period was not significantly

affected by the pretreatment received. Unlike male mice, locomotor activity in female mice did not differ significantly between genotypes but showed significant pretreatment effects (interaction of METH x pretreatment $F(3,70)= 6.58$, $p= 0.001$). Tukey's post-hoc analysis revealed female 124-pretreated mice displayed enhanced METH-induced LHA compared to saline controls ($p< 0.05$), and no significant differences were observed between saline-pretreated and 100-pretreated, and between saline-pretreated and 111-pretreated female mice (Figure 4.6).

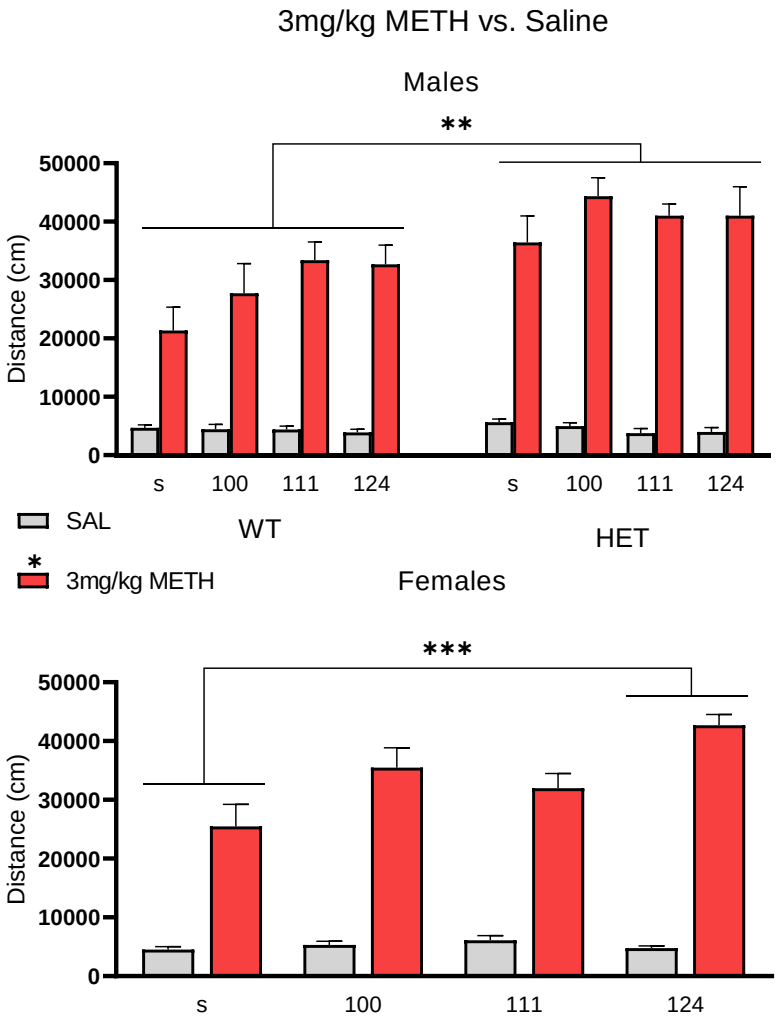

Figure 4.6 Total distance: baseline vs 3mg/kg METH. *Hyperactivity to 3mg/kg METH was compared to LMA obtained 2-hours post-injection with saline. The total distance moved increased significantly following acute METH (*P<0.001). There was no main effect of genotype in female mice, but male BDNF HET mice had significantly enhanced hyperactivity compared to male WTs (**P<0.05). In female mice, genotypes were not significantly different, so data were combined. Pretreatment with the 124-protocol resulted in enhanced hyperactivity compared to saline controls (***P< 0.05). In all cases data are mean ± SEM.*

4.4.4.1 3mg/kg METH timeline analysis: first hour

LHA to high METH challenge was greatest over the first post-injection hour and showed the largest variability between genotype and pretreatment groups during this period (Figure 4.7). In the second hour, genotype and pretreatment differences were no longer apparent, hence, data from the first hour were analysed separately to determine the impact of BDNF depletion and METH-pretreatment on peak LHA. Compared to the first hour of LMA in SAL-treated mice, mice that received 3mg/kg METH were significantly more active (main effect of METH, $F(1,148)= 968.25$, $p< 0.001$; Figure 4.8). This activity was enhanced in BDNF HETs compared to WT mice (interaction of METH x genotype, $F(1,148)= 17.96$, $p< 0.001$; Figure 4.8). In contrast to analysis of the full 2-hour post-injection time course, LHA during the 1-hour post-injection period did not differ between male and female mice, so data were grouped for further analysis. Pretreatment also had a significant effect on the locomotor response to 3mg/kg METH (interaction of METH x pretreatment, $F(3,148)= 10.84$, $p< 0.001$), and given genotype differences, WT and HET mice were analysed separately for differences between pretreatment groups. Tukey's multiple comparisons confirmed increased hyperactivity in 124- and 111-pretreated groups compared to saline control controls in WT mice ($p< 0.05$; Figure 4.8). In HET mice, Tukey's analysis reported significantly higher LHA in 124- and 100-pretreated mice compared to saline ($p< 0.05$; Figure 4.8), but no significant difference between saline and 111-pretreated mice.

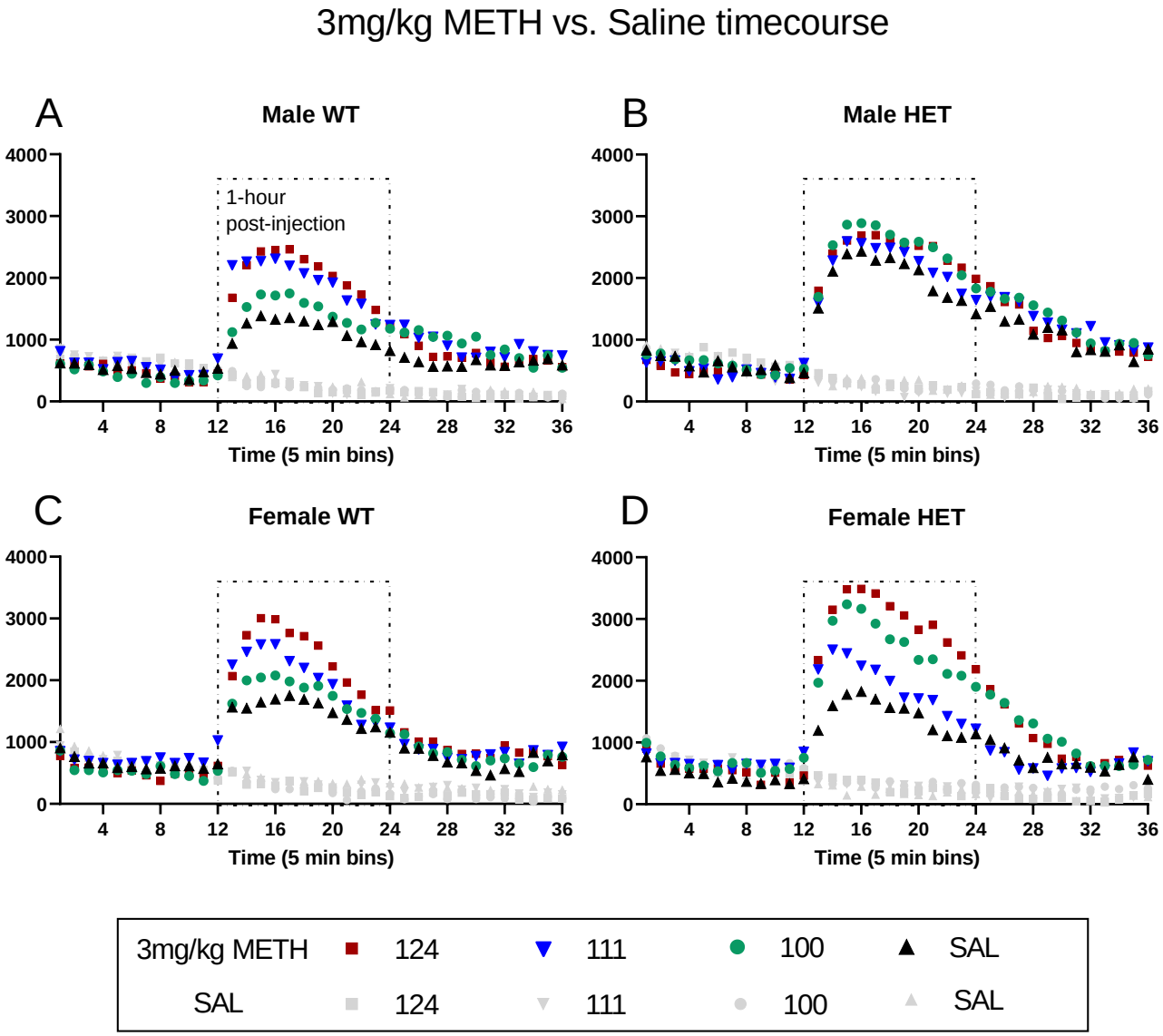

Figure 4.7 Saline and 3mg/kg METH timecourse. *Distance moved was compared between acute saline and 3mg/kg METH challenge over the first hour post-injection. Graphs compare LHA between pretreatment groups within the four sex and genotype combinations. Saline or METH was injected at time bin 12 following 1 hour of habituation to the photocell cage. In all cases data are represented as the mean.*

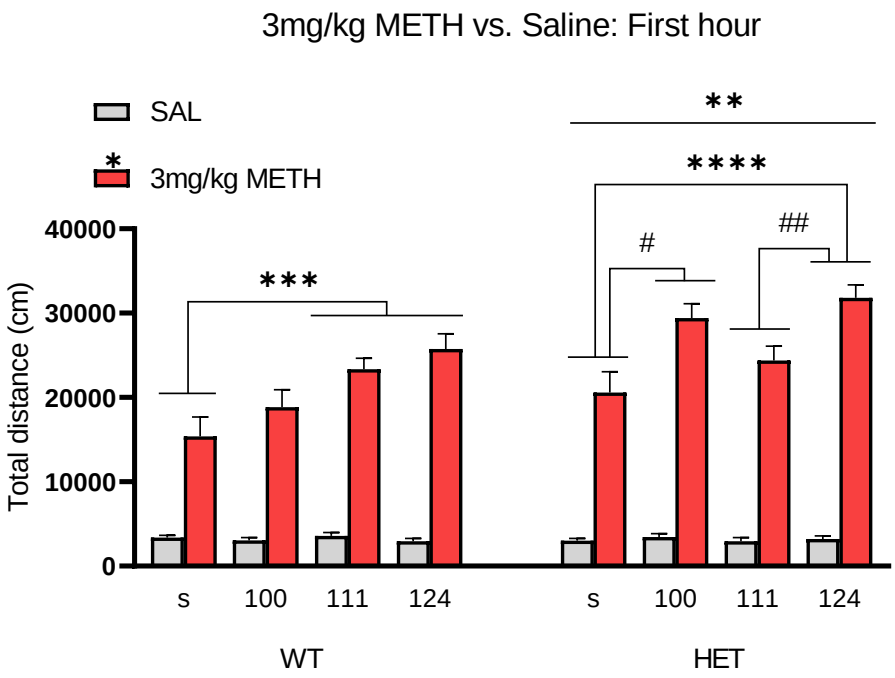

Figure 4.8 First hour total distance: Baseline vs 3mg/kg METH (sexes combined). *Hyperactivity to 3mg/kg METH was compared to LMA after saline injection over the first hour. There was no main effect of sex on hyperactivity, so male and female data were combined. The total distance moved increased significantly following acute METH (*P<0.001) and HET mice were significantly more hyperactive than WT (**P<0.001). Tukey's post hoc revealed a significant increase in WT hyperactivity in 124-, and 111- vs. saline-pretreated mice (***P< 0.05). In HET mice, 100- and 124-pretreatment groups were significantly more hyperactive compared to saline (#P<0.05; ****P<0.05). BDNF HET 124 mice were also significantly more active than 111-pretreated mice (##P<0.005). In all cases data are mean ± SEM.*

4.5 Discussion

4.5.1 Baseline LMA

There were no differences in baseline locomotion between BDNF HET and WT mice, nor any significant impact of METH-pretreatment on naïve activity. This is partially supported by previous research conducted by Manning et al. [31], who reported similar findings in one behavioural facility and some contrasting results in another. The latter identified increased basal activity in METH-pretreated mice, and female HET mice were significantly less active than WT, although these were subtle differences in activity. The discrepancy between results is likely a representation of changes in the testing environment, highlighting the importance of conducting behavioural experiments under stringent and replicable parameters.

Together, these data suggest equivalent LMA baseline between METH- and saline-pretreated BDNF HET and WT mice.

The psychomotor effects of 1mg/kg (low) and 3mg/kg (high) acute METH varied across METH pretreatments groups. As expected, the hyperactivity response to METH was mediated by BDNF heterozygosity and METH pretreatment, but this response varied between low- and high-METH challenge.

4.5.1.1 Hyperactivity response to 1mg/kg (low) METH

Low-dose challenge was compared to LMA following saline injection. There were no significant differences between METH pretreatment regimens over the 2-hour timecourse, nor differences between genotypes. This could suggest that while low 1mg/kg METH is increasing LMA, this dose may not be stimulating the DAergic system sufficiently to reveal METH-induced sensitization. Manning et al. previously examined hyperactivity in response to a subthreshold dose of 1mg/kg amphetamine in the BNDF HET 124 METH model, reaching similar conclusions [31]. Although we did not observe sex-specific effects, previous studies have reported enhanced striatal DA release in BDNF HET female mice following administration of acute 1mg/kg METH [48]. This is somewhat in line with the trend detected for enhanced baseline LMA in this study.

Alternatively, there is evidence from this study to suggest that a shorter timeframe may be more appropriate for examining hyperactivity in response to low METH. The peak of hyperactivity to the acute 1mg/kg dose occurred within the first hour post-injection and LMA was lower in the second hour of testing. If the stimulating effects of METH are largely reduced in the second hour, the analysis of the total distance moved over this period could act to ‘dilute’ the analysis of psychomotor sensitization to the low acute drug dosage and mask potential differences in pretreatment and genotype groups. Therefore, analysis of METH-induced hyperactivity over the first hour was used as an alternative measure. During this post-injection period, each METH pretreatment regimen significantly enhanced hyperactivity compared to saline-pretreated mice, suggesting METH sensitization was only significantly impacting LMA during the peak hyperactivity phase. This observation highlights neurochemical pathways mediating sensitization may only be impacting LMA during peak DA release. Furthermore, BDNF HET mice had

increased LHA compared to WT mice during the first hour. Therefore, the contribution of BDNF mediation on the low-dose METH response may be most clear during the period of greatest hyperactivity. This is further highlighted by the fact that BDNF heterozygosity and pretreatment impact sensitization at similar timepoints, suggesting a role for BDNF in mediating sensitization evoked by the different METH pretreatment regimens. Given that peak LMA is enhanced by both BDNF heterozygosity and METH-induced sensitization in mice, it could be argued that if these 'hits' create enhanced activation of DAergic pathways, in humans they could contribute to the development of psychosis-like behaviours.

Although the response to acute low METH was affected by a history of METH use, the dosage of METH in the pretreatment regimen did not significantly alter the induced hyperactivity in the first- or second-hour post-injection. This is an important result to consider for future psychomotor studies as it implies less importance can be placed on an escalating METH regimen for investigating psychosis-related behaviours, particularly to lower doses of psychostimulants. This finding also bears clinical relevance for METH addicts who use the drug infrequently or are in some way regulating their intake, as opposed to the typical dose escalation that precedes psychosis. If sensitization is required for the development of heightened psychomotor behaviours, these results highlight the potential of drug-induced psychosis following limited exposure to METH and other amphetamines. Sensitization of dopaminergic pathways may be occurring irrespective of an escalating dose regimen.

4.5.1.2 Hyperactivity response to 3mg/kg (high) METH

Combined analysis of saline (baseline) LMA and the acute high-METH challenge revealed sex-specific effects. Male BDNF HET mice were hypersensitized relative to WT controls and no effect of METH pretreatment was detected. In contrast, LHA was similar between BDNF HET and WT female mice but the 124-regimen produced significantly stronger METH-induced hyperactivity than saline-pretreated mice. These data highlight a role for BDNF in mediating sensitization to METH. Male mice appear more susceptible to the effects of attenuated BDNF expression, which points to the possible involvement of sex hormones in mediating sensitization pathophysiology.

LHA in the first hour was analysed to examine peak activity following a 3mg/kg METH challenge. Significantly enhanced LHA was detected in METH-pretreatment groups and BDNF HET mice compared to saline control and WT mice, respectively. There were LHA differences observed between METH regimens in the first hour, although hyperactivity did not follow a standard dose-response curve. 100-pretreated mice were significantly more hyperactive than saline-pretreated mice, while 111-pretreated had similar activity to controls. This was unexpected as 100-pretreated mice received less METH in the single week regimen, although it should also be noted that these mice had an additional 2 weeks between METH and behavioural testing, which may contribute to differences observed.

METH-induced locomotor variation between pretreatment groups across the timecourse may also be explained by taking into account METH half-life ($t_{1/2}$). The disappearance $t_{1/2}$ of a 2.5mg/kg METH dose injected IP in mice is approximately 66 minutes [342]. The mice in these experiments would have metabolised the only slightly higher acute 3mg/kg dose at a similar rate, resulting in disproportionate DA system stimulation between the first and second hour of the experiment. A previous study conducted in METH-treated mice during the dark phase reported increased running wheel distance and LHA, where METH concentration correlated with the magnitude of horizontal locomotion, based on pharmacokinetics of the drug [342, 343]. In the current study, the marked difference in LHA between the first and second post-injection hour suggests reduction of METH effects and the associated stimulation of DAergic activity. The lack of dose-dependent differentiation between METH-pretreatment groups over the first hour post-injection of the acute challenge may reflect saturation of the drug's effect (i.e. a 'ceiling' effect) and a resulting DAergic activation above the concentration which could show differential sensitization.

Sex-specific effects were observed on the average distance moved over the 2-hour timecourse, but not over the first hour. BDNF haploinsufficiency enhanced hyperactivity over the first hour irrespective of relative METH dose, but in the averaged 2-hour timecourse, female mice no longer showed any genotype differences. The lack of BDNF genotype effects in female mice over the 2-hour period may therefore reflect temporally sensitive sex-hormone effects that protect female mice from increased LHA in the BDNF-depleted brain. As there were no

sex differences in the initial hour, this could suggest that the neuroprotective effects of hormones, such as estrogen, take time to impact BDNF-mediated pathways. Alternatively, sex hormones may promote a tonic regulation of neurotrophic signalling, which is masked by high METH concentration in the first hour.

4.5.2 Previous findings in the BDNF HET model: Partially supported

The van den Buuse lab has previously examined the effect of an escalating METH pretreatment regimen on D-amphetamine-induced LHA [31]. These earlier experiments report similar low-dose results to a 1mg/kg amphetamine challenge. However, the previous finding of an endogenous sensitization in BDNF HET mice to a 3mg/kg D-amphetamine challenge was not supported by the results described in this chapter. Here we instead demonstrate hypersensitivity to a 3mg/kg METH challenge in METH-pretreated and BDNF HET mice with variable sex- and dose-dependency. The discrepancy could be explained by two principal differences in study design. The first is a change in testing facility and apparatus, which may play a significant role in mediating locomotor activity in mice through a host of potential environmental variables (light intensity, visual and auditory stimuli, olfactory variance), thus preventing systematic replication. Behavioural phenotypes of mice have been shown to vary depending on environmental conditions, including housing [344]. Compared to the Manning et al. study, in which mice were housed in open top cages, mice tested in this chapter were housed in IVC units. Differences between these two environments include effects of forced ventilation, relative humidity, olfactory inhibition, and temperature. Inconsistencies in study results between housing conditions has been shown to affect locomotor hyperactivity [345-347], which may reflect reduced anxiety in IVC-housed mice. However, these findings are still debated, with some studies citing no change in exploratory LMA between the two housing conditions [348, 349]. Compared to open top cages, IVC housing may also reduce schizophrenia-relevant PPI deficiencies in Nrg1 mice [345], and increase ASR of some mouse strains [350]. Consequently, housing variables may alter PPI reactivity and housing conditions may have contributed to behavioural variance in amphetamine-induced LHA and APO- and MK-801-induced PPI deficits between Manning's data and the current study.

Another factor that may have contributed to variability is the differential effect of D-amphetamine and METH on DAT-mediated hyperdopaminergia [351]. Both amphetamine derivatives stimulate the excess release of DA into the synaptic cleft via reversal of DAT function, although relative potency between the psychostimulants is debated in rodent models [351]. Behavioural studies have reported a range of equipotent [352], lower [297], and higher [353] METH- vs. amphetamine-induced behaviours, further highlighting the complexity of amphetamine-mediated DAergic activity. More recent research has demonstrated greater DA efflux, intracellular calcium ion release, and inhibition of synaptic DA clearance in the NAc of METH-treated rats compared to amphetamine-treated [351]. Therefore, it is possible that an acute 3mg/kg METH challenge could be evoking greater DAergic activation within the mesocorticolimbic circuitry than the 3mg/kg D-amphetamine, which could explain differences in LHA behavioural sensitization results between the psychostimulants.

4.6 Conclusion

The main finding of the studies described in this chapter is that an escalating METH regimen may not be required for sensitization to psychostimulant-induced LHA. Compared to controls, METH-pretreated mice displayed enhanced LHA during 1mg/kg METH challenge, but this activity was not significantly different between METH groups. In the first hour after 3mg/kg METH challenge, we observed similar differentiation of METH-pretreatment groups, with 124-pretreated mice demonstrating increased hyperactivity compared to saline-pretreated mice. This difference was also detected between 124- and saline-pretreated female mice over the 2-hour timecourse, however as before, LHA in METH groups did not differ significantly between one another. Combined with a lack of differential pretreatment effect in males to 3mg/kg METH, these results point to a ceiling effect in the high dose challenge as METH pretreatment had limited effects on LHA response. Based on these results, we conclude that DA system sensitization can occur at lower METH pretreatment regimens, although differentiation of pretreatment dose may require a greater range of challenge doses to determine potential differences in psychostimulant reactivity. As the lower challenge dose METH did not produce sensitization differences in METH groups, an intermediate

challenge between 1- and 3mg/kg may provide greater insight into METH pretreatment and how varied dose regimens contribute to sensitization.

The studies described in this chapter have also furthered our understanding of the role of BDNF in sensitization of METH-induced locomotor hyperactivity. We have confirmed both low- (1mg/kg) and high-dose (3mg/kg) METH challenges evoke increased hyperactivity in BDNF HET mice during the first hour post-injection. Male HET mice hyperactivity over the first hour was similar to 2-hour timecourse data, but female HET effects were lost following analysis of the longer post-injection phase, resulting in equal LHA between female genotypes and highlighting potential sex-specific regulation of BDNF by sex-hormones. This result partially supports previous amphetamine studies administering an escalating dose regimen and provides further evidence for BDNF-mediated effects on DA circuitry in LHA.

Chapter 5 Prepulse inhibition in the BDNF HET D3KO double mutant mouse

5.1 Introduction

As discussed in Chapters 1 and 3, attenuated PPI represents a quantifiable change in rodent sensorimotor gating that is relevant to the positive symptoms of schizophrenia and psychosis-like behaviours [216, 230]. A wide literature supports a role for both D2- and D1-receptor families in the regulation of PPI, although the majority of evidence highlights D2-like receptors as the primary modulators in rats [231] and these observations are supported by studies in mice [354]. APO and amphetamine both disrupt mouse PPI and APO-induced deficits are reversible through D2-receptor antagonism [354]. In addition, D2KO mice are resistant to amphetamine-induced PPI deficits [355] and studies on DAT KO mice that typically exhibit disrupted sensorimotor gating have demonstrated reversal of PPI deficits by D2R antagonists [356].

There is also some evidence for a role of D3R in mediating PPI in rodents. The D3R agonists, quinelorane, 7-OH-DPAT, and (+)-PD128907 disrupt PPI in the rat [357-359]. However, due to high sequence homology between D2-like receptors, these results should be interpreted with caution. D3R-agonist and antagonist compounds display poor DA receptor specificity *in vivo* and may induce parallel D2R activation/inhibition. To overcome the lack of D3R-specific ligands, here a genetic approach has been implemented to test the role of D3R activity in PPI regulation. A study in mice found no significant difference between D3KO and WT mice at baseline PPI. When treated with amphetamine, PPI was reduced, but no effect of genotype was observed [355]. In contrast, in the same study D2KO mice did not display deficits in PPI following amphetamine administration. These findings support D2Rs as the primary DAergic mediators of PPI. However, a role for D3Rs has been identified in D3KO mice treated with cocaine. D3KOs were more susceptible to the PPI-disrupting effects of acute cocaine administration, displaying heightened gating deficits [360]. To our knowledge, no study has examined the role of D3R in METH-induced PPI disruption, and given the proposed role of D3Rs in behavioural sensitization, this study could provide evidence of D3R-mediated changes in PPI in a DA sensitized system.

BDNF has also been implicated in regulation of the neurocircuitry governing PPI [32, 304, 337, 361], although the neural substrates involved remain unclear. There

is evidence to suggest BDNF regulates the DAergic system and impacts behavioural sensitization via induction of D3R synthesis [85]. As highlighted in chapter 1, binding of D3R inhibits D1/D2 receptor-mediated activity through opposing postsynaptic activity on AC and downstream signalling targets, and autoregulation of presynaptic DA release. However, it is primarily the activation of postsynaptic D3Rs that is hypothesized to put a 'brake' on hyperdopaminergic activity in the reward network, particularly within the D3R-rich NAc [290]. Consequently, D3KO preclinical models describe heightened susceptibility to the effects of amphetamines, double the extracellular DA concentration of WT animals, and reduced DA reuptake from loss of DAT regulation [78, 80, 81].

Furthermore, lower D3R mRNA and protein expression has been reported in schizophrenia patients during episodes of acute psychosis and in chronic disease pathology [362-364]. Combined with innate downregulation of D3R in BDNF HET mice [49], these findings highlight a potential pathway mediating psychosis-like behaviours in BDNF haploinsufficient mice that may contribute to disruption of neurocircuitry governing PPI. It is hypothesized that partial depletion of BDNF may promote DAergic system sensitization through loss of D3R-mediated negative feedback and drive sensorimotor gating deficits in BDNF HET mice.

5.2 Aims

Experiments thus far on BDNF heterozygote mice and rats have garnered mixed results on inhibition of startle gating in METH-pretreated neurocircuitry. While we did not observe sensorimotor gating genotype differences in studies described in Chapter 3, previous work conducted by our group has demonstrated reduced PPI at baseline in BDNF HET mice [31] and rats [40]. Consequently, this study sought to repeat PPI experiments in the BDNF HET mouse model to replicate previous results in METH-pretreated mice. APO and MK-801 were used to disrupt PPI and compare sensorimotor gating deficits between genotypes.

Here we suggest D3R expression, which is reported to be attenuated in BDNF HET mice, might contribute to changes in PPI due to DAergic system sensitization and BDNF deficiency. To our knowledge, this is the first study to combine BDNF HET and D3KO. This new BDNF HET / D3KO double-mutant (DM) model offers a platform to examine the involvement of the D3R in the role of BDNF in psychosis

and the effects of chronic METH development and provides further interrogation of the relationship between BDNF and the DA and glutamate signalling systems.

5.3 Methods

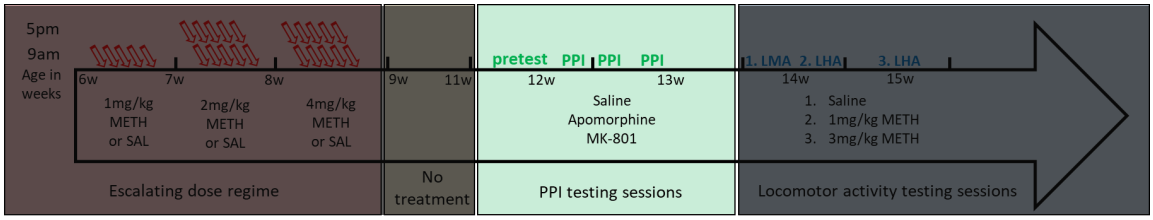

Figure 5.1 A timeline of METH pretreatments and PPI experiments outlined in this chapter. *Mice underwent three PPI sessions pseudorandomly ordered across a 2-week testing phase (highlighted in green). The three injections included: saline, 3mg/kg APO, and 0.2mg/kg MK-801.*

5.3.1 Animals and statistical analyses

Table 5.1 Number of mice used in PPI experiments outlined in this chapter

	SAL-pretreated				METH-pretreated			
	WT	BDNF HET	D3KO	DM	WT	BDNF HET	D3KO	DM
Male	12	13	10	13	11	14	10	12
Female	12	14	11	14	13	11	10	13

A total of 193 mice were used in the experiments described in this chapter. Statistical analysis was conducted on groups separated by genotype, sex, and METH pretreatment (outlined in Table 5.1) resulting in 16 experimental groups for behavioural comparison. Statistical analysis was conducted on the average PPI percentage across the 30ms and 100ms ISIs and startle amplitude, as described in chapter 2. Outliers were detected using Z scores calculated for the saline, APO, and MK-801 100ms ISI average PPI values.

We did not conduct a SHIRPA [365] screen or physical exam of the double mutants, although the BDNF HET/ D3KO mouse is a cross of two previously

established mouse lines which have previously been examined for basic motor functions and behavioural abnormalities. The BDNF HET mouse strain does not exhibit overt behavioural changes or other developmental, or reproductive traits and/or predisposition to disease. One abnormality reported in this study and others is a small but significant increase in average animal bodyweight in BDNF HET mice. In addition, this strain requires no additional care and maintains regular breeding activity when crossed with WT mice [37].Previous behavioural analysis of the D3KO mouse strain has demonstrated mixed results regarding increased basal locomotor activity [84, 366], and reduced anxiety levels in open field, forced swim test, and elevated plus maze paradigms [366, 367]. However, another study reported no differences between D3KO and WT mice in a standardised behaviour battery, including exploratory behaviour and anxiety and depressive-like symptoms [368].

Four mice were removed from analysis (saline-pretreated, 1 male BDNF HET, 1 male D3KO, 1 female BDNF HET, 1 female D3KO) based on average PPI values outside 3 standard deviations of the mean. A repeated-measures ANOVA was used to compare differences in PPI between sex, genotype, and pretreatment following separate IP injection of saline, 3mg/kg APO, and 0.2mg/kg MK-801 prior to session commencement.

5.4 Results

5.4.1 Bodyweight of mice

Mice were weighed twice per week during METH or saline pretreatment and once before every acute drug IP injection. Bodyweight is presented here at 4 timepoints: initial weight, end of pretreatment phase, end of no-treatment (NT) phase, and end of behavioural analyses (Figure 5.2). Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated for bodyweight change over the 4 timepoints ($\chi^2(5) = 349.33$, $p < 0.05$), so the Greenhouse-Geisser correction was used to adjust results. Bodyweight increased significantly in all groups across the experiment timeline (main effect of time, $F(1.397,240.31) = 419.81$; $p<0.001$). There was no effect of pretreatment on bodyweight, although male mice were heavier than female mice at each timepoint and genotype differences were identified (main

effect of sex, F(1.397,240.31)= 7.02; p= 0.004; main effect of genotype, F(4.19,240.31)= 8.69; p<0.001). To further explore genotype variability in bodyweight, data were split into BDNF and D3R variables to test the individual contribution of BDNF deficiency and D3R loss on weight variance.

Results confirmed no significant D3R interaction with mouse bodyweight, and a significant BDNF effect, in which both BDNF HET genotypes gained significantly more weight than the corresponding WT genotypes (BDNF HET and DM vs. WT and D3KO; interaction, Time x BDNF genotype, F(1.40,240.31)= 25.71; p<0.001).

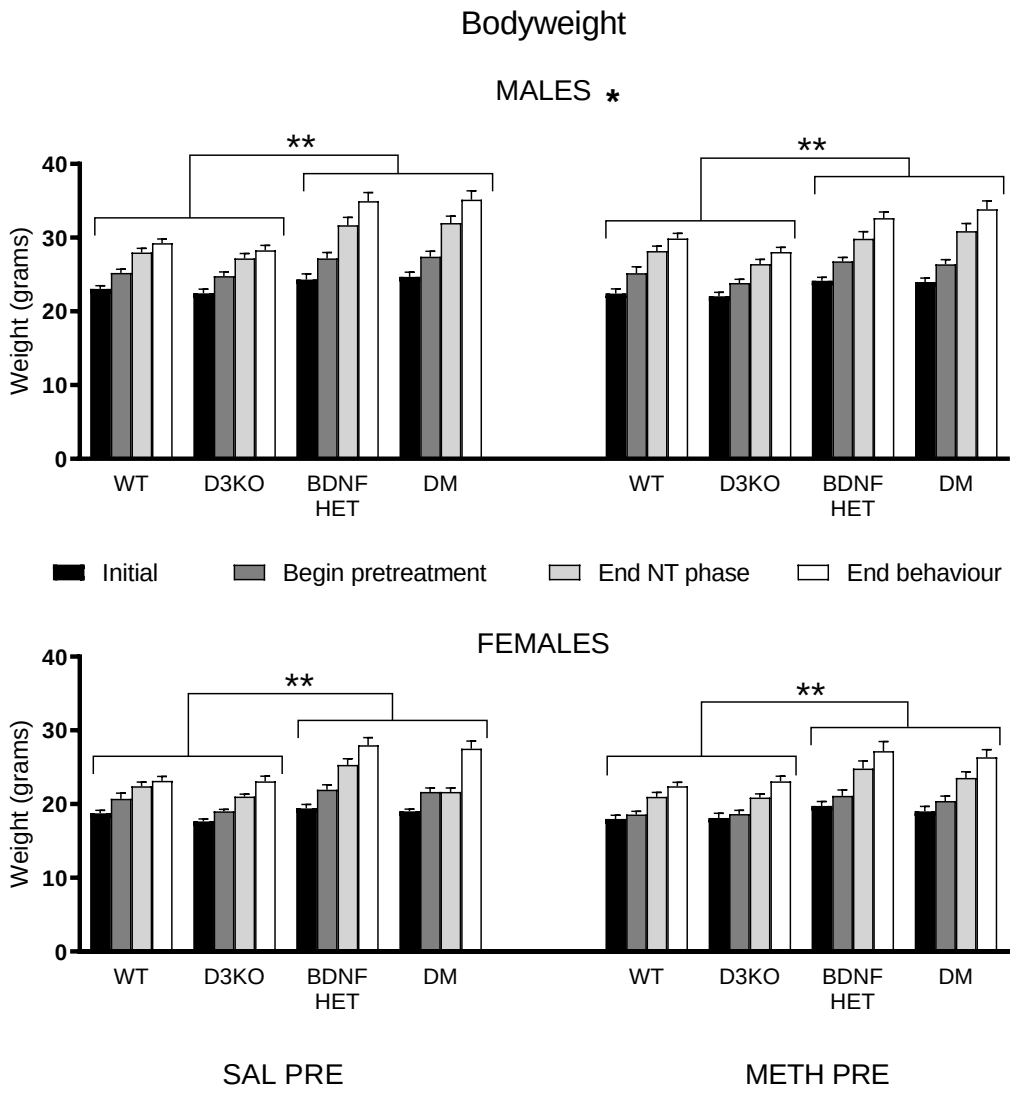

Figure 5.2 The bodyweight of mice across the BDNF HET/D3KO study. *Males were significantly heavier than females (*p < 0.05) and BDNF HET mice were significantly heavier than WT mice at all time points. No difference in weight was observed between METH and Saline-pretreatment. *= p < 0.05 for difference between males and females, ** = p< 0.05 for difference between BDNF HET and BDNF WT mice.*

During testing (end no-treatment phase to end experiments), mouse bodyweight was similarly affected by BDNF heterozygosity, with BDNF HET mice gaining significantly more weight over time (interaction, bodyweight change x BDNF, $F(1,172)= 5.54$, $p= 0.02$). No significant bodyweight differences were observed between saline- and METH-pretreatment groups.

5.4.2 Baseline PPI

Baseline PPI was measured following acute saline injection. Average PPI at 30ms and 100ms ISI was significantly affected by genotype (30ms ISI, main effect of genotype, $F(3,173)= 1.92$; $p= 0.006$; 100ms ISI, main effect of genotype, $F(3,173)= 4.30$; $p= 0.006$) but the effect of D3 and BDNF deficiency varied between the two interstimulus intervals. Baseline PPI at the 30ms ISI did not vary across sex or pretreatment groups, therefore these data were combined. Tukey's post hoc analysis of genotype at the 30ms ISI revealed differences in baseline PPI between BDNF and D3 genotype variants (Figure 5.3). Compared to D3KO, BDNF HET and DM mice had significantly lower average PPI following acute saline injection (Tukey's post hoc, $p< 0.05$; Figure 5.3), although no group was significantly different from WT mice.

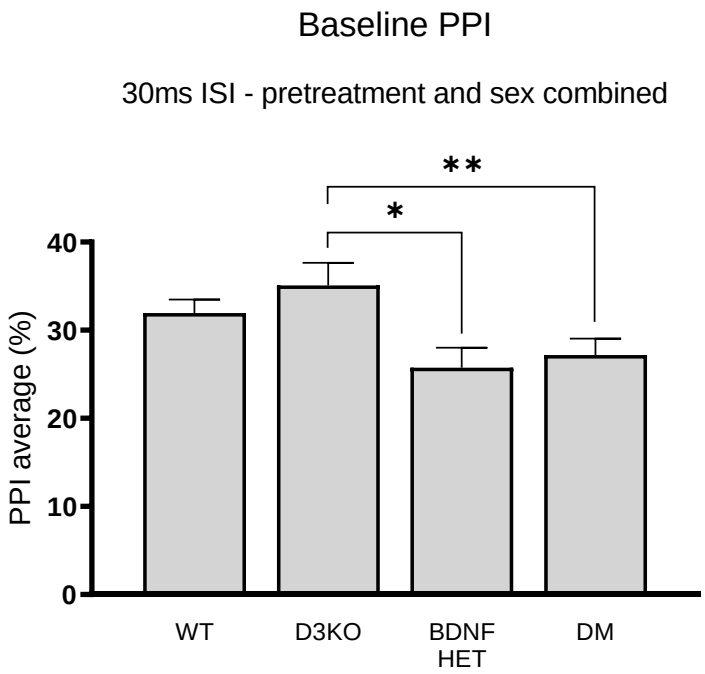

Figure 5.3 Average baseline PPI at the 30ms ISI following acute saline injection. ** = p< 0.05 difference between D3KO and BDNF HET mice, **= p< 0.05 between D3KO and double mutant mice. Data are mean ± SEM.*

At the 100ms ISI, a significant 3-way interaction was observed between sex, pretreatment, and genotype (interaction F(3,173) = 3.09, p= 0.028). To explore this interaction the data were split by genotype and sex (Figure 5.4 A-B). Saline-pretreated male baseline PPI did not differ significantly between genotypes. Contrastingly, male mice pretreated with METH displayed a BDNF genotype effect (F(3,43)= 4.32, p= 0.01). Tukey's post hoc analysis confirmed lower baseline PPI in BDNF HET and DM mice compared to D3KO mice (p< 0.05). No group was significantly different from WT mice baseline PPI (Figure 5.4 A). There was no difference between female saline- and METH-pretreated mice (Figure 5.4 B).

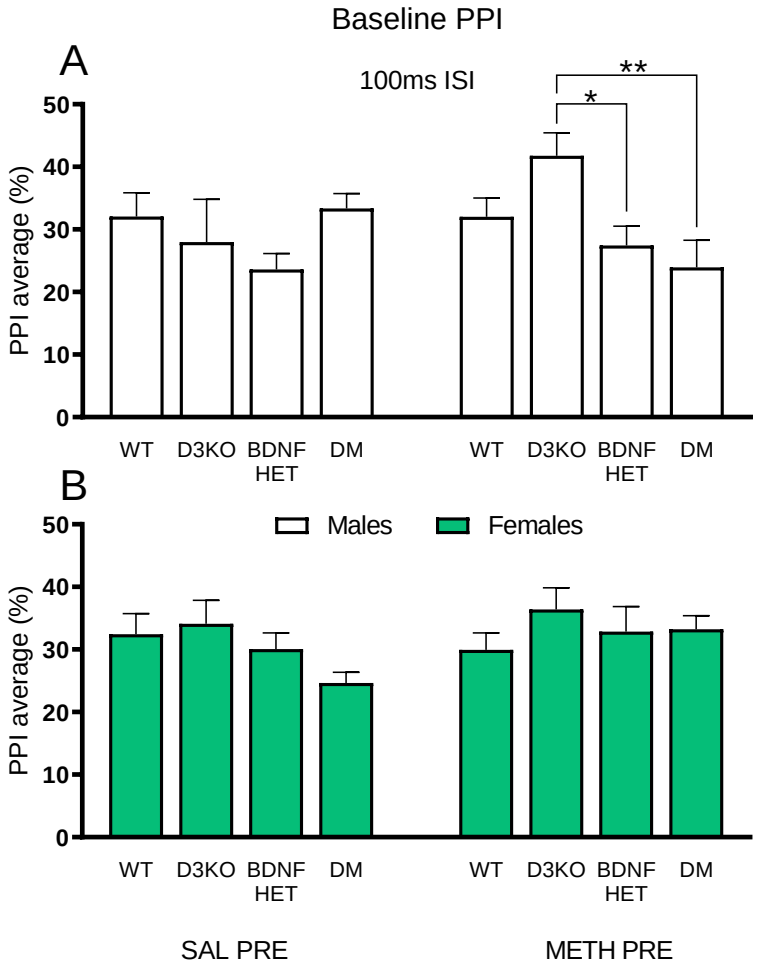

Figure 5.4 Average baseline PPI at the 100ms ISI following acute saline injection. (A)* = p< 0.05 difference between male D3KO and BDNF HET mice, **= p< 0.05 between male D3KO and DM mice. **(B)** Average baseline female mice data. Data are mean ± SEM.*

5.4.3 Baseline Startle

Baseline startle recorded over the saline PPI session was unaffected by chronic METH injection, so the METH- and saline-pretreatment groups were combined (Figure 5.5). Startle was significantly lower in female mice compared to male mice (main effect of sex, $F(1,173)= 28.62$, $p<0.001$). A significant main effect of genotype ($F(3,173)= 10.09$; $p<0.001$) prompted further analysis, in which a post hoc Tukey test identified D3KO mice had significantly lower startle compared to BDNF HET and DM genotypes, but not WT ($p< 0.05$; Figure 5.5).

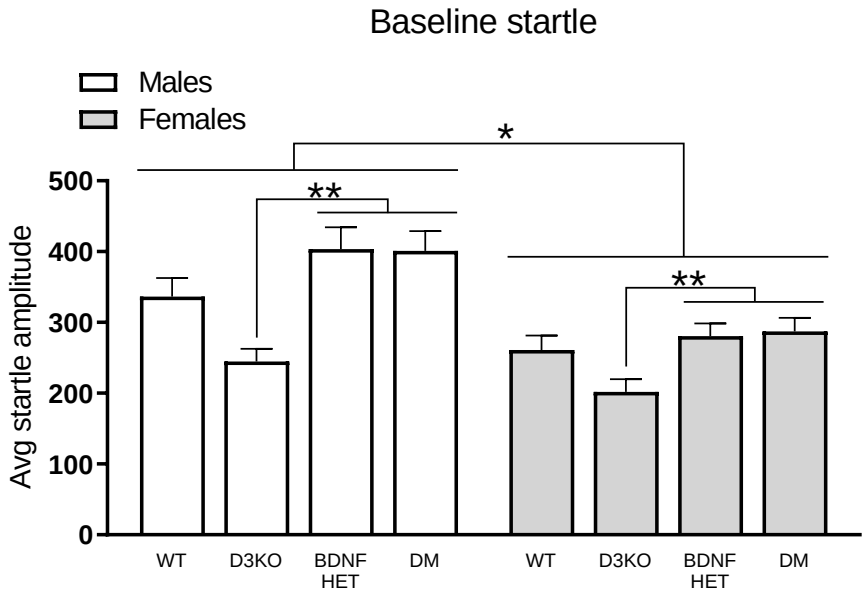

Figure 5.5 Startle amplitude of mice after saline challenge. ** = p< 0.05 difference between male and female mice, **= p< 0.05 between male D3KO and both BDNF HET and DM mice. Data are mean ± SEM.*

5.4.4 APO PPI

Compared to saline IP injection, acute APO administration significantly reduced PPI at both the 30ms and 100ms ISIs (30ms ISI, main effect APO, $F(1,173)=192.76$, $p<0.001$; 100ms ISI, main effect of APO, $F(1,173)= 115.4$, $p<0.001$; Figure 5.6 A,B,D). At the 30ms ISI, no differences were observed between saline and APO between genotype, sex, or pretreatment groups.

A significant 4-way interaction was detected at the 100ms ISI (APO x sex x genotype x pretreatment, $F(3,173)= 3.19$, $p=0.025$), prompting further investigation of the data split by sex and pretreatment. APO-induced PPI disruption varied in

male mice pretreated with METH. Compared to WT and D3KO, male BDNF HET and DM mice had an attenuated APO response (BDNF HET x APO, F(1,42)= 9.41; p=0.004; Figure 5.6 B). Post hoc ANOVA confirmed no genotype differences between male mice pretreated with saline, nor differences across genotype or pretreatment in female mice Figure 5.6 D).

Compared to baseline startle following saline injection, startle reactivity following APO challenge was significantly attenuated (main effect APO, F(1,173)= 204.34; p<0.001; Figure 5.6 C) but this reduction did not differ significantly between genotype, pretreatment, or sex.

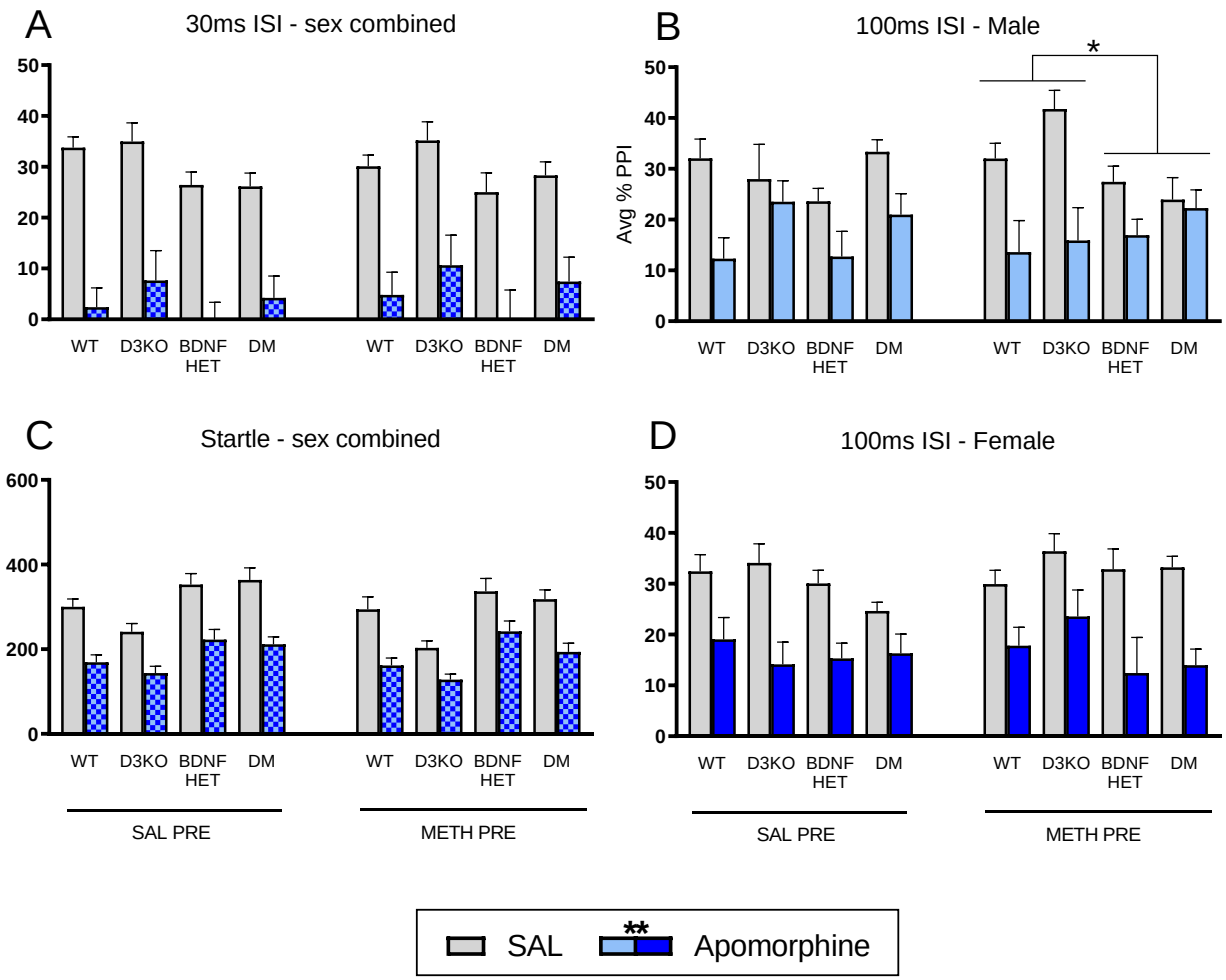

Figure 5.6 Average PPI and startle response across acute saline and APO sessions. (A) *Sex combined 30ms ISI PPI.* ***(B)*** *Male 100ms ISI PPI *= p< 0.05 for difference between saline and APO PPI in BDNF HET vs. BDNF WT mice.* ***(C)*** *Sex combined average startle amplitude.* ***(D)*** *Female 100ms ISI PPI. **= p< 0.05 difference between saline and APO PPI and startle. Data are mean ± SEM.*

5.4.5 MK-801 PPI

Compared to saline IP injection, acute MK-801 administration significantly reduced PPI at both the 30ms and 100ms ISI (30ms ISI, main effect MK-801, $F(1,173)= 192.76$, $p< 0.001$; 100ms ISI, main effect MK-801, $F(1,173)= 237.72$, $p< 0.001$; Figure 5.7 A-C). At the 30ms ISI there were no genotype-, pretreatment-, or sex-specific effects observed for the average change in PPI between saline and MK-801 sessions (Figure 5.7 A).

A significant genotype interaction was detected at the 100ms ISI (MK-801 x genotype, $F(3,173)= 3.35$, $p=0.02$). A second ANOVA was conducted with genotype split into presence or absence of BDNF heterozygosity and D3KO. The 2x2x2x2 ANOVA revealed no significant interaction or effect of loss of the D3R, but similar to the APO PPI data, BDNF HET and DM male mice displayed significantly attenuated PPI disruption compared to BDNF WT male mice (main effect BDNF, $F(1,173)= 9.60$; $p= 0.002$; Figure 5.7 B). Female mice were not significantly different across genotype or pretreatment regimens, and the genotype of saline-pretreated male mice did not affect average MK-801-induced-PPI.

Average startle amplitude was compared between saline and MK-801 PPI sessions. A significant sex effect led to separation of data into male and female groups (interaction of MK-801 x sex, $F(1,173)= 7.02$, $p= 0.009$; Figure 5.7). Sex-specific analysis of startle amplitudes showed no effect of MK-801 and no impact of genotype in male mice. A trend for higher startle in METH-pretreated males compared to saline-pretreated was detected (interaction, MK-801 x pretreatment, $F(1,85)= 3.89$, $p= 0.052$), but no interaction reached significance. In female mice, startle amplitudes following MK-801 treatment were slightly, but significantly lower than baseline values (MK-801 main effect, $F(1,88)= 8.70$, $p= 0.004$), and reduced startle amplitudes were detected in BDNF WT groups, WT and D3KO, compared to BDNF HET mice (interaction, MK-801 x BDNF, $F(1,88)= 5.19$; $p= 0.025$; pretreatment combined data not shown on Figure 5.7 E).

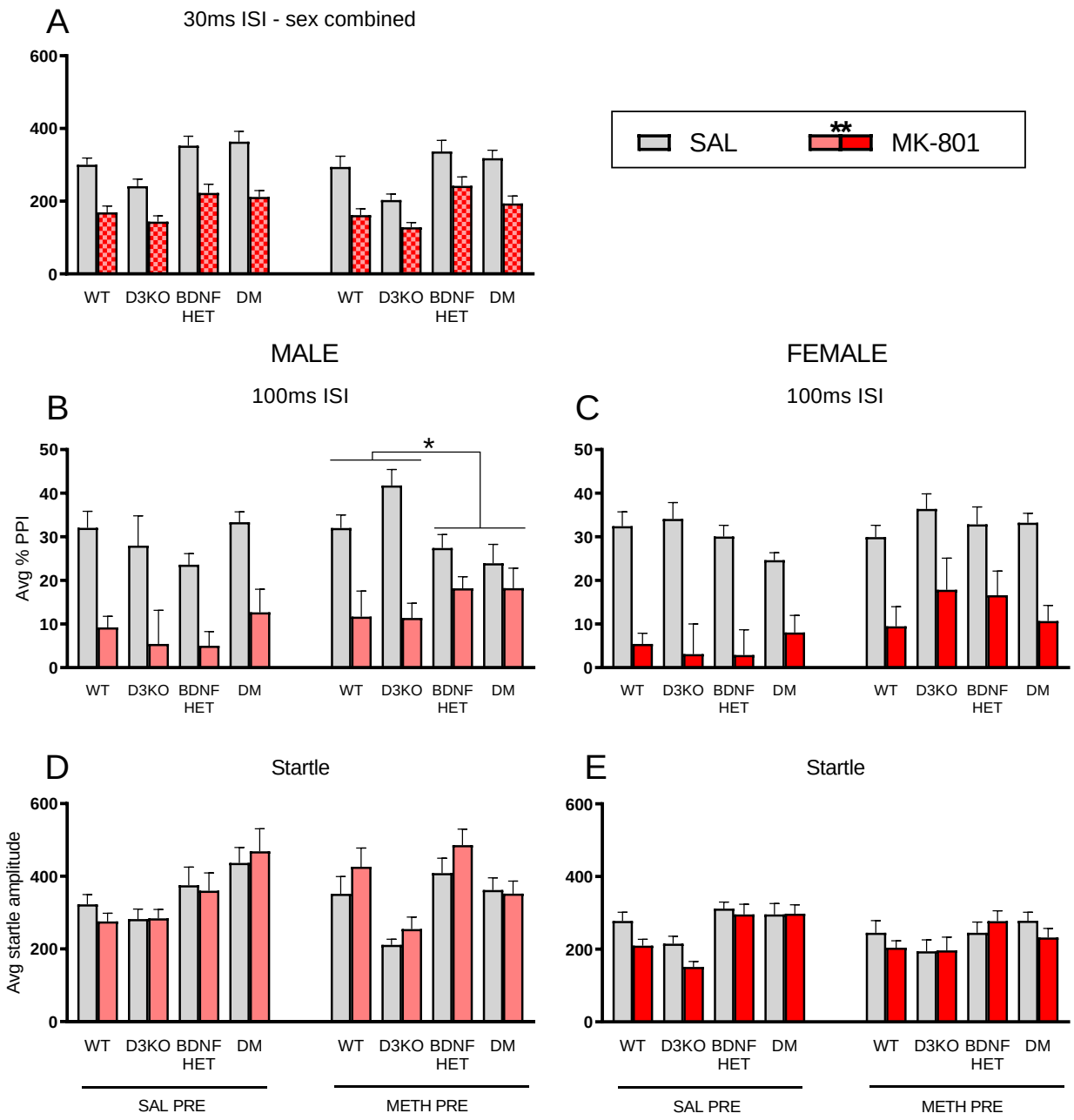

Figure 5.7 Average PPI and startle response across acute saline and MK-801 sessions. (A) *Sex-combined 30ms ISI PPI.* ***(B)*** *Female 30ms ISI PPI.* ***(C)*** *Male 100ms ISI PPI *= p< 0.05 for difference between saline and MK-801 PPI in BDNF HET vs. BDNF WT mice.* ***(D)*** *Female 100ms ISI PPI.* ***(E)*** *Male average startle amplitude.* ***(F)*** *Female average startle amplitude. **= p< 0.05 difference between saline and MK-801 PPI sessions. Data are mean ± SEM.*

5.5 Discussion

The aim of this study was to examine potential interaction between BDNF and the D3R in regulating PPI in METH-sensitized and naive mice. To examine potential differences in DAergic and NMDA receptor-mediated regulation of sensorimotor gating, PPI was recorded following administration of saline, APO and MK-801. Results from this experiment add to previous literature on the BDNF HET mouse model and provide insight into the role of D3Rs in regulating PPI.

5.5.1 Bodyweight and baseline startle

As in the low-dose experiment, BDNF HET mice gained significantly more weight over the duration of the study. This is in line with previous research demonstrating the role of BDNF in regulating eating behaviours in mice [334]. D3R loss did not affect bodyweight at any time point and saline- and METH-pretreated mice gained weight at equal rates. These data support results from the low-dose study that the escalating METH-pretreatment regimen is not having substantial or persistent neurotoxic/anorexic effects. This was not unexpected as human METH users who have substantial weight loss typically consume much higher doses (up to 32mg/kg per day) and experience great toxicity [369]; mouse models with comparable dosage have reported similar findings [369].

Baseline startle amplitudes were higher in male mice than in female mice and reduced in D3KO compared to BDNF HET and DM, but no group was significantly different from WT. Although previous PPI studies have used selective D2-like receptor antagonists and agonists, few have examined the D3KO genotype in modulation of PPI. Consequently, comparison to previous investigations is limited, however, one previous study has reported no difference in startle between D3KO and WT mice [355]. As the differences in baseline startle in our study occur irrespective of pretreatment, the reduction in D3KO mice cannot be attributed to DA system sensitization changes and must be occurring as a result of some other neurobiological mechanism.

5.5.2 Sex-specific baseline PPI deficits in METH-pretreated mice

Baseline PPI at the 30ms ISI was significantly impacted by genotype, although no group was statistically different from WT mice. Mice carrying the BDNF HET genotype had significantly lower average PPI than the D3KO group. These results suggest alternate effects of D3R loss and BDNF depletion on baseline PPI. The contribution of BDNF HET on PPI regulation appears more substantial than D3R-mediated effects, as DM mice display similar deficits as BDNF HET. These results are in line with our previous experiment in the BDNF HET low-dose study as neither report significant variance from WT PPI at the 30ms ISI.

At the 100ms ISI, PPI following METH-pretreatment was significantly different between genotypes and sexes. In males, METH-pretreated BDNF HET and DM mice had lower average PPI than D3KO mice, while METH-pretreated female and saline-pretreated animals demonstrated similar PPI. As in 30ms ISI, no group was significantly different to WT control. These data are somewhat in line with comparable baseline PPI between BDNF HET and WT mice in the low-dose study. These results are also supported by previous PPI experiments in rats demonstrating a tendency for lower PPI in BDNF HET animals that similarly did not reach significance [40], although these animals were subjected to chronic stress and not METH. These data partially support previous findings of reduced baseline PPI in BDNF HET mice [304].

5.5.3 APO-induced PPI deficits are impacted by METH pretreatment and BDNF heterozygosity

APO induced PPI deficits in all groups at the 30ms ISI. At the 100ms ISI, the majority of groups displayed similar effects of APO except in male BDNF HET and DM mice pretreated with METH, which showed no effect of the APO challenge. This result may reflect reduced baseline PPI in these mice but may also highlight potential gene x environment interactions leading to PPI dysregulation. The combination of METH-pretreatment and BDNF depletion prevented APO from effectively reducing PPI in this group, suggesting involvement of BDNF in mediating DAergic pathways governing PPI. Therefore, METH-induced sensitization of the DA system in BDNF HET mice is somehow disrupting the efficacy of D2/D1 receptor agonism in reducing PPI. Alternatively, as this

population of mice have a demonstrated reduction in baseline PPI, it may be that METH-pretreatment and BDNF heterozygosity confer substantial dysregulation of PPI networks innately, and therefore administration of APO does not produce further reduction because the system is already attenuated. These data are also in contrast to previous work in the BDNF HET model that did not identify any change in APO-induced PPI disruption [304]. However, it is important to consider these experiments were conducted in different facilities where relatively minor changes, such as housing, may impact replicability. It is also important to note that mice in the Manning et al. study were bred on the BDNF HET background and our mice had a mix of BDNF HET and D3KO genetic background, which could have contributed to the differences between study results.

5.5.4 MK-801-induced PPI deficits are attenuated following METH pretreatment in BDNF heterozygotes

Similar to APO, MK-801 significantly reduced PPI in all mice at 30ms ISI. At 100ms we observed a sex by pretreatment by genotype effect, whereby male mice carrying the BDNF HET genotype did not respond to MK-801-induced PPI disruption following METH-pretreatment. Reduced PPI 'disruptability' in these mice by MK-801 as well as APO suggests two potential hypotheses. The first is that BDNF depletion reduces MK-801-evoked PPI deficits in a sensitized DA system, but only in male mice, potentially highlighting the importance of protective sex hormone interactions in female mice. These results provide evidence for a putative role of BDNF in NMDA receptor-mediated PPI disruption in the METH-sensitized DAergic system. An alternative explanation of these results that should be recognised is that the reduction in baseline PPI in male METH-pretreated BDNF HET carriers prevented further significant reduction of PPI by MK-801. Reduced baseline PPI in BDNF HET and DM mice may reflect changes in neurocircuitry which are also involved in PPI reduction following drugs such as MK-801. This result is supported by similar findings in the APO challenge sessions.

Results obtained in these experiments differed from PPI studies in the low-dose MK-801 study. It is unclear how this occurred as mice were treated in identical testing parameters with the same apparatus and drug dosages. In order to generate mice for this study, D3KO and BDNF HET lines were interbred, therefore,

it is conceivable that the genetic makeup of these animals is not identical to the BDNF HET line used in the low-dose experiment. These findings do not fully match previous work, which found no interaction between METH pretreatment and BDNF depletion on PPI, including disruption of startle inhibition by APO and MK-801 [304].

5.5.5 D3R do not impact PPI at baseline or during drug-induced disruption

D3KO did not significantly impact APO- or MK-801-induced PPI disruption. We did not find any difference in PPI and its disruption by APO or MK-801 between D3KO and WT mice and between BDNF HET and DM animals, suggesting D3R expression does not contribute to or play a significant role in mediating DA- or NMDAR-driven PPI deficits. Results from this study are in line with previous literature, which has shown limited evidence supporting a role for D3R in PPI regulation. D3R agonists have been reported to disrupt PPI, although as discussed, these drugs are not D3-specific and may activate D2 receptors at doses used in those experiments [357]. Further support for a lack of effect of D3R receptors in modulating PPI is demonstrated by studies in knockout mice for D2, D3, and D4 receptors. Amphetamine significantly disrupted baseline PPI in D3R and D4R WT and KO genotypes. D2R WT mice were similarly attenuated, but D2KO PPI was not affected by amphetamine [355], suggesting D2R are mediating these deficits.

Interestingly, the gain of function Ser9Gly polymorphism, a common variant of the D3R gene, is associated with PPI deficits in humans. Gly-9 homozygous subjects have increased D3R DA affinity and DA-mediated cAMP response, and enhanced MAPK signalling, suggesting D3R hyperfunction [370]. In healthy male subjects, the Gly allele load has been correlated with severity of PPI. Gly9 homozygotes had the lowest PPI, followed by intermediate PPI in Ser9Gly and the highest in Ser9Ser [371]. These results highlighting reduced PPI in D3R hyperfunction propose a role for D3R in modulating sensorimotor gating in humans. Provided relatively conserved networks govern PPI across species, we could have expected D3KO to impact PPI in our 2-hit mouse model. However, as D3R were absent during development, it is possible that compensatory mechanisms develop in this model and are contributing to a lack of difference in behaviour. However, previous

observations in the D3KO model show unaltered expression of the other D1- and D2-like receptor families in the mouse spinal cord [372], suggesting no compensation by DA receptors. This is supported by observations in D3KO mouse brain tested for changes in DA receptor activity, where no difference in D1R- or D2R-binding was observed between D3KO and WT controls [373].

In combination with data presented in this chapter, these studies do not support a role for D3R in mediating PPI and suggest D2Rs as the primary DAergic mediators of METH- and amphetamine-induced PPI deficits.

5.6 Conclusion

These PPI studies have demonstrated a sex-specific role for BDNF in mediating PPI sensitivity to psychostimulant drug administration in a sensitized DA system. BDNF HET mice displayed reduced baseline PPI and diminished sensitivity to APO- and MK-801 induced PPI deficits. This provides evidence for a role of BDNF in schizophrenia aetiology, where deficits in PPI are considered an endophenotype of the disease. Importantly, PPI dysregulation was only observed in male mice, who are at higher risk of psychosis development in humans [374]. In male mice, only those who were pretreated with METH and carried the BDNF HET genotype demonstrated reduced sensitivity to PPI disruption. This supports the 2-hit hypothesis of schizophrenia development, as a gene x environment interaction was required for disruption to normal PPI regulation.

In contrast to BDNF, we conclude that these results show no appreciable role for D3R in mediating PPI at baseline or following APO or MK-801 administration. This is in line with the lack of significant difference in amphetamine-induced PPI disruption between D3KO and WT mice shown previously [355].

D3R expression is mediated by BDNF [85], thus, the lack of interaction between BDNF and D3R on PPI suggests BDNF signalling is mediating neuronal networks governing PPI via a separate pathway that is independent of D3R signalling systems. Consequently, reduced baseline PPI and loss of APO- and MK-801-induced sensorimotor gating deficits in male BDNF HET carriers cannot be attributed to reduced D3R activity and must therefore reflect a change in other neural substrates mediated by BDNF.

Chapter 6 LHA in the BDNF HET D3KO double mutant mouse

6.1 Introduction

6.1.1 METH-induced neurobiology

As discussed previously, prolonged METH use has been associated with neuroadaptive and neurotoxic changes to monoaminergic pathways within the brain. These alterations occur predominantly in dopaminergic neurons and include changes in total DA levels, VMAT and DAT function, and receptor expression and sensitivity. Combined with an increase in presynaptic DA release, sensitization to METH is thought to occur via induced neuroadaptations of the dopaminergic system. However, the aetiology is yet to be defined and the pathophysiological evidence is inconclusive. As sensitization likely underpins psychosis and addiction, a better understanding of the mechanisms involved in sensitization development will be important for elucidating the mechanisms driving drug dependence and psychosis.

6.1.2 DA system sensitisation is implicated in psychosis

Behavioural sensitization denotes the progressive and enduring enhancement of behaviour in response to a repeated stimulus. In humans, extended and repetitive use of psychostimulant drugs may lead to development of sensitized behaviours characterised by lasting hyperresponsiveness of midbrain DA neurons, auditory and visual hallucinations, paranoia, and psychomotor output [121, 375]. Sensitization of the same DAergic neural circuitry is also hypothesized to play a role in schizophrenia pathophysiology and positive symptom progression [122, 376]. A large body of evidence supports greater mesolimbic DA system reactivity in patients with schizophrenia and enhanced DA synthesis compared to unaffected controls [377]. In combination with increased amphetamine-induced DA release and greater behavioural response to psychostimulant drugs, these neuroadaptations form a state of endogenous sensitization that is hypothesized to develop in schizophrenia patients [122, 377].

While behavioural sensitization has been recognised as a central component of psychosis aetiology, the molecular underpinnings of DAergic system sensitization are only partially understood. The working theory involves two stages of development. Initially, it is hypothesized that DA receptor density in the VTA is

altered via changes in neuronal impulse activity [378]. Neuroadaptations in DAergic cells impact DA release from mesolimbic projections, notably in the ventral striatum, comprised of the NAc shell and core, and OT [378]. In response to heightened activity, e.g. following drug effects, autoreceptor subsensitivity can occur transiently on VTA neurons as well as DA postsynaptic receptor. Reduced autoreceptor signalling results in reduced negative feedback and enhanced DA synthesis and release [379]. Despite these discoveries, studies are yet to fully identify the neural substrates involved in DA system sensitization following psychostimulant use and in schizophrenia aetiology.

As BDNF is required for normal D3R expression [85], a reduction in BDNF signalling could result in attenuated D3R binding availability, promoting an enhanced rate of DA sensitization. BDNF heterozygosity may therefore promote a state of endogenous sensitization to DA-releasing agents that we have previously observed in LHA experiments [31]. This theory is supported by studies in BDNF HET rodents demonstrating D3R down-regulation [49, 69, 85], which could mediate enhanced DAergic sensitization and subsequent psychosis-like behaviours such as LHA.

6.1.3 Aims

The present study aims to investigate the interaction between D3R expression and BDNF in behavioural sensitization following METH administration, as measured by enhanced LHA to an acute dose of the drug [287]. We propose that changes in D3R signalling in BDNF HET mice cause the endogenous sensitization and altered effects of an acute drug challenge in this model [31]. To test the relationship between BDNF and D3R in DA sensitization, in this study WT, BDNF HET, D3KO, and double mutant mice carrying both genetic alterations, were administered METH in an escalating regimen during adolescence. The animals were subsequently tested for differences in LHA to low and high acute challenge doses of METH.

6.2 Methods

6.2.1 Mice and statistical analysis

Mice used in this study were the same as used for PPI studies (see Chapter 5). Approximately 1 week after completion of the PPI experiment, the mice were tested in Med Associates locomotor photocells over 3x 180-minute sessions, as outlined in the General Methods (Chapter 2.3.3; Figure 6.1). Mice received:

1. Saline vehicle in the first session.

2. 1mg/kg meth in the second session.

3. 3mg/kg meth in the final session.

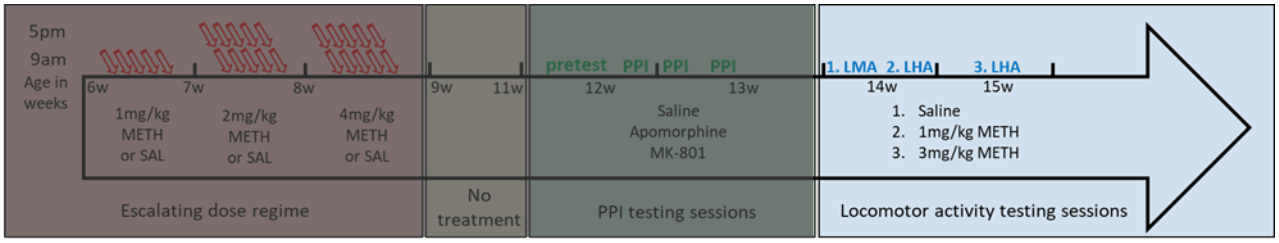

Figure 6.1 Timeline of METH experiments described in this chapter*. Baseline locomotor activity and hyperactivity were tested in weeks 14 and 15. Three separate locomotor trials were conducted starting with a baseline saline session. Three to five days later hyperactivity was measured following an acute 1mg/kg METH challenge dose, and finally after a similar wash-out period, a third trial was conducted with an acute 3mg/kg METH injection in week 15. Approximately 1-week later mice were humanely killed, and brains were dissected and fresh-frozen or perfuse fixed for future analysis.*

A total of 192 mice were used in experiments described in this chapter. Statistical analyses were conducted on 16 experimental groups, separated by sex, genotype, and drug pretreatment. Distance moved over the 120 minutes was analysed in 5-minute bins or as the total following acute injection of vehicle or METH (Table 1.1).

Table 6.1 Number of mice per experimental group tested in low-dose BDNF HET locomotor study

	Saline-pretreated				METH-pretreated			
	WT	**BDNF HET**	**D3KO**	**DM**	**WT**	**BDNF HET**	**D3KO**	**D3KO**
Male	12	13	10	13	11	14	10	12
Female	12	14	11	14	13	11	9	13

Total distance was used to determine outliers across saline, 1mg-, and 3mg METH injection data. As a result, 6 mice were excluded from further analysis based on having values outside 3 standard deviations of the mean (METH pretreated: 1 male D3KO, 2 male BDNF HET, 1 male DM, 1 female BDNF HET; saline pretreated: 1 female DM), leaving n = 186 for locomotor investigation. A repeated measures ANOVA was used to compare vehicle vs. METH-induced hyperlocomotion between genotype, sex, and pretreatment (Table 6.1).

6.3 Results

6.3.1 Bodyweight

Male mice were heavier than females and BDNF HET mice gained more weight over the duration of the experiment. METH-pretreatment did not affect mouse bodyweight. Detailed analyses of bodyweight between genotypes is described in BDNF HET/ D3KO PPI Chapter 5.

6.3.2 Baseline Locomotor

Baseline locomotor activity was measured following acute saline IP injection. Univariate analysis of the total distance moved revealed a small but significant reduction in BDNF HET mice compared to BDNF WT (combined group of WT and D3KO; main effect of BDNF genotype, $F(1,170)= 3.94$, $p= .049$). No significant

main effects of pretreatment, D3 genotype, or sex were observed, however, a significant 4-way interaction was detected (D3 x BDNF x pretreatment x sex, $F(1,170)= 6.59$, $p= 0.011$). Given this result and the trend for sex differences previously observed in Chapter 4, data were split into male and female groups, where post-hoc analyses confirmed a small but significant increase in baseline locomotor activity in male mice following METH-pretreatment (Males, pretreatment main effect, $F(3,89)= 3.97$, $p= 0.05$; Figure 6.2). Furthermore, in male METH-pretreated mice there was a D3 x BDNF interaction ($F(1,38)= 5.19$, $p= 0.028$), and inspection of the data (Figure 6.2) suggested that this interaction reflected higher activity in BDNF HET and D3KO mice compared to DM. However, post-hoc pairwise comparison showed no significant differences in LMA. No significant differences were observed in female mice' data.

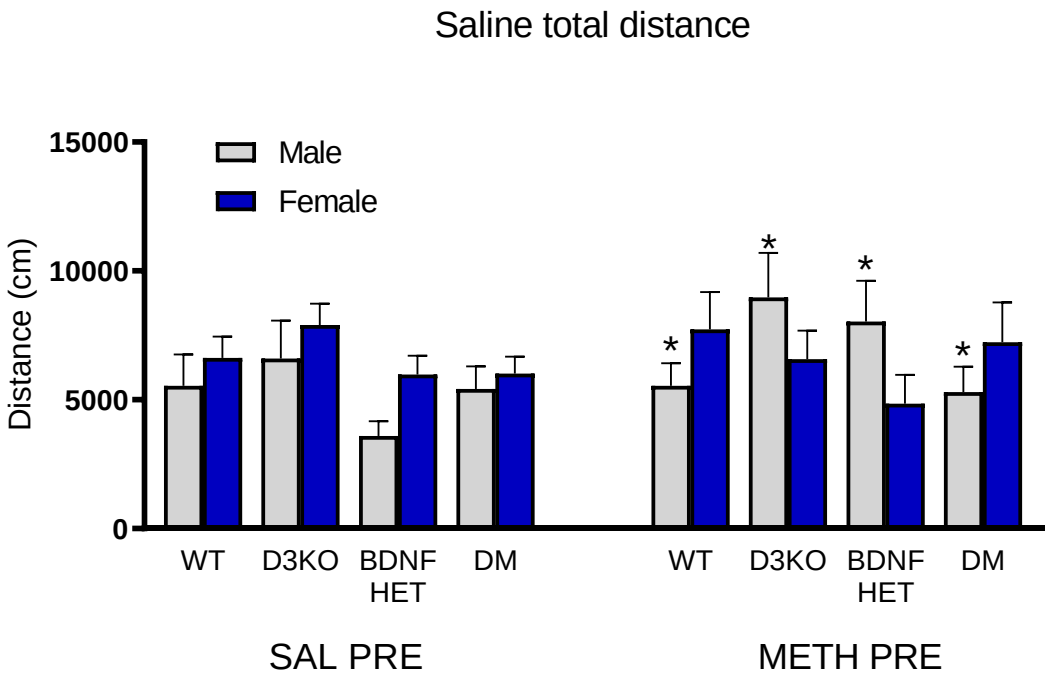

Figure 6.2 Total baseline locomotor activity post-saline injection. *Male and female BDNF HET, D3KO, DM, and WT mice injected with saline were analysed for baseline locomotor activity following METH or saline pretreatment. The total distance moved over 2-hours post injection did not differ significantly between genotype groups. Post hoc analysis revealed a significant increase in baseline locomotor activity in male METH-pretreated mice compared to saline-pretreated (*P< 0.05). In all cases data are mean ± SEM.*

6.3.3 LHA to 1mg/kg METH challenge

Compared to baseline activity, acute 1mg/kg METH produced LHA (main effect of METH, $F(1,170)= 100.26$, $p< 0.001$; Figure 6.3) and this was enhanced in mice who received METH pretreatment, suggesting sensitization to the drug (interaction of METH x pretreatment, $F(1,170)= 27.95$, $p< 0.001$; Figure 6.4). No significant sex differences were observed, nor genotype differences, on total 1mg/kg METH-induced LHA. As in Chapter 5, one-hour post-injection data were analysed separately, but no additional differences were observed (data not shown).

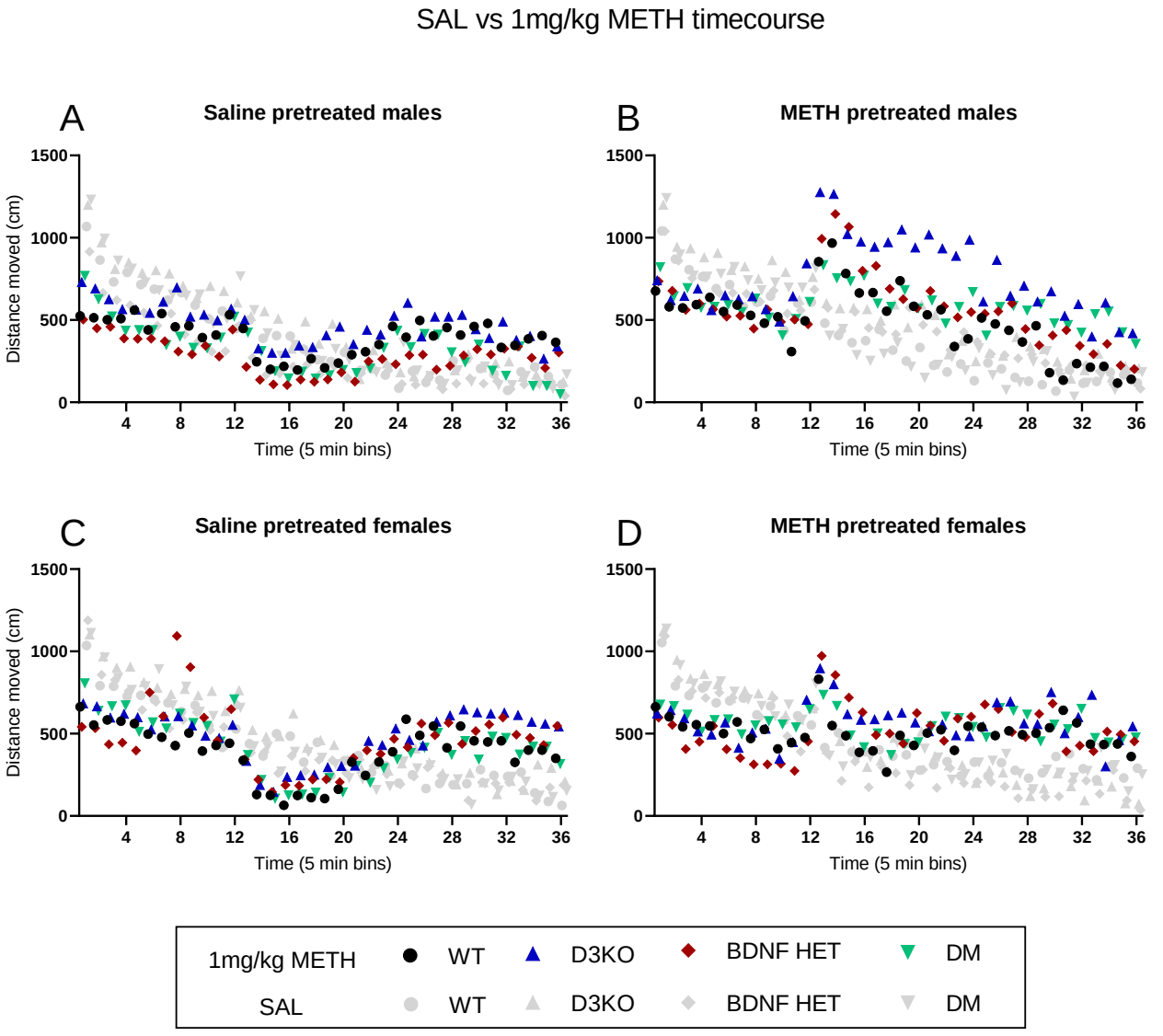

Figure 6.3 Saline and 1mg/kg METH timecourse. *Distance moved over the 2-hour timecourse was compared between acute saline (grey dots) and 1mg/kg METH (coloured dots) 2 hours post-injection. Graphs are split by sex and pretreatment groups (A-D). Colours represent different genotypes. Saline or METH was injected at time bin 12 following 1 hour of habituation to the photocell cage. In all cases data are represented as the mean.*

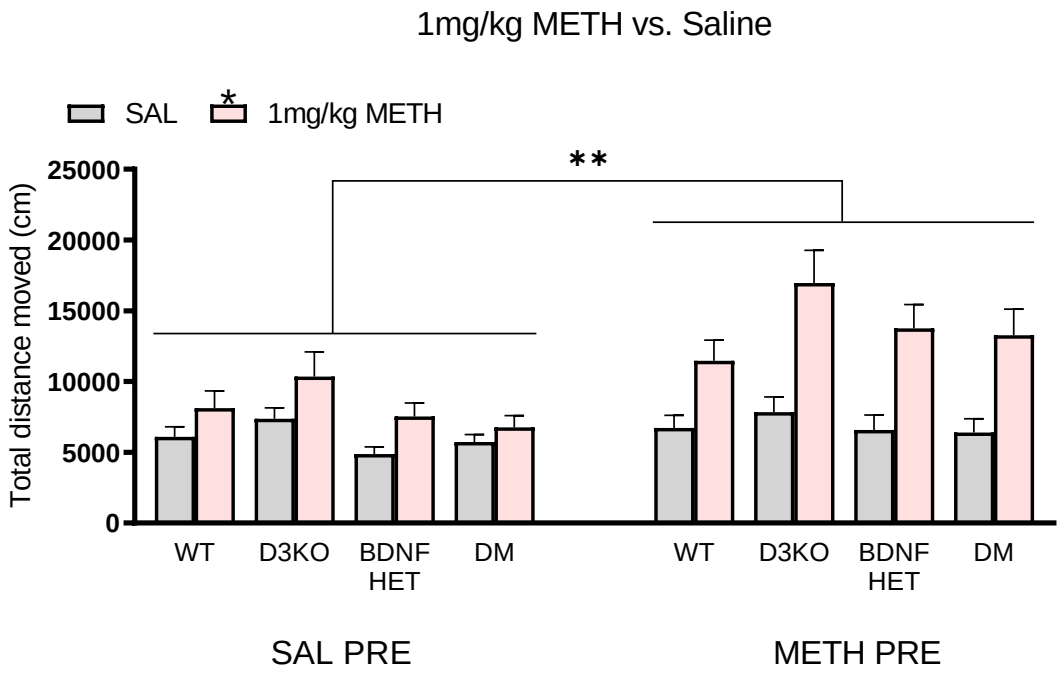

Figure 6.4 Total distance baseline vs 1mg/kg METH (sexes combined). *Hyperactivity to 1mg/kg METH was compared to LMA obtained over 2 hours post-injection of saline. The total distance moved increased significantly following acute METH (*P<0.001). There was no main effect of sex or genotype on hyperactivity, but significant sensitization was observed in METH-pretreated mice compared to saline-pretreated controls. In all cases data are mean ± SEM.*

6.3.4 LHA to 3mg/kg METH challenge

LMA to acute saline (baseline) was compared to hyperactivity following 3mg/kg METH challenge (Figure 6.5). Acute 3mg/kg METH caused significant LHA (main effect of METH, $F(1,170)= 1136.71$, $p< 0.001$; Figure 6.6), and this hyperactivity was enhanced further by METH-pretreatment (interaction of METH x pretreatment, $F(1,170)= 35.52$, $p< 0.001$; Figure 6.6). A significant 3-way interaction (METH x sex x genotype, $F(3,170)= 3.43$, $p= 0.018$) led to sex-specific analysis, which showed no significant genotype interactions in male mice. In female mice, LHA differed between genotypes (females, interaction of METH x genotype, $F(3,89)= 4.44$, $p= 0.006$), prompting further analysis according to the presence or absence of D3R and BDNF heterozygosity. Analysis of the D3KO genotype found no differences between mice with functional D3Rs (WT and BDNF HET) and D3KO (D3KO and DM) mice. Conversely, analysis of the BDNF genotype found BDNF

haploinsufficiency (BDNF HET and DM) significantly enhanced locomotor hyperactivity compared to BDNF WT (WT and D3KO; interaction of METH x BDNF, $F(1,89)= 10.46$, $p= 0.002$; Figure 6.6), and this occurred irrespective of METH-pretreatment, suggesting female BDNF HET mice were endogenously sensitized to the locomotor-stimulating effects of METH. Like analyses in Chapter 5, one-hour post-injection data were analysed separately, however, no additional differences were observed over the one-hour post-injection (data not shown).

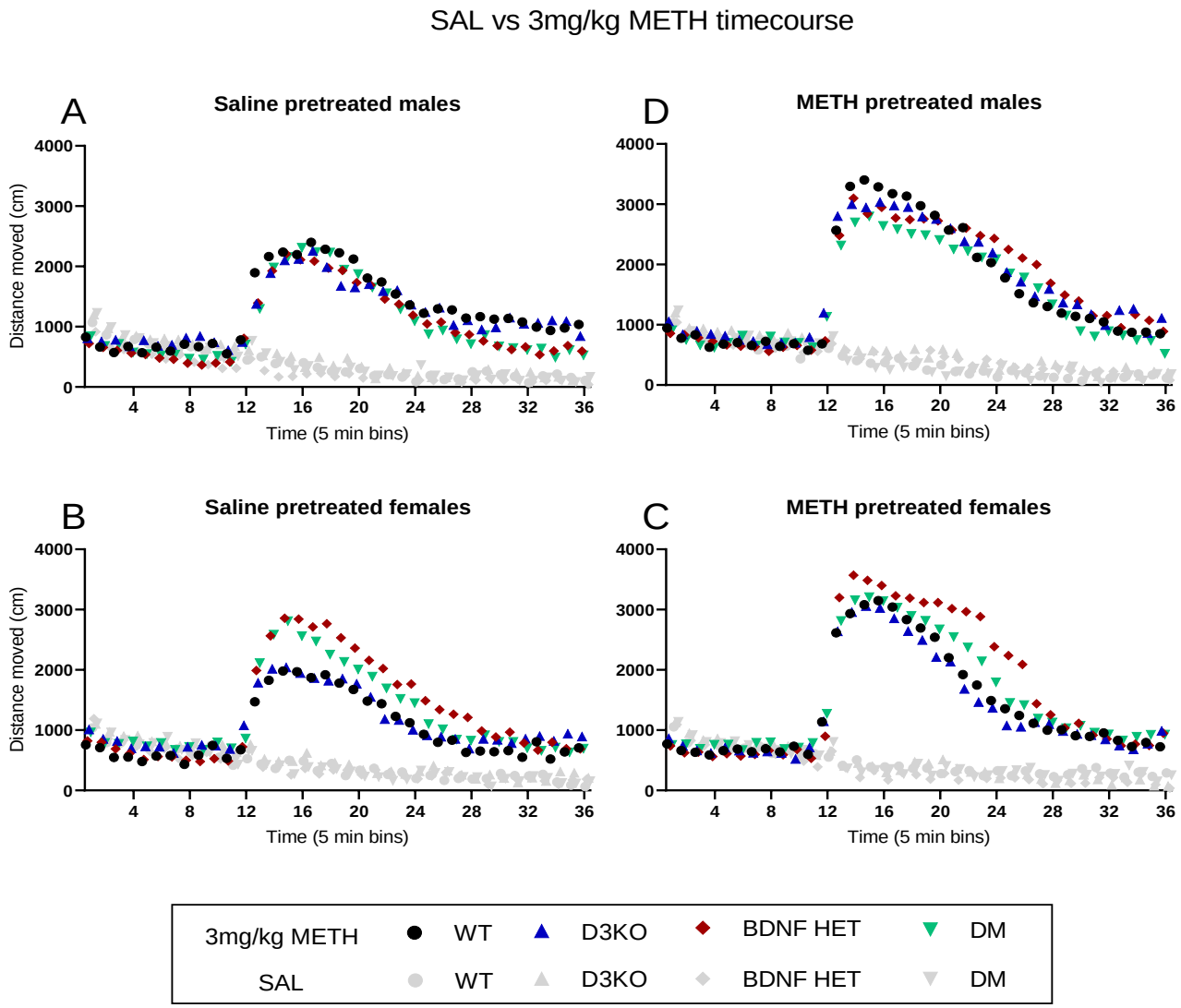

Figure 6.5 Saline and 3mg/kg METH timecourse. *Distance moved over the experiment timecourse was compared between acute saline (grey dots) and 3mg/kg METH (coloured dots) 2 hours post-injection. Graphs are split by sex and pretreatment groups. Colours represent different genotypes. Saline or METH was injected at time bin 12 following 1 hour of habituation to the photocell cage. In all cases data are represented as the mean.*

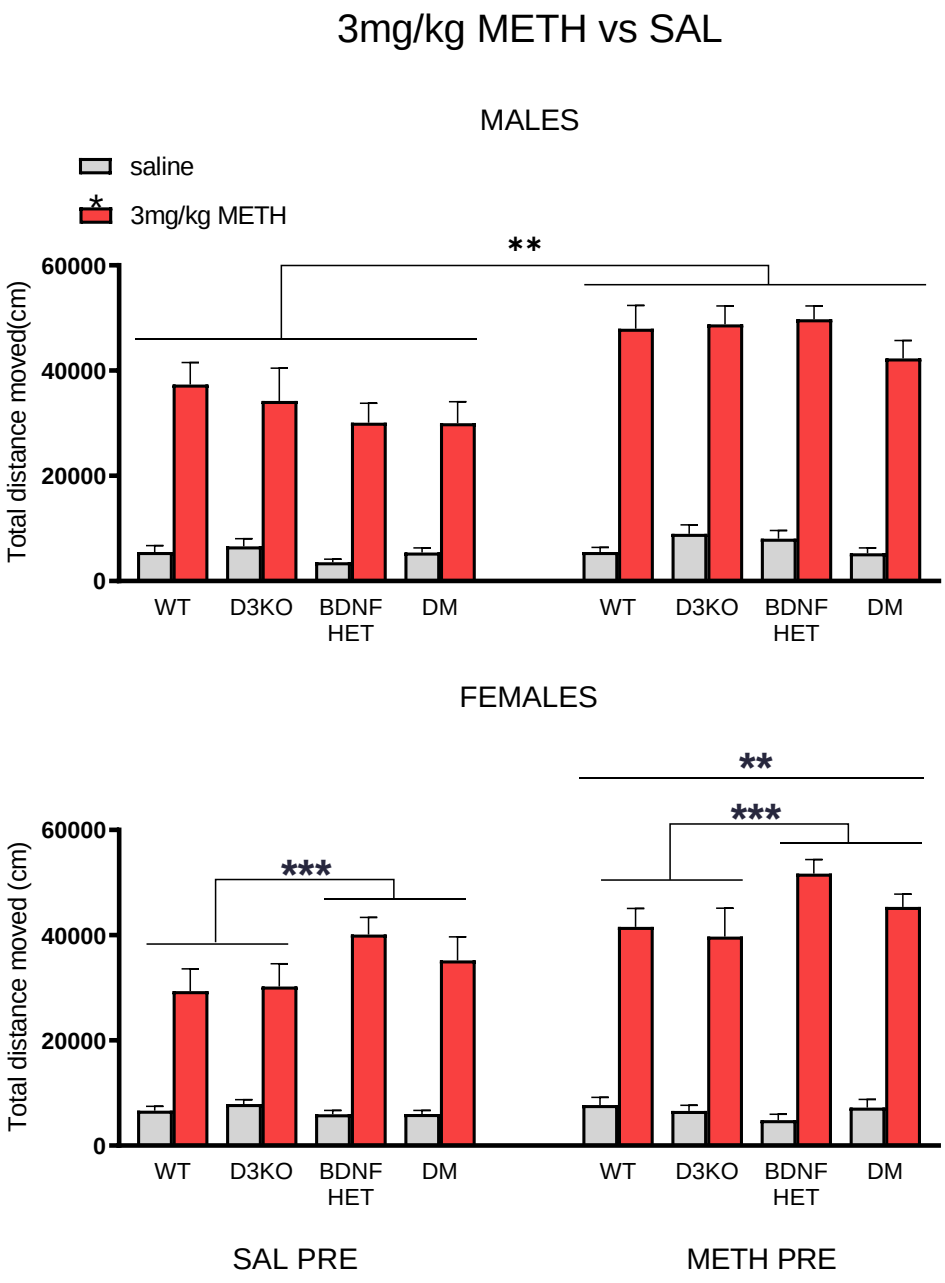

Figure 6.6 Total distance baseline vs. 3mg/kg METH. *Hyperactivity to 3mg/kg METH was compared to LMA obtained 2-hours post-injection with saline. The total distance moved increased significantly following acute METH (*P<0.001). METH-pretreated mice had significantly higher LHA than saline-pretreated mice (**P<0.05). There was no main effect of genotype in male mice, but female BDNF HET and DM mice had significantly enhanced hyperactivity compared to WT and D3KOs. In all cases data are mean ± SEM.*

6.4 Discussion

6.4.1 Baseline LMA

Average baseline locomotor activity was significantly lower in BDNF HET mice, although this was a small difference and significance was lost when data were split into male and female groups. METH pretreatment had no effect on female mice LMA. In contrast, male METH-pretreated mice had higher locomotor activity than saline-pretreated mice and displayed a D3 x BDNF interaction, although post-hoc testing revealed no significant difference in LMA between genotypes. The lack of BDNF genotype differences described in this study are supported by the experiments described in Chapter 4 and a previous LMA study [31], where similar LMA was reported between WT and BDNF HET mice. No further D3R-mediated effects were observed on baseline LMA. This is in line with a study in mice reporting no difference between WT and D3KO during habituation to a novel environment, other than a significant increase in exploratory behaviour during the first 10 minutes [380]. A later study examined D3R HET and wildtype mice baseline LMA during light/dark cycle. Similar to this experiment, there was no effect of genotype during the light phase, although D3KO and D3 HET mice were significantly more active at baseline during the dark phase of the circadian cycle [381]. The current studies were all done during the light phase of the circadian cycle. In other studies, LMA after saline challenge, defined as number of crossovers into active zones of the chamber, was similarly unaffected by D3R loss in mice [382] and D3R overexpression in the striatum had no effect on baseline LMA [383]. Lastly a study examining DA receptors and MDMA-induced LMA concluded there were no significant differences in baseline activity between WT and D3KO mice [384]. Combined, these studies provide compelling evidence that D3Rs do not play a major role in baseline LMA.

6.4.2 LHA to 1mg/kg METH

Compared to baseline LMA, a 1mg/kg METH challenge produced LHA in mice, which was further increased by METH-pretreatment. There were no sex or genotype effects on LHA. These results are similar to the experiments described in chapter 4 where a trend was detected for higher LHA in METH-pretreated mice,

but no significant sex or genotype effects. Manning et al. used the same escalating METH regimen in BDNF HET and WT mice and reported LHA in response to a 1mg/kg D-amphetamine challenge, although saline-pretreated mice did not become hyperactive in this experiment [31]. Here, we do report LHA in saline-pretreated mice that travelled approximately 36% further during the acute 1mg/kg METH challenge session compared to the saline session. Differences between these results could be explained by pharmacological differences in METH and D-amphetamine discussed in Chapter 4, or differences in mice' genetic background, testing environment and housing conditions, which may have contributed to varied locomotor response between facilities in the Manning et al. study [31].

The present study did not reveal any significant differences in LHA between BDNF HET and WT mice to low METH challenge. Similar lack of difference between BDNF HET and WT mice was reported in a prior investigation using 1mg/kg D-amphetamine [31]. Nevertheless, it had been shown previously in DA dialysis data that BDNF HET mice react to a single injection of 1mg/kg METH with significantly higher DA release than BDNF WT mice between 80-120 minutes post-injection. Interestingly, this does not correspond with the peak LHA in our experiment which occurred in the first 60 minutes post-injection of METH, suggesting extracellular DA concentration does not directly correlate with LMA. As in Chapter 4, LHA over the first hour was analysed separately, but no differences in group effects were observed between one- and two-hour total distance travelled.

Similar to findings in BDNF-deficient mice, we did not observe D3R-mediated effects on 1mg/kg METH-evoked LHA. Few studies have examined the effect of amphetamine derivatives on LHA in this model. However, other psychostimulants have been examined. In a LMA investigation, WT and D3KO mice were administered either D1-like or D2-like receptor agonists, but no difference in hyperactivity was reported between genotypes [380]. Yet, when both receptor agonists were given together, D3KO mice were significantly more active, suggesting coactivation of D1-like receptors and D2-like receptors triggers D3R-mediated suppression of LHA in WT but not D3KO mice. As METH is an indirect DA agonist, administration should coactivate D1- and D2-like receptors in the mouse brain and promote hyperactivity in D3KO mice. It is possible that a 1mg/kg METH dose did not cause substantial D3R activation in the current study. This is

supported by findings in another type of LHA, defined as crossovers into active zones, in D3KO mice following varied doses of amphetamine [382]. Results support our lack of D3R genotype effect, as investigators reported no difference in LHA between genotypes at low-dose (0.2mg/kg) or high-dose (5mg-10mg/kg) amphetamine. Interestingly, the only dose that differentiated D3KO and WT mice LHA was 2.5mg/kg, with D3KO demonstrating significant increases in hyperactivity compared to WT. These findings suggest D3R-mediated LHA is dose-dependent and may only occur in response to a certain level of DA release. As D3R act to inhibit DA release, lower concentrations of amphetamine may not trigger negative feedback mechanisms and therefore loss of D3R signalling would have minimal impact on LHA.

D3R signalling has also been investigated in behavioural sensitization to cocaine, a drug that produces dose-dependent increase in extracellular DA within the NAc and caudate. LHA following cocaine administration was comparable between D3R HET, D3KO and WT mice in one study [381], although in another investigation, suppression of D3R expression in the NAc increased locomotor stimulant effects and over-expression reduced behavioural sensitivity to cocaine [385]. Taken together, these results may indicate the need for targeted DA system manipulation that is specific to regions associated with motor function and reward. Combined, these studies do not support an appreciable role of D3R in mediating psychomotor response to lower doses of psychostimulant drugs.

6.4.3 LHA to 3mg/kg METH

As expected, METH pretreatment produced behavioural sensitization to LHA induced by high-METH challenge, resulting in locomotor hypersensitivity compared to saline-pretreated mice. High-METH challenge also uncovered sex-specific genotype effects in female BDNF HET mice and DM who were more hyperactive than WT or D3KO genotypes. Findings presented in Chapter 4 somewhat align with these results. In studies outlined in Chapter 4, we did not observe a pretreatment effect in male mice, but BDNF HET were significantly more sensitive to METH. In contrast to the present data, in female mice, genotype had no effect on LHA. Surprisingly, BDNF genotype differences appear to have opposite sex dependency across studies. These results are also partially supported by previous

studies in the escalating METH sensitization model examining amphetamine-induced LHA in BDNF HET and WT mice. Manning et al. described similar LHA to a 3mg/kg D-amphetamine challenge, where BDNF HET mice displayed increased hyperactivity to the challenge, even in the absence of METH pretreatment, suggesting development of endogenous sensitization in BDNF depleted systems. This endogenous sensitization was also identified in female saline- pretreated BDNF HET and DM mice in the present study. However, unlike Manning et al., we also report hypersensitivity to METH in METH-pretreated female BDNF HET mice. Although the reason for hyperactivity differences between our results is unclear, a lack of hypersensitivity in METH-pretreated BDNF HETs in Manning et al.'s study could reflect some form of LHA ceiling effect to D-amphetamine. This is evidenced in that study by the trend for a BDNF x METH x D-amphetamine interaction which may have reached significance at a lower challenge dose. Hypersensitivity to psychostimulant administration in our study suggests BDNF may be implicated in mediating neuronal networks governing sensitization development and psychosis and provides evidence for neurotrophic modulation of psychomotor behaviours.

Although a trend was detected for an effect of loss of D3R on LHA ($p= 0.076$), no significant differences were observed between D3KO (D3KO and DM) and D3 WT (BDNF HET and WT) mice. As mentioned in section 6.4.1, a previous LMA crossover study described hyperactivity in D3KO mice to amphetamine at a similar dose. However, it is possible these studies are not comparable based on differences between METH and D-amphetamine neurochemical effects, such as DA efflux and DAT modulation. METH is a more potent stimulant than amphetamine. Therefore, 3mg/kg METH may induce similar DAergic effects as higher amphetamine doses that were not impacted by loss of D3R in that study [351]. D3R effects on cocaine-induced LHA appear to be similarly dose-dependent in mice. A study examining LHA in response to acute cocaine treatment reported loss of significant differences between WT and D3KO mice at higher cocaine doses [380]. LMA to D3R over-expression has also been examined. Transgenic mice with elevated D3R in the striatum showed reduced LHA in response to treatment with a D1R agonist, SKF 81297 (5mg/kg) [383], although it is unclear how this dose compares to DAergic signalling induced by 3mg/kg METH challenge. In summary, any potential D3R effects on LHA appear dose-dependent. This could suggest that

the dampening effects of D3R activation on motor behaviours only occur at lower DA stimulation and are bypassed with significant stimulation. Therefore, increased extracellular DA in D3KO mice may impact LHA more significantly at lower doses of DA-enhancing drug, where small changes in extracellular DA levels could prompt greater activity in D3R depleted systems. This effect would be masked at higher psychostimulant drug doses where D3R activation is unable to dampen DAergic signalling. Nevertheless, our data did not support any significant impact of D3R loss on LHA to either low or high METH doses.

The studies described in this chapter do not support a role for D3R in BDNF-mediated changes in sensitization to METH. LHA in male mice was not impacted by genotype, but female mice displayed BDNF genotype-dependent LHA changes, demonstrating a potential role for sex hormones in mediating BDNF signalling pathways. Female BDNF HET and DM mice were hypersensitive to METH compared to D3KO and WT female mice who had similar LHA. This suggests that D3R loss did not impact LHA in BDNF WT or BDNF HET mice. If BDNF-mediated changes in LHA were attributed to downregulation of D3R, we could expect comparable hypersensitivity responses between D3KO and DM genotypes (assuming no compensation of D3R). In this interaction model, LHA in BDNF HET mice would presumably be higher than WT, but lower than D3KO genotypes (D3KO and DM), as some D3R activity may still reduce DA signalling in BDNF HET mice. As we did not observe these differences, the hypothesis that BDNF deficiency mediates sensitization through altered D3R expression is not supported. Although lower D3R expression levels have previously been implicated in suppressed negative feedback and subsequent increases in DA signalling [87, 88], here we do not report any D3KO-mediated changes in LHA. This goes against previous hypotheses that reduction in D3R expression contributes to behavioural sensitization [290] and drives endogenous sensitization to psychomotor behaviours in BDNF HET mice [31].

6.5 Conclusion

Hypersensitivity to METH in our female BDNF HET mice suggests that maladaptations in BDNF signalling may lead to sensitization changes following METH use, which could contribute to the development of METH-induced psychosis

and psychosis-like behaviours. Although the neural substrates involved in hypersensitivity to METH remain unclear, we have demonstrated a role for BDNF in mediating DA-driven hyperactivity that is independent of D3R expression and activity.

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Chapter 7 Brain-derived neurotrophic factor (BDNF) determines a sex difference in cue-conditioned alcohol seeking in rats

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Abbreviations

BDNF: brain-derived neurotrophic factor

BEC: blood alcohol concentration

FR3: fixed ratio 3 requirement

HET: BDNF heterozygous (HET) rat

IVC: individually-ventilated cages

mPFC: medial prefrontal cortex

NAc: nucleus accumbens

PR: progressive ratio

SEM: standard error of the mean

TrkB: tropomyosin receptor kinase B

WT: wildtype rat

7.1 Abstract

Alcohol use disorder is a detrimental addictive disease that develops through prolonged ethanol exposure and regular intoxication. However, the changes in the underlying neurobiology leading to alcohol addiction remain unclear. Brain-Derived Neurotrophic Factor (BDNF) is implicated in substance abuse disorders including alcoholism. As the vast majority of previous animal model studies have concentrated on males only, the aim of this study was to determine whether endogenous BDNF mediates alcohol seeking in a sex-specific manner.

We used an operant self-administration paradigm where the animals were trained in operant chambers to self-administer a 10% ethanol solution and compared male and female BDNF heterozygous (HET) and wildtype (WT) rats. Over several weeks, the animals progressed through acquisition, progressive ratio, extinction, and reinstatement phases.

There were no significant sex or genotype differences in the number of alcohol-paired lever presses during acquisition, progressive ratio, and extinction. However, a significant difference between male and female WT rats following alcohol-primed reinstatement was completely absent in BDNF HET rats suggesting a role of BDNF in sex differences in alcohol seeking after abstinence. Female BDNF HET rats showed significantly higher number of alcohol-paired lever presses during reinstatement than female WT controls.

These findings suggest that BDNF regulatory pathways are involved in sex differences in reinstatement of alcohol intake and emphasize the need to include both male and female animals to explore sex-specific interactions in addiction neurocircuitry.

Keywords: brain-derived neurotrophic factor; alcohol seeking; sex differences; reinstatement; extinction

7.2 Introduction

Alcoholism is an addictive substance use disorder that often precedes long-term chronic disease. Excessive alcohol consumption causes a variety of primary injuries through liver impairment and neurodegeneration, frequently resulting in public and domestic violence and a broad spectrum of social dysfunction disorders [47]. While alcohol intake in the short term can lead to behaviour-related injury, long-lasting dependence can alter blood pressure, cardiovascular health, mental stability, and is increasingly correlated with liver cancer progression [244]. The prevalence of alcohol abuse is high, with 5.9% of all deaths and 5.1% of the global burden of disease and injury attributed to alcohol in 2012 [386]. Understanding the neurochemical adaptations that lead to alcoholism is of great importance and may lead to amelioration of alcohol addiction though enhanced treatment and diagnosis.

Adaptations to the addiction circuitry of the brain are partially regulated through growth factors. In particular, neurotrophins, growth factors involved in neurodevelopment and neuronal plasticity, have been shown to regulate responses to drug abuse, such as alcoholism [27]. Brain-Derived Neurotrophic Factor (BDNF) is one such neurotrophin, produced in the endoplasmic reticulum and expressed within the central and peripheral nervous systems [387]. The precursor, proBDNF, is proteolytically cleaved to form the mature construct that interacts with the tropomyosin receptor kinase B (TrkB) to initiate downstream signalling on several phosphorylation pathways [47]. These pathways promote neuronal cell growth, maturation, differentiation, synaptogenesis and synapse stability, as well as playing a role in learning and long-term memory consolidation [388]. Alcohol intake has been shown to reduce BDNF expression over intermittent, repeated, moderate, and chronic exposure in rodents [261, 262]. Neuronal networks adapt to recurrent ethanol presence, altering a homeostatic balance that leads to craving in ethanol absence [263] and reducing BDNF expression.

BDNF can also regulate drug sensitization and self-administration, although this regulation appears to be regionally-specific, and differs between addictive drugs. For example, cocaine craving in rats is increased following infusion of BDNF into the nucleus accumbens (NAc) [46], while a reduction of BDNF in the same region is correlated with heightened alcohol preference [264]. BDNF in the medial

prefrontal cortex (mPFC) has a similar role with elevated endogenous activity within the mPFC reducing cocaine self-administration [265], while a reduction of levels of the neurotrophin escalates alcohol drinking [266-268]. BDNF in the dorsal striatum may act as a negative regulator for alcohol intake [27] and amygdaloidal BDNF represses anxiety-like behaviour and ethanol intake through increased dendritic spine density [269]. These similarities and variances between brain regions and between addictive substances, infer local specificity of the role of BDNF in reward pathways.

Heterozygous (HET) mutant rodents with a deletion of one BDNF gene allele exhibit a 50% reduction in endogenous protein production [35, 36]. These models have been used primarily in the two-bottle choice paradigm where BDNF heterozygous mice develop an increased sensitivity to ethanol exposure [270, 271] and a preference for alcohol consumption [270]. Rat models are favourable due to their enhanced learning capabilities and comparable neurocircuitry to humans [272]. More specifically, similar to humans, rats show a strong alcohol deprivation effect which is not as pronounced in mouse models [273]. The role of BDNF within the dorsal striatum has been studied in rats using operant, restricted, and ad libitum ethanol access [274, 275]. However, no analyses have been done in BDNF haploinsufficient rats. Furthermore, studies on the role of BDNF in alcoholism have yet to compare and acknowledge potential sex-specific interactions, with previous studies primarily testing male and rarely female animals, but never in parallel. This is despite the suggestion that female rodents have increased sensitivity to BDNF attenuation [270] and accelerated alcohol dependence [276], reflecting potential sex-specific mechanisms in alcohol addiction.

The aim of this study was to compare male and female BDNF HET rats with their WT controls to test the hypothesis that endogenous BDNF negatively regulates alcohol seeking in an operant paradigm in a sex-specific manner.

7.3 Materials and methods

7.3.1 Animals

We used BDNF HET rats and WT Sprague-Dawley control littermates obtained from a breeding colony at the La Trobe Animal and Research Teaching Facility, La Trobe University, Melbourne. All rats were genotyped by sending genetic samples to Transnetyx (Cordova, TN, USA). Rats were aged 6-8 weeks at the commencement of behavioural experiments and housed in reverse-light cycle conditions (on 8pm, off 8am) in groups of 2-4 in standard individually-ventilated cages (IVC, Tecniplast, Bugaggiate, Italy). All rats had *ad libitum* access to standard rat chow and water for the duration of the study.

7.3.2 Ethanol Preference - Operant Self-Administration

7.3.2.1 Overnight Training

Initial overnight training and subsequent experiments were conducted in operant chambers (Med Associates, St Albans City, VT, USA) as previously described [389]. A timeline of the experimental paradigm can be found in Figure 7.1. Briefly, each chamber was housed within a sound-proof cubicle with a fan to provide airflow and mask external noise. Two retractable levers (visible during operant sessions) were placed below a stimulus light and adjacent to a fluid receptacle. Levers were positioned at opposite corners of the chamber. A single drop of vanilla essence was placed onto a plastic dish under the active lever to act as an olfactory cue and the stimulus light was set to illuminate upon completion of the required number of presses of the active lever only. A controlled liquid dispenser fed each receptacle. Initially, each animal was introduced to the operant chamber for an overnight training session which ran for approximately 16 hours. During this session, rats could explore the chamber and learn to lever press for rewards of a solution containing 5% v/v ethanol and 5% w/v sucrose. Pressing the opposite lever resulted in water being dispensed. The levers were set to dispense fluid on a fixed ratio of FR2; 2 lever presses = 100 microliter reward. Rats were provided with food pellets within the chamber to ensure they had adequate access to food overnight.

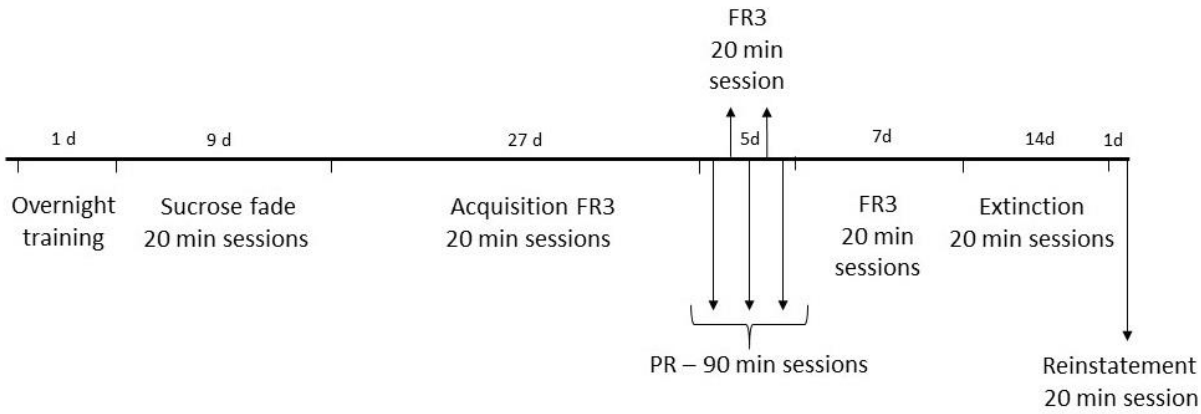

Figure 7.1 Experimental timeline depicting different stages of the operant responding paradigm. *Rats were monitored for lever presses during acquisition (including sucrose fade), extinction and reinstatement. FR, fixed ratio; PR, progressive ratio.*

7.3.2.2 Sucrose Fade

Following overnight training, rats were placed into operant chambers for daily 20-minute sessions, five times a week (Monday - Friday). Ethanol and water response levers were alternated between sessions to avoid place-preference, with a small drop of solution (either water or the ethanol-containing solution) left in each receptacle to indicate the current orientation. Similar to the overnight training, both vanilla essence and a stimulus light acted as cues to signal reward availability. A standard 'sucrose fading' protocol was administered (5% sucrose, 5% ethanol 1-5 days, 2.5% sucrose, 10% ethanol from 6-8 days). This allowed the aversive taste of ethanol to be overcome through gradual reduction of the concentration of sucrose over a 9-day period. Once sucrose was withdrawn, a 10% ethanol solution was applied to all future sessions.

7.3.2.3 Fixed ratio responding

Once 9 days of sucrose fade were complete, rats successfully responded to a 10% ethanol solution under a 3:1 fixed ratio requirement (FR3, 3 lever presses equal one reward) within a 20-minute daily exposure. Rats that did not lever press were removed from further analysis ($n=6$). For each session, the total number of ethanol and water responses were recorded. Ethanol availability was again paired with a

stimulus light and vanilla essence olfactory cue. Operant responding sessions for ethanol continued up to day 37 until a consistent baseline response was obtained.

7.3.2.4 Progressive ratio responding

Once a baseline level of responding was achieved, a progressive ratio (PR) reward system was used as previously described [390] to investigate the role of BDNF on "breakpoint". During this phase, the press requirement is progressively increased for each consecutive reward. For example, the first reward is delivered after one press, second reward delivered after three presses, and the third reward delivered after six presses). The water lever followed the same progressive ratio. Breakpoint represents the point during the PR protocol at which the animal ceases to press the active lever the sufficient number of times for a drug reward to be administered. This protocol occurred every second day for a period of one week for a total number of three 90-minute PR sessions (Monday, Wednesday, and Friday). The 20-minute FR3 condition was conducted in-between PR sessions (Tuesday and Thursday) and resumed after the last PR session for one week prior to commencing extinction testing.

7.3.2.5 Extinction

Following breakpoint analysis and a further 7 days of FR3 responding to 10% ethanol, extinction training began on day 49 on 10% ethanol. During this phase, both the ethanol solution and water, as well as the conditioned cues (vanilla essence and stimulus light) were withheld from the chamber. Sessions continued daily for 20 minutes. During the extinction phase lever pressing no longer resulted in a reward, rapidly causing a diminished propensity to lever press. Extinction sessions continued until lever pressing activity reached a low baseline, or equal to the water lever (14 days).

7.3.2.6 Reinstatement

Following extinction, the olfactory cue (vanilla essence) and stimulus light were re-introduced in conjunction with a single drop of ethanol (100µl) dispensed from the receptacle to act as a primer, however, no alcohol reward was dispensed following lever pressing. Rats typically re-instated and returned to pressing the active lever

containing the ethanol solution. Lever pressing activity was recorded for the full 20-minute reinstatement session. Reinstatement lever pressing was compared to the average lever presses taken over the last 5 days of extinction, prior to reinstatement.

7.3.3 Analysis

Statistical analysis was performed in SPSS v22.0 (Armonk, NY: IBM Corp, USA) and graphs were created using GraphPad Prism version 5.00 for Mac (GraphPad Software, San Diego California, USA). Data are presented as the mean ± standard error of the mean (SEM) and a $p \leq 0.05$ was regarded statistically significant. Data were analysed using two-way ANOVAs with repeated measures and post-hoc analysis contingent upon experimental conditions. Grubb's outlier test was used to remove any data points outside normal distribution. The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

7.4 Results

Of the 51 rats screened for operant testing, all but n=6 (n=4 WT, n=2 HET) learned to operate the levers within the chamber, resulting in n=45 for analysis. Experimental groups consisted of n=9 male WT, n=11 male HET, n=16 female WT, n=9 female HET. No significant differences were found between genotypes at the beginning of the study; however, males were significantly heavier than females (not shown). During testing, one female HET rat became unwell and was removed from the study prior to reinstatement recording, leaving n=44 for all further analysis. At the end of testing, male rats had significantly higher body weights than female rats ($F(1,41)=558.6$, $P<0.001$), whereas BDNF HET rats were slightly, but significantly heavier than WT controls ($F(1,41)=38.4$, $P<0.001$; Figure 7.2).

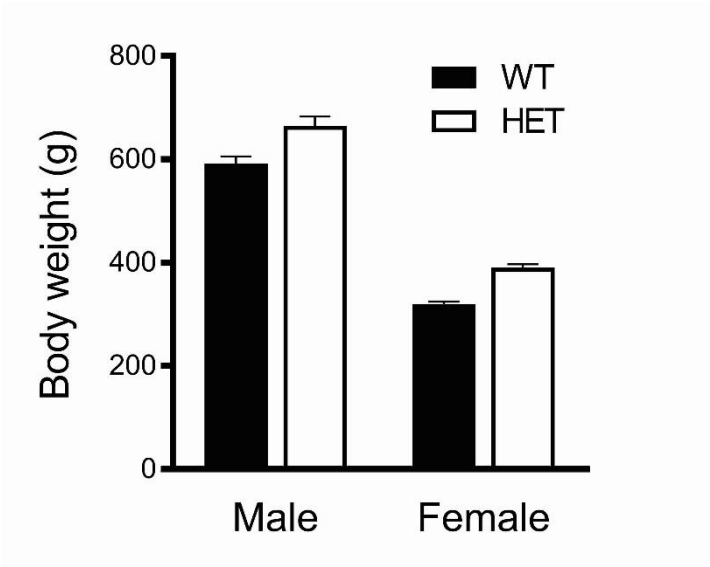

Figure 7.2 Final average body weight following reinstatement of each experimental group. *Males were significantly heavier than their female counterparts ($F_{1,41} = 558.6$; p < 0.001) and BDNF heterozygous rats (HET) were marginally but significantly heavier than their wildtype (WT) controls ($F_{1,41} = 38.4$; $p < 0.001$). Data are mean ± SEM (for number of animals per group, see text).*

There were no significant differences in operant responding between BDNF HET rats and WT controls of either sex during the FR3 normal responding phase (p=0.722; Figure 7.3). More specifically, the average number of lever presses for BDNF HET females was 33.0 ± 10.3 compared to 35.2 ± 7.0 for female WT controls. Similarly, no differences were observed in males with BDNF HET males showing an average number of lever presses of 51.3 ± 9.5 compared to 60.8 ± 14.8 for WT littermates. However, acquisition tended to be faster in the males, with a baseline established at approximately 15-20 days. Females continued to increase the number of presses across the 37-day acquisition period. Similar to FR3, no significant difference was detected between genotypes or sexes during progressive ratio breakpoint analysis (p=0.39, n=45), with each group averaging between 12 to 14 presses before abandoning lever operation (Figure 7.4).

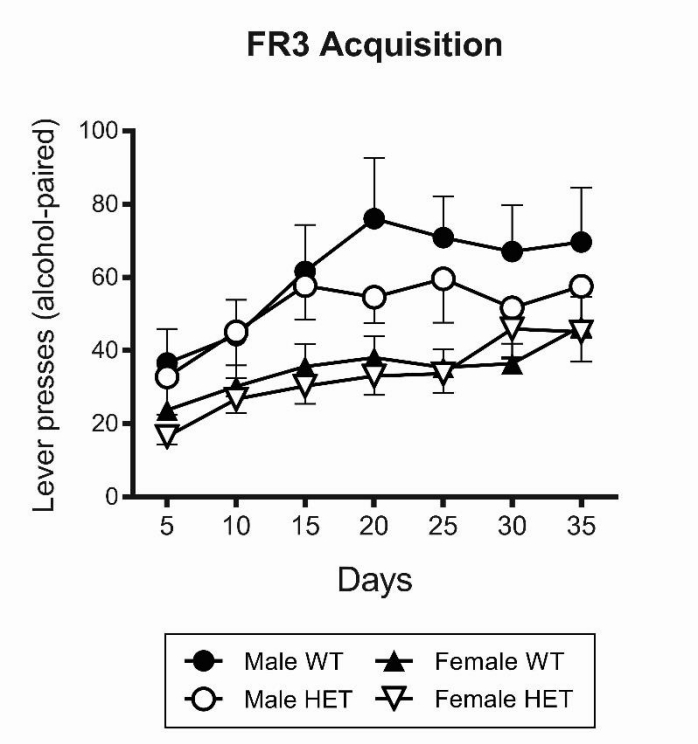

Figure 7.3 Operant responding (FR3) to a 10% v/v ethanol solution in BDNF heterozygous rats (HET) and wildtype controls (WT). *Data are expressed as average weekly alcohol-paired lever presses during 35 days of alcohol-seeking acquisition. Female animals tended to increase lever press activity across the time course while males plateaued by approximately 20 days. Data are mean ± SEM.*

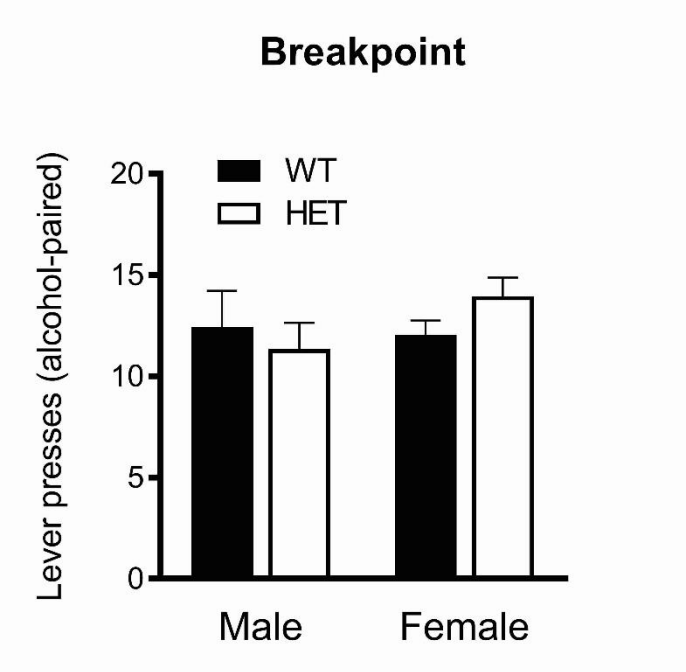

Figure 7.4 Breakpoint of BDNF heterozygous rats (HET) and wildtype controls (WT). *Data are expressed as average highest number of successful alcohol-paired lever presses to a 10% v/v ethanol solution during a progressive ratio protocol. No difference between breakpoint was observed between any genotype or sex combination. Data are mean ± SEM.*

All rats successfully extinguished active lever pressing over a 14-day period and active lever pressing was reduced to a low baseline or equal to the non-active lever (Figure 7.5). All experimental groups significantly increased active lever operation during reinstatement of the stimulus cues (main effect of Day: $F_{1,38}$=50.4, p<0.001; Figure 7.6), however this depended both on genotype and sex of the animals (Day x Strain x Sex interaction: $F_{1,38}$=8.06, P=0.007). Further analysis revealed that in WT rats, reinstatement was sex-specific, with WT males showing significantly higher numbers of alcohol-paired lever presses than WT females ($F_{1,22}$= 9.9; p=0.005, n=24). In contrast, this interaction was absent amongst male and female HET rats (p=0.386, n=18). This was mostly caused by increased reinstatement in female HET rats, as this group showed an average 31.3 lever presses above their WT littermates ($F_{1,21}$=8.11; p=0.010, n=23; Figure 7.6). In contrast, there was no genotype difference between male groups (p=0.178, n=19).

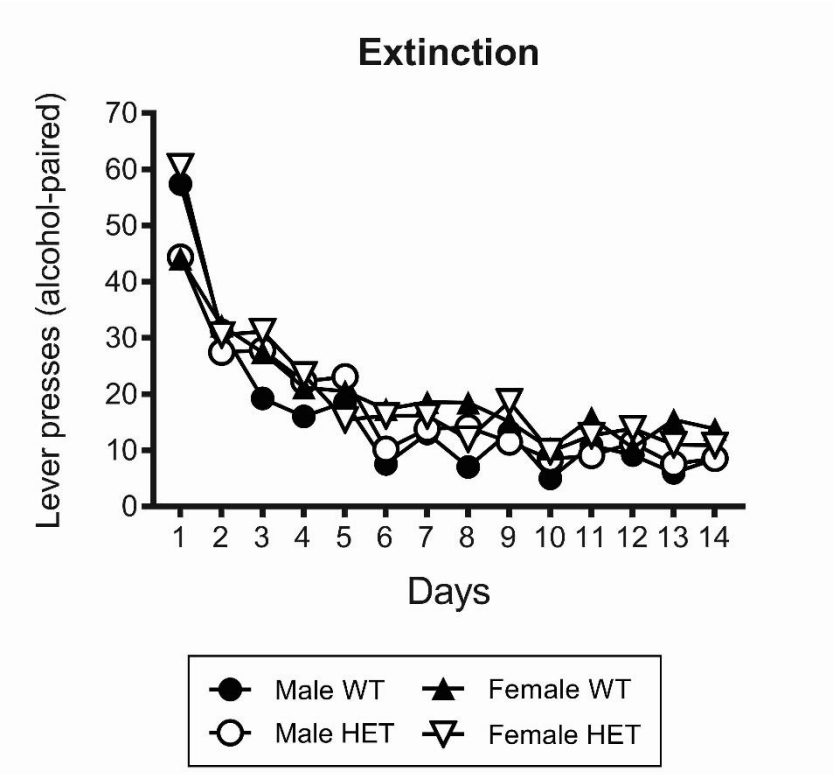

Figure 7.5 Operant responding (expressed as number of alcohol-paired lever presses) in male and female BDNF heterozygous rats (HET) and wildtype controls (WT). *Extinction was measured over 14 days following removal of the stimulus cues and ethanol and water solutions.*

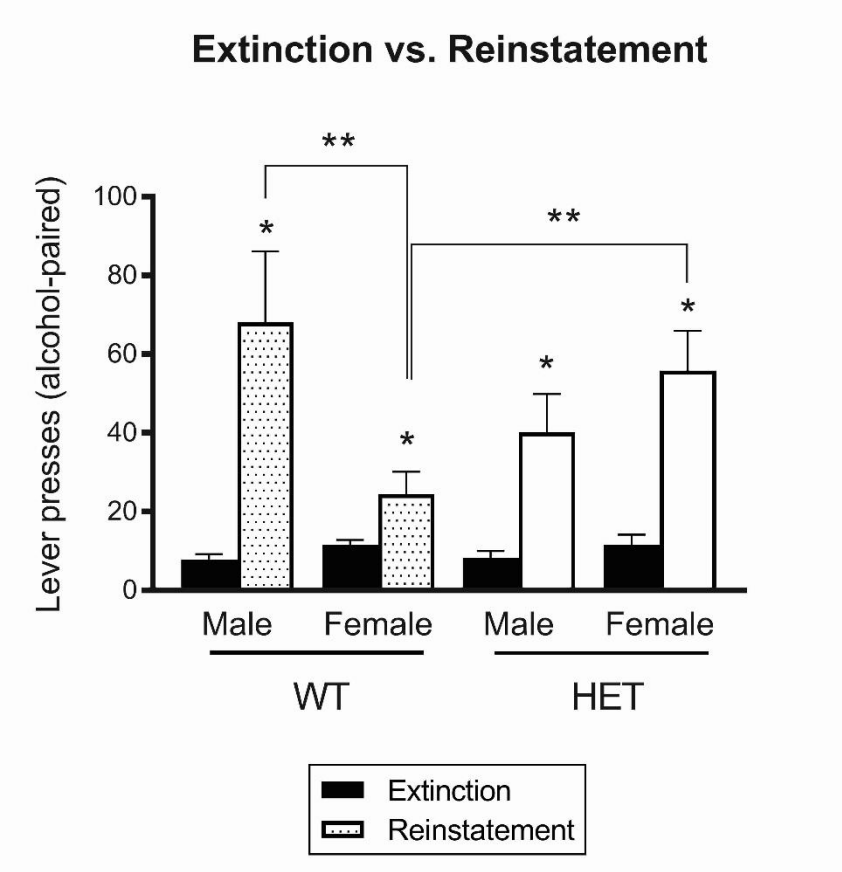

Figure 7.6 Average active lever pressing after restoration of stimulus cues demonstrated in male and female BDNF heterozygous rats (HET) and wildtype controls (WT). *All groups significantly increased lever pressing following reinstatement compared to extinction (average lever presses over the 5 days prior to reinstatement), * = p < 0.05. Male WT rats showed a significantly greater number of reinstated lever presses compared to WT female rats (** = p < 0.05) but there was no sex difference in BDNF HET rats. Female HET rats reinstated significantly higher than female WT rats (** = p < 0.05). Males: n = 9 WT, n = 11 HET; Females: n = 16 WT, n = 9 HET.*

7.5 Discussion

This study demonstrates that, similar to rat strains commonly used to model alcohol-seeking, BDNF HET rats successfully respond to alcohol-predictive cues, extinguish lever pressing following removal of these cues, and reinstate following re-exposure to these stimuli. However, there were sex differences in reinstatement in WT controls which were absent in BDNF HET rats.

BDNF HET and WT littermate rats displayed similar FR3 acquisition for ethanol self-administration in an operant paradigm. This was unexpected as previous work portrays BDNF as a negative regulator of alcohol consumption in mice across

exogenous and endogenous manipulation [267]. Previous rat studies include the use of BDNF antisense oligonucleotides, which increase alcohol intake and cause development of an anxious phenotype [391], as well as alcohol-preferring rats (P rats) that have inherent reductions in BDNF mRNA in the medial and central amygdala [392]. Results are inconsistent regarding the effect of alcohol on levels of BDNF mRNA and protein in the NAc of rodents, with one study reporting unaltered concentrations between P and non-P rats [392], and another suggesting overall reduced levels in these animals [264]. Though there is evidence for a role of BDNF mRNA and protein augmentation in alcoholism, to our knowledge, this is the first study to examine rat BDNF heterozygosity in the context of alcohol-seeking behaviour.

While our findings on the acquisition of alcohol in an operant paradigm failed to show a difference between genotypes, it is possible this may reflect a form of intrinsic compensation as recently highlighted [32]. More specifically, we have previously shown that TrkB phosphorylation in the hippocampus is unaltered in these BDNF heterozygous rats [40], suggesting normal BDNF signalling in this brain region and possibly others. Therefore, it is possible that lifelong attenuation of BDNF levels elicits compensatory mechanisms that maintain TrkB activation and downstream phosphorylation pathways, irrespective of a 50% endogenous reduction of the levels of the neurotrophin.

Similarly, these data suggest the motivation to obtain ethanol was preserved across both sexes and BDNF heterozygosity, as suggested by the fixed-ratio analysis, where no significant differences were found in lever pressing across male and female groups in either genotype. Although no significant difference in breakpoint was detected, the average 12-14 presses reached in this study are similar to those reported in P rats under progressive ratio analysis [393]. This is further testament to the presence of a drug-seeking phenotype in the BNDF HET and WT rats, suggesting animals learnt quickly to increase the number of sequential presses to get a reward. Consequently, modulation of the underlying neurochemical pathways governing motivation driven alcohol drinking, between BDNF HET and WT rats may be largely unchanged.

The most intriguing finding of the present study was the variance we observed in alcohol reinstatement between male and female WT rats. While at first glance this interaction suggests heightened reinstatement in WT males relative to WT females, it is important to note analysis was conducted on lever presses alone. In the context of alcohol craving this is potentially confounding as the relative amount of alcohol consumed varies with overall body mass. Average body weights for each genotype and sex (Figure 7.2) highlight the stark difference between male and female animals' body mass. In future studies, the analysis of blood ethanol concentrations (BEC) following operant sessions would help to unmask the effect of weight differences between rats and allow possible metabolic variances between animals to be measured and compared.

One conclusion that can be drawn from the lack of disparity between heterozygous animals during reinstatement is that relative to body weight, BDNF HET females have heightened alcohol reinstatement, and consequently seek alcohol compared to other genotype/sex combinations. We found males to be approximately 50-100% heavier than their age-matched female counterparts, rendering males' relative alcohol consumption lower than female rats. This is in line with studies suggesting human females become addicted to alcohol more easily, escalate drug use at a faster rate, and have amplified susceptibility to relapse [394, 395]. Furthermore, animal studies have been conducted that highlight female rats' sensitivity to addiction. Moore et al. demonstrated that female ethanol-preferring P rats had higher consumption and preference for ethanol than males, as well as an accelerated path to addiction [276]. This discovery is intriguing, as it could imply sex hormone-mediated addictive protection mechanisms are lost following endogenous BDNF down-regulation. This was emphasised in the acquisition phase, where females steadily increased their ethanol intake and males appeared to rapidly reach a threshold. Our findings are in line with other studies reporting increased alcohol craving in female rats in an alcohol deprivation paradigm [396], and heightened cue-induced alcohol reinstatement in female rats compared to male counterparts, as reviewed by Becker et al. [397].

The observed alcohol craving proclivity observed in our female HET rats in this study may be contributed to by the ovarian hormones and the oestrous cycle. Estradiol, the most active estrogen, is a neurochemical modulator of addiction circuitry including the dopaminergic and glutamatergic signalling systems [398, 399]. It has been hypothesized that estrogens elicit downstream signalling pathways analogous to those of BDNF; activating the MAPK cascade to initiate anxiolytic and neuroprotective effects [400, 401]. Estradiol is also capable of upregulating BDNF synthesis through putative genomic and inhibitory pathways [402]. High levels of estradiol increase BDNF expression in the cortex through GABAergic interneuron inhibition, leading to upregulated BDNF synthesis and release from newly uninhibited BDNF-expressing neurons [403]. The BDNF gene contains a motif highly analogous to the estrogen response element sequence, activated by estradiol to regulate gene expression [404]. Binding of estradiol to the homologous BDNF sequence has been hypothesized to increase transcription and translation of the neurotrophin. Furthermore, the BDNF receptor, TrkB, is inhibited by estradiol-stimulated PI3K cascade initiation, demonstrating a regulatory interaction between estradiol and BDNF-mediated trkB function [405].

As estrogens appear, in part, to modulate their trophic effects indirectly through BDNF regulation, this study suggests a potential relationship between sex hormones, BDNF, and alcohol intake. As BDNF HET rats have marked reduction in endogenous BDNF production, it may be that reduced efficacy of estradiol in promoting BDNF release leads to a more anxious and depressive heterozygous female animal, that consequently craves more alcohol following withdrawal. This is evident in female Sprague Dawley rats that develop deficits in the forced swim test following chronic alcohol exposure [406]. This depressive state is associated with a reduction in hippocampal BDNF and could imply an anxiety-driven urge to “self-medicate” with ethanol. Female mice examined during the acute phase of alcohol withdrawal show upregulated BDNF expression [407]. Therefore, it is feasible the efficacy of this upregulation is partially lost in BDNF heterozygosity, rendering female HET mice more susceptible to relapse and craving than their WT littermates. Future studies should therefore more closely investigate anxiety- and depression-like behaviours in male and female BDNF HET rats to elucidate the role of these behaviours in the reinstatement differences observed in the present

study. Previously, we have already found that male BDNF HET rats chronically treated with corticosterone, to mimic chronic mild stress, showed reduced anxiety-like behaviour on the plus-maze but no changes in depression-like behaviour in the forced swim test [36]. However, those studies did not include alcohol self-administration or female rats.

In terms of sex-specific interactions, it is important to briefly acknowledge the fluctuations in female ovarian hormones that can impact behavioural testing. There is evidence suggesting female rats have reduced alcohol self-administration during the proestrous and estrous phase of hormone cycle compared to diestrous [408], most likely as a result of varying circulating levels of female sex hormones such as progesterone and estrogen. There are also marked cycle-dependent differences in dopamine concentration within the PFC following ethanol administration, suggesting a role for estrogen in ethanol-mediated cortical signalling in dopaminergic neurons [409]. It is possible that estrogen cycling is dysregulated following ethanol intake in female HET rats. Disruption to normal hormone rotation could increase ethanol consumption if hormone regulation results in a more prominent diestrous state. However, as it is probable that the estrous cycle in our female rats was not synced, it is unlikely the effects of the estrous cycle systematically affected lever pressing during reinstatement. Nevertheless, future studies should use a reinstatement value averaged over several days to ensure no estrous cycle effect is present between experimental and control groups.

There are limitations within this study that should be addressed. Firstly, future work could focus on a different technique for elucidating alcohol preference, such as the two-bottle choice paradigm, where fluid intake is monitored daily. Although it forgoes the concept of motivation-based behaviour, two-bottle choice allows for face and construct validity of habitual drinking through daily recording, and a non-operant representation of ethanol preference [410]. Future studies should be undertaken in conjunction with weight and blood alcohol concentration measures to assess ethanol metabolism between animals. Furthermore, to elucidate the role of sex steroid hormones on BDNF HET alcohol craving, reinstatement should be averaged over multiple trials to prevent estrous cycle stage bias.

7.6 Conclusions

The findings presented in this study support the hypothesis that sex-specific BDNF interactions exist within the rat neurocircuitry regulating alcohol addiction. This outcome has prospective face validity for alcohol use disorder in humans, where female alcoholics are increasingly diagnosed with heightened disease symptomology, and a negative phenotype that worsens at an exponential, or “telescopic” rate with increased alcohol consumption. This response is poorly understood and leads to a proportionally larger craving propensity in females compared to their male counterparts. Our results also indicate that BDNF may play a role in alcohol seeking through hormone regulatory pathways, such as the neurotrophin’s potential upstream activation by estradiol. Continued examination of the mechanisms governing sex differences in BDNF HET rats in alcohol seeking and reinstatement could help to elucidate the underlying neurobiological changes principal to alcohol addiction development, and further highlight the cellular alterations leading to dysregulated BDNF expression in prolonged ethanol exposure.

7.7 Acknowledgements

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Chapter 8 7,8-Dihydroxyflavone enhances cue-conditioned alcohol reinstatement in rats

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8.1 Abstract

Alcohol use disorder (AUD) is a detrimental disease that develops through chronic ethanol exposure. Reduced brain-derived neurotrophic factor (BDNF) expression has been associated with AUD and alcohol addiction, however the effects of activation of BDNF signaling in the brain on voluntary alcohol intake reinstatement and relapse are unknown. We therefore trained male and female Sprague Dawley rats in operant chambers to self-administer a 10% ethanol solution. Following baseline acquisition and progressive ratio (PR) analysis, rats were split into drug and vehicle groups during alcohol lever extinction. The animals received two weeks of daily IP injection of either the BDNF receptor, TrkB, agonist, 7,8-

dihydroxyflavone (7,8-DHF), or vehicle. During acquisition of alcohol self-administration, males had significantly higher absolute numbers of alcohol-paired lever presses and higher PR breakpoint. However, after adjusting for body weight, the amount of ethanol was not different between the sexes and PR breakpoint was higher in females than males. Following extinction, alcohol-primed reinstatement in male rats was not altered by pretreatment with 7,8-DHF when adjusted for body weight. In contrast, in female rats, weight-adjusted potential amount of ethanol, but not absolute numbers of active lever presses, was significantly enhanced by 7,8-DHF treatment during reinstatement. Analysis of spontaneous locomotor activity in automated photocell cages suggested that the effect of 7,8-DHF was not associated with hyperactivity. These results suggest that stimulation of the TrkB receptor may contribute to reward craving and relapse in AUD, particularly in females.

Keywords: Brain-derived neurotrophic factor (BDNF); alcohol use disorder; reinstatement; relapse; operant self-administration

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8.2 Introduction

Alcohol Use Disorder (AUD) can be defined as the compulsive and escalating consumption of alcohol in spite of severe detrimental consequences. AUD affects approximately 240 million people, or 4.9% of the global population [411], and has a significant impact on socioeconomic frameworks within society. Poor societal outcomes in AUD manifest as a result of social dysfunction, reduced workplace productivity, and physical illness that typically results in high healthcare expenses [412]. The exact mechanisms underlying AUD remain largely unknown and current intervention strategies are limited [413]. Consequently, understanding the mechanisms which contribute to neurochemical adaptations to excessive alcohol consumption is vital in the development of novel therapeutic treatment options.

The mesolimbic and mesocortical neuronal pathways projecting from the ventral tegmental area to the nucleus accumbens and prefrontal cortex have been identified as core components of addiction development [414]. These

neurocircuitry systems are regulated by neurotrophins, which are responsible for neural maintenance, development and plasticity, and their expression is altered in response to drugs of abuse such as alcohol [415]. Of the four neurotrophins expressed within the mammalian central nervous system (CNS), brain-derived neurotrophic factor (BDNF) is the most abundant and widespread and has been implicated in drug-induced neuroadaptations in AUD and regulation of regions of the brain crucial for addiction development [416].

BDNF is generated as the precursor, proBDNF, where it is cleaved to the mature form (mBDNF) [417]. Upon release, mature BDNF binds to the tropomyosin receptor kinase B (TrkB) to initiate downstream signalling pathways which regulate gene expression encoding proteins involved in neuronal cell survival, axon and dendrite growth, and plasticity [417]. Alcohol intake alters BDNF expression in rats and mice over acute and chronic ingestion [415]. For example, moderate ethanol consumption in mice has been shown to promote BDNF expression within the dorsal striatum [270], and prolonged exposure to alcohol results in a reduction in cortical levels in the brain [418]. Furthermore, a reduction in BDNF within the nucleus accumbens (NAc) has been detected in alcohol-preferring rats [264], and lower levels of the neurotrophin in the medial prefrontal cortex (mPFC) are correlated with escalated alcohol consumption [267]. The BDNF val66met polymorphism, which results in reduced activity-dependent BDNF release in the brain [417, 419], is associated with enhanced alcohol intake in mice [420]. We recently used an operant alcohol self-administration paradigm to test the reinstatement/relapse propensity of BDNF heterozygous (BDNF HET) rats, which have a 50% endogenous reduction in BDNF levels in the brain [421]. We found that female, but not male BDNF HETs displayed enhanced reinstatement potential compared to their sex-matched wildtype counterparts. These findings suggested that BDNF is a negative regulator of alcohol intake and AUD and this role may be sex-dependent.

While changes in BDNF signalling have been associated as potential mediators of alcohol use, few studies have attempted to induce alterations in neurotrophic signalling and assess the effect on alcohol intake. Such studies might inform on the potential efficacy of treatments aimed at BDNF signalling in AUD. In a previous study in rats, BDNF infusion into the dorsolateral striatum gated alcohol intake and

terminated drinking episodes [275]. In mice, the TrkB receptor agonist, LM22A-4, attenuated enhanced alcohol consumption [419]. From these findings, it can be hypothesized that the activation of TrkB, either with BDNF or a TrkB agonist, could be a potential therapeutic lead for the prevention of AUD. 7,8-Dihydroxyflavone (7,8-DHF) is a selective and potent small-molecule agonist of TrkB that can permeate the blood brain barrier (BBB) and is orally bioavailable [422]. This potent TrkB agonist has recently been implemented in a range of behavioural intervention models including studies in spatial memory [423], intracerebral haemorrhage [424], obesity [424], Parkinson's disease and Alzheimer's disease [425]. However, there has been no previous investigation into the effects of 7,8-DHF on alcohol use and addiction pathophysiology, particularly relapse, where we found an involvement of BDNF in our previous studies [421]. Following this previous study in BDNF HET rats, we therefore examined the effect of subchronic 7,8-DHF administration on reinstatement/relapse in an operant alcohol self-administration paradigm in male and female rats.

8.3 Materials and Methods

8.3.1 Animals

Male and female wildtype Sprague Dawley (SD) rats were obtained from the Animal Resource Centre (ARC; Murdoch, WA) at 5 weeks of age and housed at the La Trobe Research and Teaching Facility, Melbourne, for the duration of the study. Forty-eight rats were kept in sex-matched groups of 4 in individually-ventilated cages (Tecniplast, Italy) and allowed to habituate for 1 week prior to the commencement of behavioural testing.

8.3.2 Operant ethanol self-administration

Twenty-four male and 24 female rats were trained in operant chambers (Med Associates, St Albans City, VT, USA) as previously described [389]. Each chamber contained two levers at opposite corners of the arena. A cue light was positioned above each lever and indicated when the sufficient number of presses was reached to obtain a reward, resulting in either 100 microlitres of ethanol solution or water being dispensed into a fluid receptacle adjacent to each lever. Vanilla essence was placed under the active lever as an olfactory cue to indicate the side of the cubicle

containing ethanol solution. Rats were first habituated to the operant chambers over a 16-hour overnight session and allowed to press the levers for a reward containing sucrose and a 5% v/v ethanol solution (2 presses per reward). Food pellets were placed in the bottom of the chamber to prevent any food restriction. Following overnight training, rats were habituated to the aversive taste of alcohol in daily 20-minute sessions over 2 weeks using a previously described sucrose fade protocol [421]. Upon complete withdrawal of the sucrose solution the active lever dispensed a 10% ethanol solution for the remainder of the experiment (Figure 8.1).

8.3.3 Acquisition

Following successful completion of the sucrose fade, rats were trained daily (Monday-Friday) in 20-minute operant sessions, at a fixed-ratio response of three (FR3, three presses equals 1 reward). During this acquisition phase the number of lever presses recorded per session increased over time until the majority of rats reached a stable baseline within 25 days of acquisition training (Figure 8.1). Similar to overnight training and sucrose fade protocols a drop of vanilla essence was placed under the active lever as an olfactory cue to indicate the ethanol containing receptacle.

8.3.4 Progressive ratio

To ascertain that the same protocol was used as in our previous study [421], once lever pressing had stabilized, 90-minute progressive ratio (PR) sessions were included to measure the breakpoint of responding to the active lever (Figure 8.1). This was conducted every second day over a 1-week period in which the number of lever presses required to obtain a reward was incrementally increased with every subsequent reward. For example, the first reward occurred after one press, the second reward after 2 additional presses and the third after 3 additional presses. The water lever followed the same pattern of accumulative presses. The three PR sessions that were conducted were subsequently averaged to give a mean value per animal for breakpoint analysis. The breakpoint of each rat represented the number of total presses or g ethanol per kg body weight that the animal reached before giving up on obtaining a reward within a 90-minute period [390].

8.3.5 Extinction and reinstatement

After completing PR rats were re-baselined over a 1-week FR3 period before commencing the extinction phase (Figure 1). Extinction training began on experiment day 35 and involved the removal of both the ethanol reward and water solutions from the operant chamber. The stimulus light and olfactory vanilla essence cues were also removed, and rats were allowed to lever press for 20-minute sessions over 2 weeks until the number of active presses became low or equal to the nonactive lever. On the 5th day of extinction, daily intraperitoneal (IP) injections of 5mg/kg 7,8-DHF (TCI Chemicals, Tokyo, Japan) in 10% DMSO/saline were introduced to half of the male and female rats directly following the operant session. The remaining rats received a vehicle IP injection with the same 10% DMSO/saline solution. The treatment regimen of 7,8-DHF was chosen on the basis of previous studies, showing that subchronic administration of 7,8-DHF leads to behavioural changes [426-428] and prolonged central TrkB activation [429, 430].

Following active lever extinction, rats were reinstated to alcohol in a final 20-minute operant session. Thirty minutes prior to testing, rats that received daily 7,8-DHF injections were given a final IP injection of 10mg/kg 7,8-DHF. Rats that were given a vehicle IP injection over the last 2 weeks were similarly given a vehicle injection 30 minutes prior to testing. The vanilla essence and stimulus light cues were reintroduced to the chamber and a single drop of ethanol was placed within the receptacle that typically dispensed the alcohol solution in previous phases of the testing protocol. Active presses were recorded in this final session and compared to the extinction values to determine alcohol craving and reinstatement/relapse propensity.

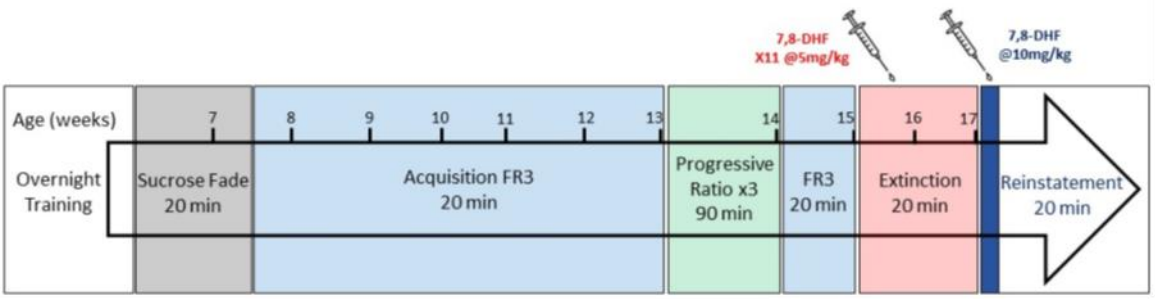

Figure 8.1 Experimental timeline of treatment protocol depicting the stages of operant training and response. *Rats were trained in 1 overnight session followed by a sucrose fade, and lever presses were recorded during acquisition, progressive ratio, extinction, and reinstatement periods. FR = Fixed ratio.*

Spontaneous locomotor activity was recorded immediately following the final reinstatement session to examine whether 7,8-DHF treatment had any effect on overall behavioural activity, such as hyperactivity or lethargy, which could impact lever pressing activity. All rats were tested in locomotor photocells (H:31 × W:43 × L:43cm; MED Associates, St. Albans, VT, USA) over a single 20-minute session, directly following the end of the reinstatement session.

8.3.6 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was done with Statistical Package for Social Sciences (SPSS, IBM Corp, USA). There were initially four groups of 12 rats: male vehicle-treated, male 7,8-DHF-treated, female vehicle-treated, and female 7,8-DHF-treated; final numbers of animals per group are indicated in the figure legends. Data were compared using mixed analysis of variance (ANOVA) with Sex and Treatment (7,8-DHF vs. vehicle) as between-group factors and, where appropriate, Time (FR3) or Session (comparison of extinction vs. reinstatement) as within-group factors. The Greenhouse-Geisser correction was applied where appropriate. Univariate ANOVA was used for between-group comparisons only if the mixed ANOVA showed significant interactions. A p value ≤0.05 was considered statistically significant.

8.4 Results

All 48 rats successfully learned to operate the levers within the chamber during the overnight training and sucrose fade (data not shown). There were no significant weight differences between treatment groups although, as expected, males weighed significantly more than females throughout the experiment (main effect of Sex: $F(1,46)=437.7$, $P<0.001$; Weight change × Sex interaction; $F(2.5,114.7)=19.3$, $P<0.001$; Figure 8.2A). Baseline acquisition of lever pressing for a 10% ethanol solution at FR3 was achieved after approximately 25 days of testing.

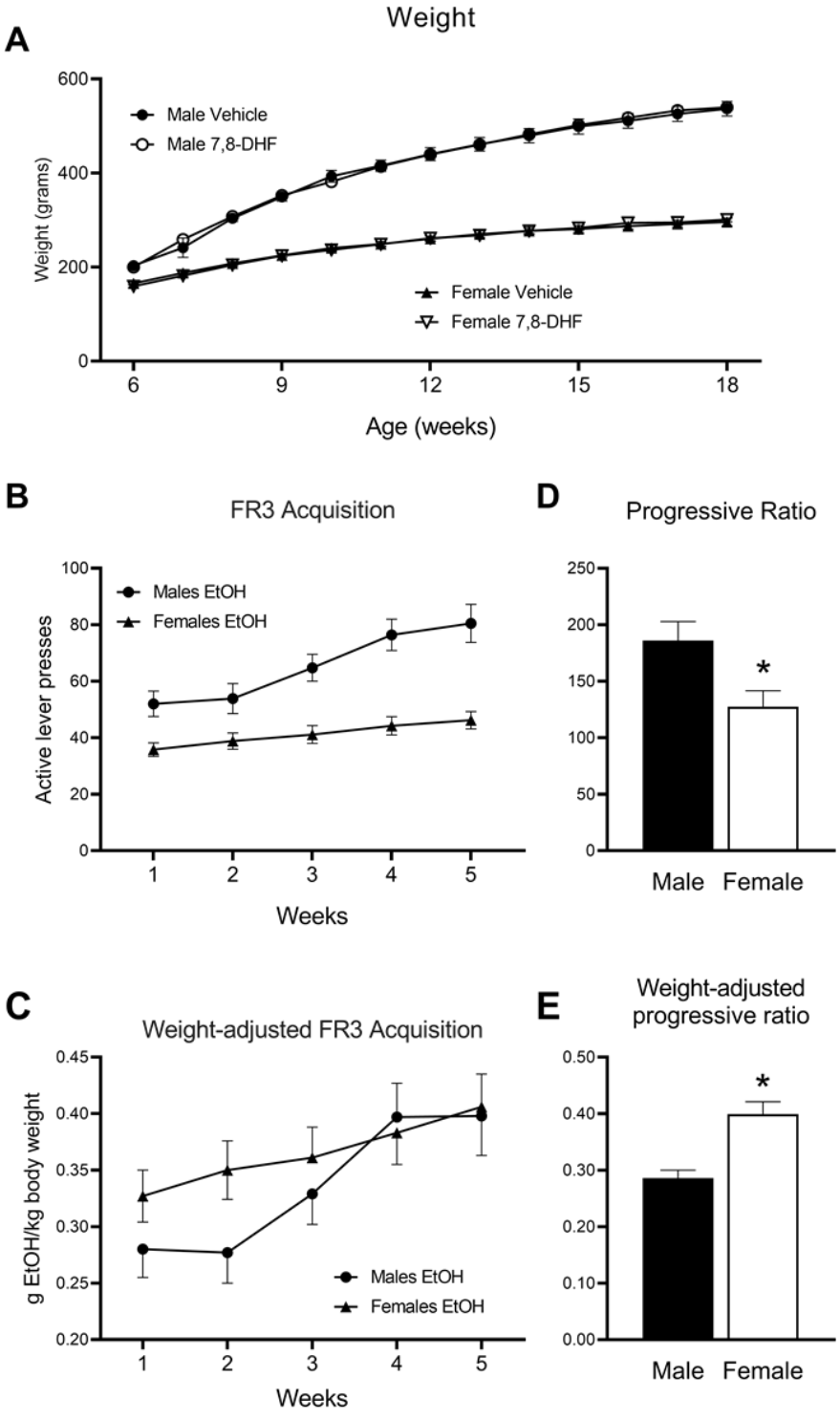

Figure 8.2 *(A) Average weight of male and female SD rats across the duration of operant ethanol self-administration. Male rats were significantly heavier than female rats at all time points, but no significant differences were detected between vehicle and 7,8-DHF treatment groups. (B) Operant responding at FR3 to a 10% v/v ethanol solution in SD rats. Acquisition rate was similar across sexes although males had significantly higher numbers of active lever presses. (C) Weight-adjusted amount of ethanol ingested at FR3. Acquisition rate was faster in males than in females but at 5 weeks there were no significant sex differences. (D) Breakpoint number of active lever pressing for a 10% v/v ethanol solution was significantly higher in male compared to female rats. (E) Weight-adjusted breakpoint amount of ethanol ingested per body weight was significantly higher in female rats than in males. In all cases data are mean ± SEM. The number of animals was n=12 in each group in 2A and n=24 in each group elsewhere.*

During this acquisition period, operant self-administration, expressed as the number of active lever presses, gradually increased (main effect of Time: $F(2.7,124.4)=41.6$, $P<0.001$) and was consistently higher in males than in females (main effect of Sex, $F(1,46)=19.5$, $P<0.001$; Time x Sex interaction: $F(2.7,124.4)=12.5$, $P<0.001$). At Week 5 males pressed an average of 80.5 ± 6.7, compared to females 46.2 ± 3.1 ($F(1,44) = 22.9$, $P<0.001$). Despite marked differences in lever pressing numbers, both male and female rats appeared to reach stable acquisition within a similar timeframe, with only marginal increases in both groups' responding between the 4th and 5th week of training (Figure 8.2B).

To take into account the significant sex differences in body weight (Figure *8.2*A), data were also analysed as the ratio of the amount of ethanol ingested by body weight (Figure *8.2*C). This amount increased over time (main effect of Time: $F(3.1,140.8)=25.2$, $P<0.001$) and, while there was no main effect of Sex, this number increased more rapidly over time in males than in females (Time x Sex interaction: $F(3.1,140.8)=3.64$, $P=0.014$). At Week 5, there was no sex difference in the amount of ethanol ingested expressed as a ratio of body weight (Figure *8.2*C). Males ingested an average of 0.398 ± 0.035 g ethanol/kg body weight, compared to females 0.406 ± 0.029 g/kg.

Significant differences were observed between male and female rats for progressive ratio (PR). Male rats pressed the active lever significantly more during the 90-minute session than female rats ($F(1,46) = 7.14$, $P=0.010$; Figure *8.2*D), suggesting a higher breakpoint and persistence to consume alcohol in male rats. However, when accounting for the significant difference in body weight between male and female rats, the amount of ethanol ingested as a ratio of body weight showed a significantly higher breakpoint in females than in males ($F(1,46)=18.4$, $P<0.001$, Figure *8.2*E).

Following PR and a one-week period of FR3 re-acquisition, rats successfully extinguished lever pressing in response to reward and stimulus cue removal (Figure 8.3A). Active lever presses markedly fell from the first day of extinction (average 66.1 ± 5.3 in males, 44.5 ± 4.3 in females) to low stable levels during the last five days of the protocol (average 10.5 ± 1.1 in males, 11.9 ± 1.3 in females), although a small, but significant, preference for the previously rewarded lever remained (average previously inactive lever presses 3.5 ± 0.6 in males, 4.7 ± 1.2

in females, main effect for comparison with previously active lever: $F(1,44)=128.7$, $P<0.001$). Daily injections of 5 mg/kg 7,8-DHF or vehicle were introduced from Day 5 onwards. There was no significant difference in operant responding during extinction between the drug treatment groups (data not shown).

Of the 48 rats that completed the extinction protocol, 39 reinstated their alcohol drinking behaviour following reintroduction of the stimulus cues. The 9 rats that did not respond to the reinstatement session were removed from further analyses (2 male vehicle, 2 male 7,8-DHF, 2 female vehicle, 3 female 7,8-DHF) based on activating ≤2 rewards (5 or fewer presses) over the 20-minute period. This cut-off prevented accidental activation of the lever impacting the mean reinstatement value of each group. All groups showed significantly higher numbers of active lever presses during the reinstatement session compared to the extinction session (main effect of Session; $F(1,35) = 26.9$, $P<0.001$). Importantly, a significant Treatment x Session interaction was observed ($F(1,35) = 5.2$, $P=0.029$) reflective of enhanced reinstatement following administration of 7,8-DHF compared to vehicle (Figure 8.3B). However, there were no statistical interactions with Sex (Figure 8.3B).

To take into account the significant difference in body weight between male and female rats, active lever press data were converted to the potential amount of ethanol ingested and expressed as a ratio of body weight (Figure 8.3C). Analysis of these data again showed significant reinstatement (main effect of Session: $F(1,35)=29.6$, $P<0.001$) which was significantly greater following 7,8-DHF treatment (Drug x Session interaction: $F(1,35)=6.97$, $P=0.012$). There was also a main effect of Sex ($F(1,35)=13.1$, $P=0.001$) and a Drug x Session x Sex interaction ($F(1,35)=5.48$, $P=0.025$) suggesting differential effects between males and females. Separate analysis of data from males and females revealed that male rats showed significant reinstatement (main effect of Session: $F(1,18)=14.6$, $P=0.001$) but there was no effect of 7,8-DHF treatment (Figure 8.3C). Female rats also showed significant reinstatement ($F(1,17)=15.6$, $P= 0.001$) but this was significantly greater following 7,8-DHF treatment compared to vehicle (Treatment x Session interaction: $F(1,17)=8.46$, $P=0.010$).

Post hoc analysis showed significant reinstatement in female rats treated with 7,8-DHF ($F(1,8)=17.2$, $P=0.003$) but not vehicle (Figure 8.3C). Thus, a differential effect of 7,8-DHF on reinstatement between male and female rats was shown but only following analysis of reward data as the ratio of amount of ethanol potentially ingested per body weight (Figure 8.3B vs. 8.3C).

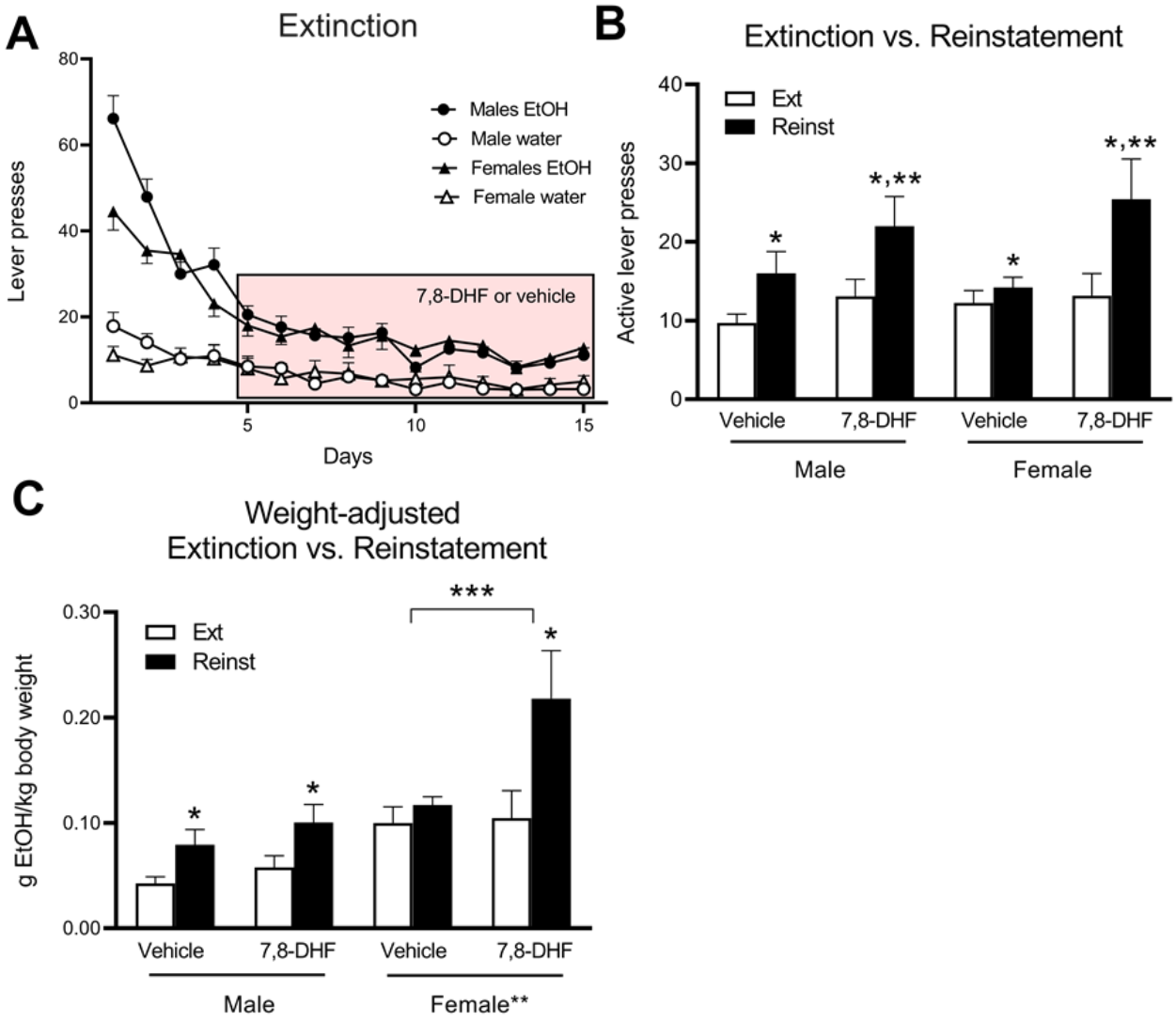

Figure 8.3 *(A) Operant responding in male and female SD rats over the 15-day extinction protocol. The red box indicates the injection period of either 5mg/kg 7,8-DHF or vehicle. (B) Average active lever pressing in the last week of extinction (Ext) vs. the reinstatement (Reinst) session. *P<0.05 for difference between number of active lever presses during reinstatement vs. extinction based on main ANOVA effect of Session. **P<0.05 for enhanced reinstatement in 7,8-DHF treated compared to vehicle-treated rats based on main effect of Treatment. There were no sex differences. (C) Average potential amount of alcohol ingested expressed as ratio of body weight (kg). **P<0.05 for main ANOVA effect of Sex indicating higher amount of g ethanol/kg in females than in males. *P<0.05 in males for main ANOVA effect of Session with no effect of 7,8-DHF; difference only observed after 7,8-DHF treatment in females. ***P<0.05 for ANOVA Session x Treatment interaction showing higher weight-adjusted g ethanol/kg vs. extinction in female rats following 7,8-DHF than vehicle treatment. All data are mean ± SEM of n=10 for males and n=9 for females.*

Spontaneous locomotor activity was not significantly different between control and 7,8-DHF treated rats following reinstatement testing (Figure 8.4). Females rats had significantly higher locomotion over the 20-minute testing session (sex main effect; $F(1,35) = 5.3$, $P=0.027$), covering an average distance of 59.6 ± 17.4m compared to males 48.6 ± 11.1m.

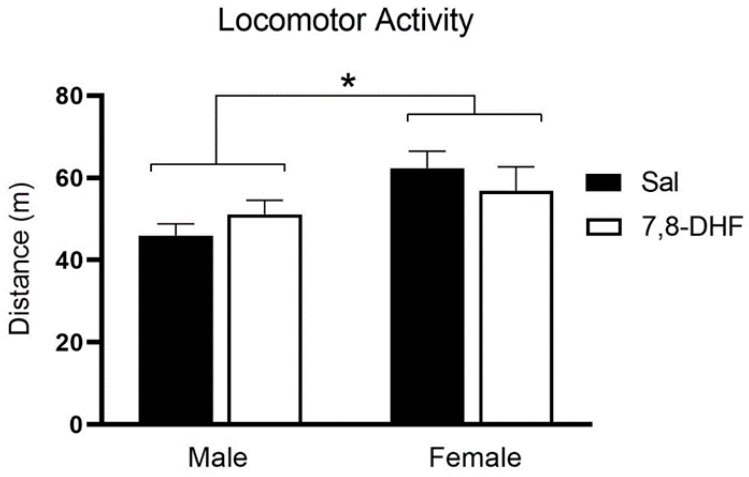

Figure 8.4 Average spontaneous locomotor activity during a 20-min post-reinstatement session. *Rats received either 10mg/kg 7,8-DHF or sham injection 50 minutes prior to locomotor testing. Female rats showed higher spontaneous locomotor activity than male rats but there were no differences between 7,8-DHF-treated and vehicle-treated rats. Data are mean ± SEM; * = p <0.05 for difference between males and females.*

8.5 Discussion

In line with our previous ethanol self-administration protocol, SD rats successfully learned to respond to stimulus cues and preferentially activate the alcohol-paired lever. Furthermore, lever pressing frequency increased with the enhanced difficulty of obtaining a reward during a progressive ratio protocol, extinguished in response to cue and reward withdrawal, and reinstated with the reintroduction of these stimuli. The absolute number of active lever presses during reinstatement was significantly higher in male and female rats treated with 7,8-DHF compared to vehicle, suggesting a potential role for TrkB activation in alcohol relapse. When expressed as weight-adjusted amount of ethanol ingested, female rats showed significantly higher reinstatement following 7,8-DHF treatment compared to vehicle, whereas this was not observed in male rats. Spontaneous locomotor activity, assessed immediately following the reinstatement session, showed no significant difference between 7,8-DHF and vehicle-injected rats, suggesting any

variation in lever pressing between the groups cannot be attributed to non-specific drug-induced hyperactivity.

The data confirm a role of BDNF signalling in reinstatement of alcohol self-administration after extinction. Previous literature on neurotrophic factors and their role in alcohol addiction highlights an upregulation of BDNF mRNA and protein expression in response to prolonged alcohol intake, followed by a gradual decrease of expression. Previous studies in rats have shown a reduction in BDNF expression typically accompanies increased alcohol craving. This has been demonstrated using BDNF antisense oligonucleotides microinjected into the central amygdala (CeA), which provoked anxiety-like behaviours and increased alcohol preference in a two-bottle choice paradigm [391]. Further evidence of a role for CeA BDNF in alcohol use disorders has been demonstrated by innate differences in BDNF expression between alcohol-preferring (P rats) and non-preferring rats, with P rats having naturally lower BDNF levels within the medial temporal lobe [392]. The same trend in BDNF expression change is seen within the hippocampus, as BDNF levels are enhanced following acute intermittent alcohol exposure but reduced during chronic intake [431].

The mesocorticolimbic dopamine system is a central pathway governing learning processes associated with stimulus reward [47, 432]. BDNF mediates dopaminergic activity via trophic modulation of neuron maintenance and differentiation [433]. Therefore, the disruption of BDNF expression and TrkB activation within dopamine-driven reward regions may be critical for AUD development. A study in alcohol naive P rats demonstrated reduced BDNF protein in the NAc [264], while a different study was unable to detect significant levels of BDNF in the ventral striatum, instead reporting on reductions in amygdaloidal BDNF mRNA and protein expression [392]. These findings demonstrate that BDNF may act on VTA-NAc pathways mediating alcohol addiction and craving propensity. However, because in this study we injected 7,8-DHF systemically, effects of the drug outside of the dopaminergic pathways cannot be excluded.

Based on previous research we anticipated 7,8-DHF-mediated TrkB activation would reduce cue-conditioned alcohol reinstatement. The finding that the TrkB agonist had the opposite effect and promoted reinstatement to alcohol was

therefore unexpected. However, recent research has demonstrated BDNF expression recovery following prolonged drug abstinence. BDNF expression within the cortex returns to basal levels during abstinence following exposure to ethanol <6 weeks in mice [418]. Consequently, the introduction of 7,8-DHF may have enhanced activation of the BDNF receptor above baseline levels and enhanced the addiction phenotype that is previously also described in cocaine studies [434]. If BDNF had normalised in our alcohol-extinguished rats, TrkB activation may have been enhanced and the homeostatic balance between TrkB and P75NTR signalling disrupted, leading to potential addiction / craving reinforcement. This has been demonstrated with other substances of abuse, namely heroin, cocaine, and morphine, where exogenous BDNF within the VTA increased the likelihood of rats becoming drug dependent [47].

Another possibility to explain the apparent discrepancy between enhanced reinstatement in BDNF heterozygous rats in our previous study [421] and the present results is that prolonged treatment with 7,8-DHF resulted in desensitization of the TrkB receptor, effectively inducing reduced BDNF-TrkB signalling comparable to BDNF heterozygosity. However, previous studies have suggested 7,8-DHF treatment increases TrkB phosphorylation even after prolonged treatment [429, 430]. Future studies will need to confirm if this is the case in animals treated and tested according to the protocol used in the present study.

In our previous study in BDNF HET rats we found enhanced reinstatement in female but not male rats [421]. Previous studies have shown a close interaction of sex steroid hormones and BDNF signalling [402]. The estrous cycle is known to impact BDNF mRNA expression levels in the hippocampus and frontal cortex of rats, with both reduced and enhanced levels observed during periods of high estradiol circulation [435, 436]. The finding that 7,8-DHF enhanced the absolute number of active lever presses similarly in both male and female SD rats was therefore unexpected. However, while BDNF HET rats have significantly reduced BDNF levels in the brain, we previously demonstrated unaltered TrkB phosphorylation in these animals, suggesting possible compensation due to the lifelong deficit in BDNF levels [40]. It is possible that this compensation is different in male and female BDNF HET rats, contributing to sex-specific changes in reinstatement following prolonged BDNF dysregulation in that model. In the

present study, 7,8-DHF treatment was continued for a more limited time, possibly preventing global and sex-specific compensatory mechanisms.

Surprisingly, when expressed as the amount of ethanol potentially ingested as a ratio of body weight, a sex-specific effect of 7,8-DHF was found. Compared to vehicle, in female rats 7,8-DHF significantly increased the potential weight-adjusted amount of ethanol per body weight, a treatment effect no longer significant in male rats. Thus, expressing the data this way produced the same sex-specificity as in our previous study [421] although, as discussed above, the direction of effect of BDNF heterozygosity and 7,8-DHF treatment was unexpectedly similar. Although analogous to the majority of the literature we did not express our data adjusted by body weight in our previous study, other reports have shown the importance of this adjustment when evaluating age and sex differences in operant alcohol self-administration [437-440]. This finding is consistent with a large literature on sex differences in addiction to a range of psychoactive substances [397, 439]. The reason for the apparent discrepancy in sex-specificity between the absolute number of lever presses and body weight-adjusted amount of ethanol (potentially) ingested in our study remains unclear, but could be related to the higher level of weight-adjusted responding during the late phase of extinction in female rats compared to male rats (Figure 8.3C), representing higher activity of brain circuitry involved in operant responding for alcohol and a resulting greater sensitivity to treatments such as 7,8-DHF. However, in the absence of further studies, this explanation remains speculative.

There are a number of limitations regarding study design that should be addressed in future studies. It should be acknowledged that the use of operant self-administration, as opposed to the two-bottle choice paradigm, restricts alcohol access to daily 20-minute intervals as opposed to 24-hour access. However, the benefit of operant administration is that it permits analysis of motivation-based behaviour, which provides the opportunity to examine potential BDNF-mediated alterations in alcohol craving, where we previously observed difference in BDNF HETs. Another important limitation to this study is the method of TrkB administration. Although 7,8-DHF is orally bioavailable and capable of rapid Blood-Brain-Barrier diffusion, IP injection would not have resulted in region-specific enhanced TrkB signalling. To address possible divergent effects of BDNF

expression within different neuronal pathways, future studies could target the administration of 7,8-DHF to areas predominantly associated with reward pathways within the brain. This would allow examination of the effect of enhanced TrkB activation in different functionally significant regions such as the cortex, VTA, and NAc. Although this was a behavioural study, future experiments should also incorporate manipulation check to ensure 7,8-DHF was enhancing TrkB phosphorylation, and blood alcohol testing to determine intoxication levels in rats. Finally, given the 2-week abstinence from alcohol over the extinction period, rats' BDNF levels may have normalised. Therefore, in future it may be beneficial to commence 7,8-DHF treatment during FR3 testing to examine the escalation of habitual alcohol consumption in response to enhanced TrkB activation. This is arguably as important as attempting to prevent alcohol reinstatement, as early intervention is often more efficacious than attempting to undo the persistent neurocellular adaptations leading to prolonged addiction.

8.6 Conclusions

In conclusion, our findings support a role for BDNF in AUD, with activation of the BDNF receptor, TrkB, enhancing relapse/reinstatement. When the number of active lever presses were adjusted for body weight, the effect of 7,8-DHF was specific for female rats. Further work is required to understand the mechanisms involved in the effect of 7,8-DHF, including the regions critical in mediating the involvement of BDNF and TrkB in AUD development and addiction pathophysiology. These studies could lead to targeted neurotrophic interventions to reduce AUD prevalence, particularly in females.

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The authors declare no conflict of interest.

Chapter 9 General Discussion

9.1 Introduction

Improved treatment strategies for METH psychosis and AUD require a greater understanding of the neurocircuitry governing addiction and psychosis-like behaviours. The same neuronal networks associated with psychostimulant-induced psychosis and addiction are also implicated in schizophrenia aetiology, which is thought to develop through a combination of genetic and environmental risk factors. Consequently, greater understanding of the neurocellular perturbations leading to psychosis and addiction will provide further insight into schizophrenia and AUD pathophysiology and improve clinical outcomes for those suffering with these diseases. Animal models present a platform for testing potential environmental risk factors that play a role in addiction and psychosis development. In the current studies, this was achieved through repeated drug exposure and targeted genetic modification to induce vulnerability to DA sensitization. Effects of chronic METH or alcohol use were assessed in BDNF HET mice and rats, respectively, forming a two-hit model of psychosis and addiction. Although attenuated BDNF signalling and protein expression have been identified as risk factors for drug addiction, psychostimulant-induced psychosis, and schizophrenia, the neural substrates mediating BDNF influence on these diseases remain unclear. As BDNF regulates DAergic activity, and DA sensitization is considered central to both addiction and psychosis pathophysiology, we hypothesized that reduced modulation of mesocorticolimbic DA networks in BDNF HET mice and rats would increase psychosis-like behaviour and alcohol addiction behaviours, respectively.

The overarching aim of this thesis was to further our understanding of the role of BDNF in mediating psychosis and addiction development through METH- and ethanol-induced changes in behaviour. Psychosis chapters describe the effect of BDNF heterozygosity on schizophrenia endophenotypes following METH sensitization, while addiction chapters describe the role of BDNF in alcohol self-administration behaviours including acquisition, extinction, and reinstatement.

9.2 Confirming previous results in BDNF HET mice and exploring METH pretreatment regimen

METH-dependent users often develop an acute psychosis that is comparable to FEP and can progress, in some cases, to the onset of schizophrenia. Importantly, risk of disease development varies between individuals, highlighting genetic vulnerability and environmental interaction as mediators of schizophrenia development. Mutations in the BDNF gene have been hypothesized to confer susceptibility to the onset of schizophrenia. This likely occurs through a reduction in BDNF expression and signalling that is also associated with enhanced behavioural sensitization to psychostimulant drugs, highlighting common neural pathways governing sensitization and psychosis. We have previously examined the relationship between reduced BDNF protein levels and chronic METH intake on psychosis-like behaviours, where we described endogenous sensitization to the acute effects of D-amphetamine challenge in BDNF HET mice. Based on these investigations, the studies described in Chapters 3 and 4 aimed to replicate previous findings in the BDNF HET model by examining the long-term effects of an escalating METH pretreatment regimen during adolescence; a critical timepoint for neurotrophic signalling during brain development.

In the first experimental chapter we examined the impact of reduced BDNF expression on APO- and MK-801-induced PPI deficits, modelling subcortical DAergic hyperactivity and hypoglutamatergic function, respectively. These drugs were compared between saline- and METH-pretreated BDNF HET and WT mice to assess changes to sensorimotor gating associated with DAergic sensitization. In the second 'low-dose' chapter, METH-induced LHA was examined in the same mice. Disruption of these behaviours has been shown to model sub-cortical hyperdopaminergic signalling and hypoglutamatergic function and is reversed by antipsychotic medications. Overall, few effects of BDNF heterozygosity were observed in PPI experiments, which is consistent with previous research in the BDNF HET model. In LHA studies, we confirmed both a low- and high-dose METH challenge produced greater hyperactivity in BDNF HET male mice compared to WT over the two-hour timecourse, but these genotype differences were absent in females. Results were partially supported by previous experiments using a D-

amphetamine challenge and the same escalating METH regimen. Combined, these data provide evidence for a role of BDNF in mediating psychostimulant-induced sensitization neurocircuitry.

In addition to replicating previous experiments, additional METH-pretreatment regimens were tested, including a lower dose and less frequent administration. PPI experiments at lower METH doses (Chapter 3) reported similar effects on sensorimotor gating compared to the escalating METH regimen. The most significant finding was a reduction in baseline PPI in 111-pretreated mice, but this difference did not translate to changes in PPI in response to APO or MK-801 challenge. In Chapter 4, LHA to a low- and high-dose METH challenge was similar between METH-pretreatment groups, demonstrating that an escalating regimen may not be required for enhanced sensitization and the development of psychomotor behaviours.

9.3 Psychostimulant-induced PPI deficits and LHA are mediated by BDNF but not D3R

BDNF has been identified as a key regulator of D3R expression and both proteins are implicated in sensitization and psychosis pathophysiology and are dysregulated in schizophrenia patients. Based on these findings, we aimed to test the relationship between BDNF and D3R in mediating schizophrenia endophenotypes following METH-sensitization. As part of this thesis, we were the first to combine the BDNF HET and D3KO genotypes. This double-mutant model provided a platform to examine the role of the D3 receptor in BDNF-mediated changes in sensitization and allowed for further interrogation into the relationship between BDNF and the dopamine system on psychosis endophenotype behaviours. Despite once more observing deficits in PPI and enhanced LHA in BDNF HET mice, no major contribution of D3R was observed in these experiments (Chapter 5 and 6) suggesting BDNF-mediated changes in sensitization and behaviour may be occurring via a D3R-independent mechanism. While D3R expression appears dependent on BDNF signalling [85], the lack of genotype interaction described here suggests that the two systems act independently on sensitization and behavioural response to METH. Furthermore, based on the lack

of genotype effects between D3R WT and D3KO mice, we find no appreciable role of D3R in mediating disruptions in PPI, nor psychostimulant-induced LHA.

There are alternative theories on the functional significance of disrupted D3R signalling in sensitization that can be discussed. The D3R sensitization hypothesis attributes compensatory down-regulation of D3R to increases in tonic DA concentration [87]. In this process, the D3R are hypothesized to undergo tolerance to DA which reduces the inhibitory activity of the receptor on DA neurotransmission and results in DAergic hyperactivity and behavioural sensitization [290]. Provided this was occurring in our experiment, D3R loss may have had less impact on METH-pretreated animal behaviour as these mice may already display the reduced D3R expression inherent to sensitization development. Therefore, genetic ablation of D3R may have had greater impact on saline-pretreated mice. That being said, these mice may not have displayed D3R-deficiency effects because loss of the receptor has only been associated with subtle changes to the behavioural phenotype of mice, and 1- or 3mg/kg METH challenge in LHA experiments may have been above D3R inhibition thresholds. In fact, studies have demonstrated that, based on D3R high-affinity state for DA [77, 290], these receptors are spontaneously activated by basal DA activity and respond only to subtle fluctuations in phasic DA concentration. Due to sustained DA binding, D3R has been hypothesized to play more of a role in mediating tonic DA neurotransmission, rather than responding to acute elevations in neurotransmitter release [82]. Conversely, D2 receptors are typically in a low-affinity state for DA and are therefore more likely to regulate spikes in DA release that occur after stimulant exposure. Consequently, acute METH challenge may induce D2R-dependent functional changes in hyperactivity that are unaltered in the D3KO brain. The lack of D3R effects on APO- and MK-801-induced PPI deficits also suggests a limited role for the receptor in mediating sensorimotor gating, regardless of DAergic sensitization.

Although we did not observe significant differences between D3KO and D3R WT mice in endophenotype studies, previous studies show a decrease in receptor mRNA in the cortex of schizophrenia post-mortem tissue [363], suggesting dysregulation of basal DA signalling and loss of inhibitory control within disease pathophysiology. Furthermore, reduced D3R mRNA corresponds with lower BDNF

expression in the cortex of schizophrenia patients, supporting findings that D3R expression is regulated by BDNF [85]. However, multiple pathways govern D3R levels and include more than just neurotrophic modulation. For example, the D3R gene is capable of producing seven alternatively spliced proteins, five of which do not bind DA and are hypothesized to impact DAergic activity through receptor dimerization [441]. Of the five, the D3nf splice variant has attracted attention in preclinical schizophrenia research. D3nf is a truncated version of the D3R protein, missing two transmembrane domain required for DA binding [442]. Importantly, the D3nf variant is conserved across human and rodent species, suggesting functional importance. D3nf acts to inhibit DA binding to the D3R by dimerising with full-length D3 receptor sub-units which internalises the receptor away from the cell membrane, preventing DAergic inhibition [443]. In schizophrenia post-mortem tissue, D3nf splicing efficiency is increased within the cortex [364]. Presumably, heightened D3nf expression would result in a lower D3R/D3nf ratio, acting to promote DAergic signalling within the cortex. D3R isoform expression is thought to be an adaptive response that is sensitive to changes in dopamine signalling and is sensitive to amphetamine and D2-like receptor antagonism [444]. Consequently, increased D3nf splicing may be an adaptive mechanism accommodating for reduced cortical DA expression in schizophrenia. Future experiments should include quantification of different D3R splice variants by RT-PCR, to determine relative ratios of D3R/D3nf in BDNF HET mice and the double-mutant model.

9.4 Evidence supporting BDNF deficiency in AUD

While BDNF has been implicated in AUD and alcohol addiction, effects of BDNF signalling on addiction pathophysiology are poorly understood. To address gaps in the literature, publications included in this thesis describe differences in operant self-administration of ethanol between WT and BDNF HET rats (Chapter 7), and subchronic 7,8-DHF vs. saline administration in WT rats in a follow-up investigation (Chapter 8).

In the first operant study, we trained BDNF HET and WT rats to self-administer a 10% ethanol solution and tested for sex and genotype differences in baseline alcohol-paired lever pressing, progressive ratio, extinction, and reinstatement. No differences in alcohol-paired lever presses were detected between BDNF HET and

WT rats across acquisition, progressive ratio, or extinction. However, following alcohol-primed reinstatement of alcohol seeking after abstinence, female BDNF HET rats showed a significantly higher number of alcohol-paired lever presses than female WT controls, highlighting a potential interaction between BDNF and sex hormones in regulating alcohol seeking behaviour. In the follow up study, WT rats were administered a subchronic regimen of the TrkB agonist, 7,8-DHF, or vehicle injection, and tested across the same operant protocol (Chapter 8). As in the BDNF HET study, no significant differences between sexes were observed for alcohol-paired lever pressing during acquisition or extinction, but progressive ratio/breakpoint was significantly higher in female rats, highlighting greater motivation in these animals to obtain alcohol. The main finding of this study was that 7,8-DHF treatment increased reinstatement in female, but not male rats compared to controls. This was unexpected given BDNF deficiency produced larger reinstatement in the HET vs. WT female rats in studies outlined in Chapter 7. Nevertheless, in conjunction, these findings suggest that BDNF regulatory pathways mediate sex differences in reinstatement of alcohol intake, therefore emphasizing the need to explore sex-specific interactions in addiction neurocircuitry.

9.5 Investigating sex-dependent BDNF effects

During adolescence, vulnerability to psychosis and addiction development coincides with sex-specific neurodevelopmental changes driven by elevated sex hormone expression [445]. Thus, dysfunctional sex hormone signalling is postulated to play a role in schizophrenia and AUD pathophysiology. Theories on sex-specific psychosis-like behaviour note differential schizophrenia symptom presentation and onset between sexes, with heightened risk in men and a secondary vulnerability period in women that coincides with menopause and the associated reduction of estrogen levels [446]. Relevant to studies described in this thesis, sex-specific effects have been observed in PET investigation of DA release following amphetamine and ethanol administration in humans. In imaging studies using [11C]raclopride, amphetamine- and alcohol-induced striatal DA release is higher in males [447, 448], while contrastingly, psychostimulant-evoked sensitization of behaviour in animal models appears to be more pronounced in

females [449, 450]. Alongside differences between male and female psychosis symptomatology and sex-dependent response to antipsychotics [451], these findings emphasize a role for sex hormones in mediating neuronal networks affected by addiction and psychoses within the human brain.

While animal models cannot reflect higher cognitive characteristics that drive drug abuse in humans, the underlying neurobiological mechanisms differentiating male and female systems can be explored. Like in humans, variance between male and female rodent behaviour is typically attributed to the effects of sex hormones [402]. Consequently, measuring the contribution of these hormones in addiction and psychosis development may provide greater insight into the differences we observed between sexes following BDNF depletion and after 7,8-DHF treatment. In psychosis endophenotype studies we reported increased LHA (Chapter 4), reduced baseline PPI and lower PPI disruptability by psychostimulant drugs after METH pretreatment in male BDNF HET mice compared to BDNF HET female (Chapter 5). On the other hand, in our second LHA study (Chapter 6) female BDNF HET mice were endogenously sensitized to METH-induced LHA, displaying heightened sensitivity to acute METH regardless of pretreatment. Operant study results also displayed sex-specificity. BDNF HET female rats were more susceptible to cue-induced alcohol reinstatement, and after 7,8-DHF treatment, female WT rats had significantly higher active-lever pressing activity in relapse compared to vehicle controls (Chapter 7 & 8). From these data, we can conclude that some form of interaction is occurring between BDNF and sex steroid hormones to produce behavioural differences between male and female mice and rats.

Like BDNF, sex hormones are important in shaping the adolescent brain and have also been implicated in addiction and schizophrenia development [452, 453]. Androgens and estrogens make up the two classes of signalling molecules comprising male and female sex hormones, respectively. While androgens like testosterone may regulate aspects of BDNF signalling [454], the majority of preclinical research into the relationship between sex-hormones and neurotrophin activity has focussed on the estrogens [455]. The most biochemically active estrogen, estradiol, is produced in the ovaries but is also synthesized by neurons and astrocytes, where it is known to modulate addiction and psychosis neurocircuitry including DA and glutamate neurotransmitter systems [456]. Other

than associated reproductive functions, estradiol is considered a neuroprotective hormone that has antioxidant and anti-apoptotic effects [457, 458], and can regulate plasticity and synaptic density in the CNS through initiation of downstream signalling cascades [402]. It is also broadly accepted in the literature that estradiol is capable of regulating BDNF [402]. This is supported by the presence of an estrogen response element motif on the BDNF gene promotor region, suggesting direct regulation of BDNF expression by estradiol [459]. Studies on ovariectomized female rats report depleted BDNF mRNA, as measured by in situ hybridisation experiments, and increased BDNF expression following replacement of estradiol [460]. A similar effect of female sex hormone treatment on BDNF mRNA activity was reported in neonatal male rats with gonadectomy, highlighting regulation of the growth factor by estradiol during development [461].

These results are further supported by numerous previous sex-dependent observations in the BDNF HET model [402]. One study reported male BDNF HET mice in young adulthood develop an aggressive phenotype and hyperphagia in conjunction with elevated body mass [462]. In another study, selective deletion of forebrain BDNF produced hyperactivity in male mice with normal depression-related behaviours, while female mice with this conditional knock out displayed normal LMA but marked increase in depression-like symptoms [463]. The van den Buuse lab has described spatial memory deficits in male, but not female BDNF HET mice treated with corticosterone during young adulthood, which corresponded with increased expression of NMDA receptor subunit NR2B in the hippocampus [337]. In addition, hippocampal NMDA receptor expression has recently been associated with BDNF-mediated sex hormone effects. BDNF HET and WT female mice were gonadectomised and treated with estradiol and progesterone, while the corresponding gonadectomized males received either testosterone or dihydrotestosterone. Western blot analysis confirmed female-specific changes in GluN1 and GluN2 in the dorsal hippocampus that were mediated by BDNF [464], highlighting estradiol-BDNF dependent signalling in NMDA receptor subunit expression.

In previous PPI experiments, MK-801 challenge revealed sex-specific startle deficits in female mice [337], while a more recent study by Manning et al. described hypersensitivity to PPI deficits following amphetamine challenge in BDNF HET

male mice, and no differences between BDNF HET and WT female mice [304]. We have also described changes in TrkB signalling within the mouse striatum that were hypothesized to underpin sex-specific stress effects in this model [35]. Given the widespread findings of sex-dependent effects on behaviour in BDNF HET mice and rats, future experiments should seek to determine how sex hormones and BDNF signalling interact in mediating mesocorticolimbic activity.

9.6 Limitations

There are a number of limitations to experiments described in this thesis, many of which are inherent to the field of preclinical behavioural research. This section will address a number of experimental constraints that may have impacted the validity of our behavioural models and analysis of underlying changes in BDNF HET mice and rats.

9.6.1 System-wide BDNF deficiency

The BDNF HET model is useful for assessing the effects of reduced BDNF expression during DA sensitization. Marked reduction in BDNF protein expression in the BDNF HET system is particularly important in early preclinical investigations, where subtle phenotypes, that may otherwise remain uncovered, can be examined under exaggerated global BDNF deficiency. That being said, heterozygous mutants are a relatively blunt instrument for determining changes in BDNF signalling during schizophrenia. BDNF HET mice are genetically modified to possess a global reduction in protein expression, while neurotrophic deficits observed in schizophrenia are primarily localised to changes in hippocampal and cortical expression [199, 465]. Consequently, studies on psychosis described here hold relatively poor construct validity to the region-specific neurotrophic deficits observed in the pathophysiology of schizophrenia. As the mice examined in this thesis possess global reduction in BDNF expression, depletion of this neurotrophic factor may have differential effects on reward neurocircuitry and behaviour, compared to region-specific deficits in a more targeted model of development. Therefore, in future studies, a region-specific approach could be implemented (highlighted in 9.7.3) and may also accommodate for system-wide compensatory mechanisms that would otherwise dampen behavioural phenotypes.

The analysis of BDNF heterozygosity on alcohol-seeking behaviours faces similar problems with construct validity. Several studies have indicated that BDNF levels are reduced in AUD within distinct brain regions including PFC, hippocampus, amygdala, and the striatum (reviewed in [466]). Therefore, a targeted approach focussing on individual regions of BDNF depletion could provide greater insight into the networks that are implicated in addiction development.

Another way of improving construct validity could be through the examination of schizophrenia endophenotypes in BDNF Val66Met polymorphic mice, and alcohol seeking in the recently developed BDNF Val68Met rat model. The Val66Met SNP is a widely studied functional polymorphism that has been associated with a reduction in activity-dependent release of mature BDNF [467], and there is some evidence that 66Met allele load is associated with clinical features of schizophrenia [213]. The effects of METH pretreatment in this model of disrupted BDNF sorting and release are currently being investigated by the van den Buuse laboratory and may provide some insight into genetic vulnerability to psychosis-like behaviour in these rodent models and in human patients.

Regarding addiction aetiology, the evidence is mixed. In alcohol-dependent patients, higher risk and earlier occurrence of relapse has been associated with 66Val allele carriers. Contrastingly, 66Met has been linked to increased alcohol intake and altered cortisol response [468], suggesting reduced localisation of BDNF to synapses may contribute to AUD. In summary, these studies highlight the need for further investigation of the role of the polymorphism in an animal model of alcohol seeking behaviours.

9.6.2 Compensation in genetically-modified animals

Another limitation that should be considered when examining behavioural paradigms in genetically-modified animals is the potential for system compensation. As genetic alterations were present during development, changes in D3R and BDNF expression may have prompted compensatory mechanisms in mutant animals that could mask the intended neurocellular deficiencies. In this way, genetic modifications that ablate protein function can cause upregulation of related genes that act to reduce the effects of the targeted protein loss [469]. For example, previous work has highlighted the possibility of compensatory

mechanisms in the striatum of BDNF HET mice who exhibit increased NT-4 expression [35], which has been hypothesized to compensate for reduced BDNF signalling [40, 470].

Further evidence for potential compensation comes from a previous study in the van den Buuse lab in Val66Met polymorphic mice with deficient activity-dependent BDNF secretion. Met allele load is associated with reduction in activity-dependent secretion of BDNF (18% for one 66Met allele, 29% two 66Met alleles) [213]. In PPI experiments, mice with the intermediate Val66Met genotype displayed the largest stress-induced sensorimotor gating deficits, while the genotype with the greatest reduction in BDNF secretion, Met66Met, had normal PPI [212]. The lack of PPI disruption in the most genetically 'vulnerable' group was hypothesized to be a product of functional compensatory mechanisms that were activated in response to the more significant BDNF disruption, but not in response to the intermediate Val66Met genotype [32].

In further support of intrinsic compensation, another study has reported unaltered TrkB expression and phosphorylation in BDNF HET rats, despite marked reduction in BDNF levels in these animals [40]. As TrkB activity was not altered, this could suggest normal function has been maintained at the BDNF receptor due to some form of compensatory adaptation. Taken together, these findings demonstrate that reduction in global BDNF expression may induce marked neuronal re-balancing to accommodate for the loss of neurotrophic activity in the rodent brain. To test this hypothesis, immunohistochemical investigation of possible neurocellular compensation targets could be included in future studies. If changes in expression of related proteins are detected, a more targeted, or inducible BDNF deficiency model could prevent compensatory mechanisms from masking possible behavioural phenotypes.

There is also potential for compensation to occur in D3KO mice, although there is little evidence supporting this theory. In D2KO models, D3R expression has been shown to increase during later stages of postnatal development, highlighting potential compensation mechanisms balancing the lack of D2R mediated signalling [471]. However, in more recent observations, D3KO mice have demonstrated no difference in any of the DA receptor expression profiles during development [372],

suggesting no change in DA receptor neurotransmission to accommodate for system-wide D3R loss. In contrast, it has been suggested that D3KO mice display only brief hyperactivity to novel environments [380] as rapid compensation to DA hyperexcitability dampens LHA response and counters functional loss of the inhibitory D3R [472]. Thus, only the first 10 minutes of LMA is elevated in D3KO mice within a novel environment. Although these studies provide largely speculative theories on compensatory mechanisms, it is important to consider the caveat of compensation in system-wide genetic modifications versus targeted and time-dependent inducible models of disease. Future experiments should aim to reduce the likelihood of compensatory mechanisms interfering with behaviour.

9.6.3 Molecular investigation of mesocorticolimbic DA reward systems

One of the primary limitations of experiments described in this thesis is that we did not conduct any molecular interrogation of mouse and rat DA systems. Although a wealth of information can be obtained from the analysis of animal behaviour, the neural substrates and pathways governing phenotype cannot be identified without subsequent mRNA and protein analyses. Rodent behaviour is dynamic and adaptative [473, 474] and environmental factors such as time of testing, experimenter handling and interaction, order of tests, housing, and enrichment are capable of influencing result reliability and replicability [475-477]. This is evidenced by differences in psychostimulant-induced LHA and PPI disruption in the BDNF HET model between our results and the Manning et al. study [31]. In earlier experiments, data were collected from mice that were housed and tested in a different facility to studies described here, highlighting the sensitivity of behaviour to environmental stimuli. In further support, comparable mouse LHA experiments conducted by Manning et al. in two separate institutes also showed variability in results, albeit the differences were relatively minimal. Considering these caveats and the heterogeneity of behavioural data, subsequent molecular investigation of the brain can strengthen results and help to identify neuronal changes that correlate with phenotype.

Mouse brains were collected as part of this thesis, and we intended to examine neurocellular changes that may have occurred due to prolonged METH exposure and genetic modification of BDNF and D3R. This is a future direction for the project

and may also shed light on potential compensatory mechanisms that occur after neurotrophic depletion and loss of inhibitory DA receptors.

9.6.4 Assessing behaviour across multiple paradigms

The investigation of consecutive behavioural paradigms within animal models is common practise in neuroscience and permits the greatest and most ethical use of biological material. However, it is important to highlight the potential limitation of conducting behavioural batteries in mice. Following 3 weeks of chronic injections, mice in this study were tested across two behavioural tests consisting of seven separate sessions (4x PPI, 3x LMA). Therefore, it can be assumed with some confidence that mice experienced a moderate level of stress across experiments, which could have confounded behavioural outcomes based on prior testing experience. Consequently, the assessment of both endophenotypes may have impacted our results, as mouse behaviours and phenotype are sensitive to previous test parameters and conditions. In addition, due to the invasiveness of PPI, behavioural test batteries in transgenic and knockout mice typically conduct PPI after LMA experiments [478]. However, as METH is known to have sensitizing effects, the order was reversed and LHA experiments were conducted after PPI experiments to avoid long-lasting drug x drug interactions impacting sensorimotor gating results, thereby mitigating effects of prior test history [479]. PPI and LMA/LHA experiments were conducted at least 48 hours apart to reduce carryover effects from drugs administered in prior tests. Furthermore, PPI drug administration order was pseudorandomised across the 3 sessions to ensure a different sequence in each consecutive cohort of mice, which should have negated any effects of drug interaction on the data. Finally, in PPI, mice were given a pretest trial to habituate animals to the acoustic chamber and reduce the impact of stress on the testing days. Ideally, it would be optimal to test PPI and LHA in separate cohorts of test-naive mice, in parallel with mice tested over both paradigms to determine whether past experience impacts behavioural outputs. In support of our design, one previous study assessing mice in large behavioural test batteries found no difference in PPI and LMA between tested vs. naïve mice, providing evidence of resilience to the effects of test history in these paradigms [479]. Nevertheless, consecutive behavioural tests in the same animal may confound results and should be considered when interpreting these findings.

9.6.5 Experimental design improvements

In the 7,8-DHF experiment in rats, we used a 10mg/kg acute dose of drug prior to the operant session. This is double the dosage that rats received over the 10 days of subchronic injections. Ideally, a 5mg/kg dose should have been implemented on the day of operant testing. The majority of studies examining 7,8-DHF-mediated changes in behaviour test at 5mg/kg (see Chapter 8.3.5) and this dose would have been more appropriate for challenge prior to the reinstatement operant session. The incorporation of two doses of 7,8-DHF adds some complexity to the experiment and to the interpretation of the results. A higher concentration of the drug is also more likely to illicit receptor tolerance, which has the potential to inhibit increased BDNF receptor activity.

The second limitation involves METH-pretreatment protocols in the first BDNF HET study. Mice pretreated in the 100-regimen received one week of daily 1mg/kg METH injections starting at 6 weeks of age. In hindsight, it may have been more appropriate to start the 100-pretreatment at the beginning of week 8 so that regimen groups had equal “no treatment” phases between METH pretreatment and behavioural analysis. In this experiment, 100-pretreated mice had a 4-week resting phase between pretreatment and behaviour rather than the usual 2-week period. This may have promoted differences in sensitization to METH between regimens that could confound behavioural results and may explain why 100-pretreated mice displayed hypersensitization compared to 111-pretreated in some cases. On the other hand, if 100-pretreated mice were injected in the last week of the 3-week regimen, they would receive METH at a different developmental age to the 111- and 124-pretreated mice. Based on the temporal importance of adolescent DAergic network disruption, initiating pretreatment at an earlier age may be less detrimental to the comparison of pretreatment effects on sensitization than a larger period of abstinence between METH and behaviour in 100-pretreated mice. A future experiment could seek to clarify the importance of METH-pretreatment occurring over the 6-9-week adolescent period in mice.

While there is evidence supporting vulnerability to psychosis during adolescence, no direct comparisons have been made in animal models of psychosis-like behaviour or study of endophenotypes of the disease. Furthermore, based on the

individual parameters of drug use, initiating METH pretreatment protocols at different ages, and comparing the effects on behaviour and neurochemistry would improve the degree of construct validity to schizophrenia and METH-psychosis development. While adolescence is considered a particularly vulnerable period for addiction and psychosis development [480, 481], to our knowledge there has been no investigation comparing METH use in adolescence and at varying stages of adulthood. This could be a promising avenue of research considering the heterogeneity of psychosis and schizophrenia development.

Finally, we did not account for bodyweight differences between BDNF HET mice in PPI and LHA experiments. This could be considered an oversight considering some, but not all, BDNF HET mice are typically heavier than their WT counterparts. Variability in BDNF HET mice bodyweight may therefore have affected some experiment parameters such as distance moved in LHA and added a degree of restraint stress during PPI tests. Therefore, bodyweight variability should be considered a confounding variable that has some potential to disrupt genotype differences across our behavioural tests. This was evidenced by a previous LMA study categorising BDNF HET mice into heavy vs. normal bodyweight. Analysis showed significant LMA variance in BDNF HET animals and increased activity in “normal” bodyweight BDNF HET mice relative to WT and heavy BDNF HETs [334]. In future experiments, accounting for bodyweight differences between BDNF mice and WT controls may shed light on the potentially confounding effects of heavier animals skewing behavioural data. This could be achieved with some form of percentage-based classification of mice into different bodyweight categories, or analysis of adipose tissue deposits to determine overweight vs. healthy bodyweight animals.

9.7 Future Directions

9.7.1 Sex-hormone analyses

Given the array of sex-specific behaviours observed in the BDNF HET model (section 9.5), further studies are needed to determine the impact of sex hormones on psychosis and addiction. These experiments could include PPI and LHA experiments in BDNF HET mice, and operant studies in BDNF HET rats following

gonadectomy and treatment with individual hormones, such as testosterone and estradiol. Interestingly, a recent study examining the effects of testosterone and dihydrotestosterone in BDNF HET male mice found testosterone-specific changes in amphetamine-induced hyperlocomotion, where testosterone administration reduced LHA [454]. However, as testosterone can be converted to estrogen by aromatases, this result may also reflect estradiol activity as well as androgen signalling. Importantly, dihydrotestosterone-treated mice, whose androgen levels are unaffected by aromatase, did not respond differently to control LHA, suggesting androgen receptors may not be driving the phenotype.

Similar methodology could be implemented in our psychosis and addiction studies to measure the relative contribution of sex hormones to behaviour. Future studies examining androgen signalling could also include aromatase inhibitors which would block conversion of testosterone into estradiol. That being said, although manipulation of estradiol and testosterone are important future directions, differences between sexes cannot be attributed to a single protein, gene or hormone, and realistically, the differences between males and females involve numerous signalling systems and neuronal processes. Nevertheless, understanding the interaction between sex steroid hormones and BDNF during DA sensitization is a step toward uncovering the substrates governing addiction and psychoses sexual dimorphism and may one day contribute to targeted sex-specific disease interventions.

9.7.2 Antipsychotic administration

Psychosis studies presented in this thesis focussed on the acute behavioural response to psychomimetic drugs in BDNF HET and WT mice, that, in otherwise healthy subjects, can produce a psychosis similar to the positive symptoms of schizophrenia. This approach has good predictive validity when examining the behavioural effects of drugs such as METH, APO, and MK-801, which produce hyperdopaminergia (METH, APO) and hypoglutamatergic function (MK-801) which are characteristic schizophrenia pathophsyiologies. Regarding predicative validity, an approach that was not adopted in this thesis, and could be included in future studies, is the assessment of antipsychotic medications on animal behaviour. Antipsychotic treatment in METH-pretreated and BDNF HET mice could provide

additional predictive validity if behavioural differences in PPI disruption and LHA were reduced by carefully timed administration of neuroleptics. Treatment response could be similarly assessed between D3KO and D3 WT mice. Antipsychotics are generally graded by D2-like receptor blockade efficacy, and compared to WT, D3KO mice have been shown to develop differential structural changes to long-term antipsychotic treatment strategies, such as differences in striatal volume. This effect was driven by altered glial cell density, highlighting that D3R may play a role in mediating antipsychotic treatment effects on glial cells [482]. This is to be expected, given the largest D3R densities have been identified on glial cells within the brain. Although it is unclear if D3KO and D3 WT mice would differ in their response to antipsychotic treatment, previous studies have reported some differences in D3KO mice' response to typical (haloperidol) and atypical (clozapine) administration [482]. Nevertheless, these findings require replication, as authors note small sample sizes and older mouse age may have contributed to subthreshold significant findings. To address these issues, antipsychotic treatment could be incorporated into a future experiment examining psychosis-like behaviour in a sufficiently powered D3KO study, or within an inducible model of D3R deficiency, which is yet to be explored.

9.7.3 Inducible genetic modifications

Given the relatively poor *in* vivo receptor selectivity of available D3R ligands, mutant mice are the most effective model for understanding the molecular changes that occur in response to elevated DA during psychostimulant induced sensitization. The ability to control DA receptor gene expression temporally and spatially could aid in uncovering the potential role of D3R in sensitization aetiology and help to explain reductions in D3R in schizophrenia patients. As we did not observe significant differences between D3KO and D3R WT mice, psychosis endophenotypes should be investigated in an inducible D3KO model. In this system, receptor loss could be targeted to developmentally vulnerable periods which may stop system-wide compensatory mechanisms from preventing DA signalling deficits in the D3KO. The ability to switch receptor expression on and off could provide additional insight into potential D3R-mediated METH sensitization and would be a useful tool in determining whether disruption at critical stages of development contribute to greater behavioural differences compared to lifelong

D3R loss. Similar to inducible D3R loss, region-specific regulation of BDNF expression may also provide more targeted information on the circuitry implicated in neurotrophic deficiency. For example, targeting gene expression changes to brain circuitry where altered D3R expression was observed in post-mortem schizophrenia tissue, such as reduction in BDNF within the cortex and hippocampus, would provide a more sophisticated preclinical model of disease and prevent long-term compensatory mechanisms.

9.7.4 Immunohistochemical analyses

As mentioned in study limitations, immunohistochemical and molecular analyses of D3KO mice and BDNF HET mouse and rat brains should be undertaken as subsequent experiments to behavioural tests described here.

Aside from examining intrinsic compensation targets, highlighted in section 9.6.2, follow-up studies to METH behavioural experiments could also include immunohistochemical analysis of DA neuron populations between mice genotypes. Based on the excitotoxic effects of amphetamines, there is potential for DA neuron loss within reward networks of mice chronically pretreated with METH, particularly following an escalating drug regimen. Especially the higher doses of METH in the third week of chronic pretreatment may have contributed to excitotoxic effects such as oxidative stress and neuroinflammation that can cause physical damage to neurons. Repeated exposure to METH, combined with an acute 3mg/kg dose in the final LHA experiment may therefore have had significant effects on DAergic neuron populations. To determine whether significant neuronal apoptosis occurred, a future experiment could include immunohistochemical staining of VTA nuclei for TH, the rate limiting enzyme for DA synthesis, and NeuN, a neuronal nuclear antigen/marker [483]. As the majority of neurons within the VTA are either DAergic or GABAergic [484], dual-labelled cells in this region would indicate TH positive neurons, providing a measure of relative DAergic neuron density within the VTA that could be assessed across pretreatment, sex, and genotype groups.

Another molecular study that could be explored in subsequent METH psychosis projects is immunohistochemical analysis of the transcription factor, Fos. The protein, Fos, is the product of the immediate early gene, *c-fos*, which is expressed in neurons following activation by a range of stimuli [485]. More specifically,

changes in neuron activity drive second messenger signalling cascades within the cell that activate *c-fos* and drive Fos protein expression. Consequently, the detection of Fos protein can be used to map patterns of neuronal activity in response to stimulation [486]. This is a powerful technique for determining neural networks involved in METH-induced sensitization, as the expression of *c-fos* indicates brain regions that are active following administration of the drug and may also shed light on the connectivity of reward pathways mediating this response. A future project could examine Fos immunohistochemical staining in relevant mesocorticolimbic regions, such as the NAc and mPFC.

9.8 An alternative hypothesis to BDNF deficiency

Based on results from double mutant studies (Chapter 5-6), we found little evidence supporting D3R-mediated disruption of PPI and LHA in METH-sensitized mice. Few interactions were observed between BDNF and D3R genotypes on schizophrenia endophenotypes, which implies BDNF mediation of behaviour does not involve changes in D3R expression and occurs via a D3R-independent pathway. Therefore, future experiments should examine an alternative hypothesis on how depleted BDNF contributes to METH-induced psychosis and schizophrenia. Furthermore, if BDNF depletion is driving changes in sensitization and development of psychosis-like behaviours, further work is needed to identify the mechanism leading to reduced neurotrophic signalling in these systems during disease.

Other than a genetic deficiency in activity-dependent secretion of BDNF in 66Met allele carriers, one potential environmental mechanism that could decrease BDNF activity is psychostimulant-induced loss of DAergic fibres within the mesocorticolimbic DA system. Physical damage to neuronal cell populations often follows acute METH use, where perturbations such as oxidative stress, excitotoxicity, and neuroinflammation can contribute to METH-induced neurotoxicity [487]. This results in disruption of neuronal structure and function caused by neurotoxic affects, including serotonergic and DAergic nerve terminal damage, activation of microglia neuroinflammatory response, and neuronal cell apoptosis within the brain. Studies focussing on long-term exposure to METH

report degeneration of neurons, impairment of DAergic markers and DA depletion [488].

In addition, in microarray experiments, a history of METH use and pretreatment prior to acute METH challenge has identified striatal BDNF mRNA downregulation [489], disrupting signalling that would otherwise provide protection against trans-neuronal degeneration of DA neurons [490]. As neurotrophins are involved in regulating cell survival, downregulation of BDNF could further disrupt DAergic fibres within the reward network as well as other cell populations innervated by these pathways. Somewhat contrastingly, a similar experiment by the same group reported opposite effects of METH pretreatment on BDNF mRNA expression within the midbrain [491]. Real-time PCR on RNA, extracted from a region of the midbrain encompassing VTA and SN nuclei, demonstrated upregulation of BDNF mRNA in response to METH challenge in rats administered an escalating METH regimen [491]. To synthesize results, investigators concluded that METH pretreatment primed the BDNF promotor to acute METH challenge in the midbrain causing enhanced BDNF expression, whereas in the striatum, increased BDNF protein resulted in compensatory downregulation of mRNA [489]. These dynamic changes in expression may partially explain why differences in BDNF activity are not consistently described in midbrain and striatal networks in psychostimulant-induced psychosis and schizophrenia pathologies.

On the other hand, reduced BDNF expression within the cortex and hippocampus is more consistently reported in schizophrenia post-mortem tissue [199, 465], and these region-specific BDNF deficits may be explained by changes in DA afferents within the mesocorticolimbic network. Previous literature has demonstrated anterograde transport of BDNF along mesocorticolimbic DA circuity, where BDNF expression correlated with firing rates of VTA DA neurons within the midbrain [115, 492, 493]. Consequently, a METH-induced reduction in midbrain DA neuron population could, in theory, reduce BDNF signalling and expression in innervated areas along the mesocorticolimbic projection, such as the hippocampus and PFC. This is one potential mechanism explaining hippocampal and cortical BDNF depletion in METH-induced psychosis.

What remains unclear is whether a reduction in BDNF in these regions is responsible for aspects of symptomatology and a causal factor of disease or is merely a by-product of early DA neuron loss. Interestingly, there is some evidence for cortical BDNF involvement in mediating subcortical DAergic hyperactivity. The PFC is primarily comprised of excitatory glutamatergic neurons and inhibitory GABAergic interneurons that transmit signals through inotropic and metabotropic GABA receptors to inhibit neuronal activity [494]. This is achieved through the activation of ionotropic $GABA_A$ receptors, which permit chloride entry into the neuron, and metabotropic $GABA_B$ receptors, which reduce the frequency of action potentials via stimulation of potassium channels [495]. This is relevant to subcortical DA signalling as the NAc receives descending GABAergic and glutamatergic inputs from the PFC [496], and GABA neurons are relayed from the NAc to the VTA. Given BDNF-TrkB signalling has been shown to control GABA synapse formation and neurotransmission [497, 498], it could be hypothesized that a reduction in neurotrophic modulation in the PFC could reduce GABAergic function. Provided glutamatergic / GABAergic neurons innervating the reward system were dysregulated, basal inhibition of DA neuron activity may be reduced, leading to subcortical DA hyperactivity. Somewhat in line with this hypothesis, dysfunction of $GABA_A$ receptors has been associated with a range of psychiatric conditions, including schizophrenia [499, 500]. Post-mortem analysis of schizophrenia patient tissue has uncovered a reduction in glutamate decarboxylase (GAD67), the enzyme that catalyses decarboxylation of glutamate to GABA and determines synthesis of the neurotransmitter within the cortex (reviewed in [501]). Furthermore, in situ hybridisation studies in schizophrenia patients have demonstrated that dysfunction of inhibitory GABA neurons within the PFC is correlated with reduced BDNF-TrkB expression [29], suggesting reduction in TrkB activation may underlie these disruptions. This is also evident in studies examining hippocampal function, where DAergic dysfunction is hypothesized to occur following a reduction in BDNF in the hippocampus of patients with schizophrenia [502]. It is also postulated that reduced BDNF may attenuate inhibitory GABAergic interneuron activity leading to hippocampal excitability, which consequently drives hyperdopaminergia [503]. In summary, reduced BDNF expression and activity in the cortex and hippocampus could translate to significant neuronal dysfunction across multiple neurotransmitter systems, which may

underlie some schizophrenia pathophysiology, including positive symptoms and prefrontal cognitive deficits. Based on these previous findings in schizophrenia patients and the lack of D3R results observed in this thesis, future experiments should seek to clarify changes in DAergic activity, as measured by TH immunohistochemistry or microdialysis, that may be driven by altered GABAergic neurotransmission in BDNF HET mice, rather than changes in DA receptor expression. Furthermore, localisation of BDNF deficiency to cortical regions would provide greater construct validity to this model, provided disruption to endophenotypes is still evident.

A BDNF-GABA hypothesis is also in line with our observed sex-dependent BDNF effects, as some GABAergic interneurons also express estrogen receptors. Estradiol has been shown to regulate the development and maintenance of specific subsets of GABAergic hippocampal and cortical interneurons, defined by their expression of the calcium-binding protein, parvalbumin (PV) [504, 505]. PV interneurons are also modulated by BDNF, demonstrating an overlap in regulation between sex hormones and BDNF that may further contribute to sex-dependent effects in the BDNF HET. Together, these findings highlight GABA as a potential regulator of psychosis-like behaviours. Furthermore, if PV interneurons are sensitive to BDNF and estradiol, an experiment targeting this interaction in a repeat study could provide evidence for BDNF-mediated modulation of the GABA system and may explain how a reduction in BDNF expression can indirectly produce subcortical DA hyperactivity. Alternatively, brain tissue collected as part of this study could be analysed for changes in GABAergic interneuron populations in combination with DAergic neuron quantification. Immunohistochemical staining of the cortex and hippocampus could reveal differences in cell density across experimental groups and provide evidence of BDNF-GABA mediated changes in sensitization (not withstanding compensatory mechanisms).

Considering the double mutant study results, polymorphic or targeted and inducible models of BDNF deficiency comparing GABAergic and DAergic function are a logical progression for determining the role of BDNF in psychosis. These experiments would provide greater construct validity for schizophrenia pathophysiology and enable the relationship between BDNF and GABA signalling

to be investigated. As GABA signalling is also heavily implicated in AUD, these models may also be relevant to changes in mesocorticolimbic DA during addiction.

9.9 Final conclusion

In conclusion, studies described in this thesis have investigated the role of BDNF deficiency in mediating psychosis-like behaviour and addiction. In psychosis chapters, BDNF HET mice were tested for genotype specific schizophrenia endophenotypes, including disrupted PPI and LHA. These studies expanded on previous work in the “two-hit” model by comparing an escalating METH regimen to lower and less frequent doses of the psychostimulant, and by testing the relationship between BDNF and the D3R in the BDNF HET/ D3KO double mutant. Combined, “low-dose” METH experiments demonstrated that an escalating pretreatment regimen may not be required for psychostimulant-induced sensitization. Given the heterogeneity of METH use in humans, this result bears clinical significance to users with limited drug exposure, who may be at equal risk of developing neuronal adaptations leading to psychosis pathophysiology. Collective analysis of baseline and psychostimulant-induced PPI found sex-specific dysregulation of sensorimotor gating in male BDNF HET mice, while LHA experiments demonstrated hypersensitivity to METH-sensitization in female BDNF HET mice. These results add to a growing body of literature supporting the two-hit hypothesis of schizophrenia where a combination of risk factors determines disease onset.

Findings also emphasize the importance of examining the interaction between BDNF and the sex hormones in future research, to determine the influence of these signalling molecules on psychosis and addiction aetiology. Importantly, no interaction or significant contribution to behaviour was identified in D3KO (D3KO and double mutant) vs. D3R WT (WT and BDNF HET) mice, suggesting BDNF heterozygosity is determining behaviour via a D3R-independent pathway, as opposed to reduced control of D3R expression. Future studies could therefore investigate the putative relationship between BDNF and the GABAergic system and how these inhibitory neuronal populations contribute to psychosis and the more broadly defined schizophrenia symptomatology.

Addiction chapters focussed on BDNF deficiency and the effect of TrkB agonism in rats, using an alcohol self-administration operant paradigm. In both investigations, female reinstatement was more sensitive to the effects of BDNF system manipulation, with heightened relapse in female BDNF HET and 7,8-DHF-treated rats. Once again, these results demonstrate a relationship between the sex hormones and BDNF that should be investigated in future preclinical experiments to determine how estradiol and testosterone interact with neurotrophic signalling systems in adolescence and young adulthood. These findings also underline the importance of conducting preclinical research in both male and female animals and builds on previous literature implicating disrupted BDNF expression and activity in AUD and addiction pathophysiology.

Furthermore, results obtained in this thesis support shared neurocircuitry governing addiction and psychosis. This is supported by evidence suggesting genetic predisposition to schizophrenia overlaps with heightened drug addiction susceptibility. This has been demonstrated in Neuregulin 1 (Nrg1) HET mice who display enhanced sensitivity to the acute effects of tetrahydrocannabinol (THC) on various schizophrenia-related behaviours including LHA and PPI [506]. Nrg1 is a growth factor important for LTP and formation of glutamatergic synapses [507]. It was concluded that variation in the Nrg1 schizophrenia-susceptibility gene alters the behavioural effects of cannabinoids. In addition, a genetic study in humans identified a SNP on the schizophrenia susceptibility gene, NRG1, which showed genetic linkage to cannabis dependence [508], highlighting potential shared neurocircuitry governing drug addiction and psychosis.

Overlapping vulnerability can also occur through mGlu5 receptor dysfunction, which has been associated with schizophrenia pathophysiology and alcohol-related behavioural pathologies [509-511]. mGlur5 is a postsynaptic metabotropic glutamate receptor highly expressed in the mesocorticolimbic system, within the NAc, striatum, and hippocampus [512]. Mice lacking the mGlu5 receptor display schizophrenia-like behaviours such as disrupted PPI [513] and hyperlocomotion [514], take longer to extinguish METH self-administration, and have heightened reinstatement [515]. These potential genetic links between mental health and drug addiction largely impact glutamatergic and dopaminergic signalling along the

mesocorticolimbic reward network, emphasizing the importance of further studies targeted toward these signalling systems.

In conclusion, results obtained from this thesis support previous findings associating BDNF dysregulation with addiction and psychosis development and highlight sensitization of the mesocorticolimbic DA system as a central pathway governing behavioural maladaptation to disease. These studies also provide evidence for dysregulated BDNF signalling as a risk factor for the development of the positive symptoms of schizophrenia and enhanced vulnerability to alcohol seeking and relapse. Nevertheless, our findings are preliminary and further investigation is required to elucidate the underlying neurocircuitry impacted by BDNF deficiency. Consequently, preclinical research focussing on sex- and region-specific BDNF activity, combined with molecular analyses of the brain, are logical future directions for developing a better understanding of the role of BDNF in psychotic illnesses and addiction.

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Appendix

Table 1 Average Low-dose PPI at every PP Intensity measured over saline, APO, and MK-801 challenge sessions

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
MALE WT SAL										
SAL	2.7	17.2	30.3	59.2	**27.4**	14.2	6.2	29.3	57.4	**26.8**
	±6.7	±5.0	±4.5	±4.3	**±3.9**	±4.2	±4.4	±3.8	±4.5	**±2.5**
APO	-19.9	-12.0	16.9	25.3	**2.6**	-14.9	-9.4	20.6	50.7	**11.8**
	±6.1	±7.9	±9.1	±12.3	**±8.1**	±8.5	±7.0	±7.8	±5.9	**±5.3**
MK-801	-15.3	0.3	14.8	33.5	**8.3**	-0.7	1.7	8.7	22.5	**8.0**
	±9.4	±7.3	±7.1	±6.7	**±6.0**	±7.6	±4.6	±5.1	±6.2	**±4.1**
MALE WT 100										
SAL	6.6	22.7	30.9	51.4	**27.9**	12.7	16.0	29.1	49.1	**26.7**
	±4.9	±4.6	±5.4	±5.3	**±3.7**	±3.1	±2.8	±5.3	±3.6	**±2.7**
APO	-13.7	4.8	20.6	40.8	**13.1**	-4.9	2.6	22.8	48.4	**17.2**
	±4.3	±7.1	±5.3	±10.7	**±5.4**	±5.6	±4.8	±4.1	±5.0	**±3.0**
MK-801	-5.0	4.9	20.5	43.7	**16.0**	4.8	-7.7	18.0	37.6	**13.2**
	±6.8	±4.5	±6.1	±5.9	**±3.9**	±5.1	±6.8	±4.4	±6.3	**±4.0**
MALE WT 111										
SAL	-23.8	3.6	25.9	54.1	**15.0**	-0.8	6.6	25.9	50.8	**20.6**
	±7.4	±7.0	±3.1	±5.4	**±2.3**	±7.4	±2.4	±6.5	±4.2	**±2.4**
APO	-30.3	-24.5	12.5	34.2	**-2.0**	-14.2	-5.6	19.8	53.8	**13.5**
	±13.1	±25.5	±5.7	±4.7	**±9.4**	±3.2	±4.8	±3.5	±4.0	**±1.7**
MK-801	-1.0	5.6	13.6	30.7	**12.2**	5.1	0.9	10.0	23.1	**9.8**
	±13.0	±5.0	±6.4	±5.6	**±2.9**	±4.0	±3.4	±4.4	±6.3	**±3.9**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
MALE WT 124										
SAL	4.9	25.5	37.7	58.8	**31.7**	12.9	25.0	41.2	57.1	**34.0**
	±9.1	±5.5	±6.8	±5.4	**±4.7**	±3.2	±6.3	±5.9	±5.3	**±4.6**
APO	-0.6	4.9	14.6	44.8	**15.9**	5.7	-7.3	36.7	56.2	**22.8**
	±9.0	±7.1	±10.0	±7.5	**±7.1**	±3.9	±5.6	±8.2	±6.8	**±5.4**
MK-801	-3.1	10.9	18.9	40.0	**16.7**	12.1	2.8	10.8	36.8	**15.6**
	±6.2	±10.3	±5.8	±5.4	**±5.9**	±5.8	±7.1	±5.5	±6.2	**±4.4**
MALE HET SAL										
SAL	1.9	17.6	30.0	48.5	**24.5**	10.9	14.2	35.1	48.1	**27.1**
	±3.9	±5.5	±4.4	±4.7	**±4.0**	±4.5	±4.0	±4.4	±4.3	**±3.3**
APO	-6.0	5.2	7.2	23.6	**7.5**	2.2	-0.1	14.7	41.7	**14.6**
	±6.7	±4.8	±7.0	±5.4	**±3.4**	±4.7	±5.9	±3.0	±4.4	**±2.6**
MK-801	-22.7	1.0	13.9	26.9	**4.8**	10.7	4.8	8.5	23.4	**11.8**
	±19.0	±4.5	±3.8	±11.5	**±8.7**	±4.7	±5.1	±4.0	±11.1	**±3.3**
MALE HET 100										
SAL	2.6	20.4	30.6	51.0	**26.2**	1.3	13.2	28.7	43.6	**21.7**
	±7.0	±5.7	±6.4	±7.6	**±4.6**	±3.3	±3.9	±5.7	±5.3	**±4.0**
APO	-6.0	0.1	19.7	34.1	**12.0**	-0.8	-5.2	18.0	41.5	**13.4**
	±4.8	±10.0	±8.0	±8.0	**±5.7**	±2.9	±9.5	±5.3	±7.8	**±4.9**
MK-801	-12.5	2.0	20.9	35.9	**11.6**	-7.4	-5.5	4.7	22.8	**3.7**
	±6.7	±5.1	±5.8	±5.1	**±4.6**	±6.8	±9.9	±4.9	±5.4	**±5.6**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
MALE HET 111										
SAL	-6.7	15.3	30.3	52.0	**22.7**	10.6	18.2	31.3	46.4	**26.7**
	±6.1	±7.7	±4.9	±5.3	**±4.7**	±3.9	±3.0	±5.1	±4.7	**±3.1**
APO	-19.5	1.8	22.6	42.5	**11.8**	-5.4	3.0	19.9	49.3	**16.7**
	±6.7	±4.4	±6.2	±6.4	**±4.1**	±5.5	±4.9	±3.9	±4.8	**±2.5**
MK-801	-20.9	-11.9	4.3	33.6	**1.3**	-8.5	-15.3	-6.1	23.8	**-1.5**
	±9.8	±7.0	±6.5	±6.2	**±5.6**	±9.8	±7.6	±7.9	±5.3	**±6.6**
MALE HET 124										
SAL	0.4	26.7	30.2	49.1	**26.6**	14.2	16.0	28.1	47.6	**26.5**
	±3.1	±4.8	±3.9	±5.7	**±2.8**	±4.8	±3.7	±4.2	±5.4	**±4.0**
APO	-11.5	0.6	12.8	33.1	**8.7**	-1.6	6.6	21.8	43.5	**17.6**
	±4.9	±6.7	±6.1	±5.6	**±4.1**	±7.5	±2.9	±5.6	±5.3	**±4.2**
MK-801	-15.9	-2.9	5.3	31.5	**4.5**	-5.4	-5.8	4.7	16.0	**2.4**
	±7.3	±4.2	±7.1	±5.8	**±4.0**	±5.2	±6.7	±4.3	±4.6	**±3.8**
FEMALE WT SAL										
SAL	12.3	27.3	49.7	73.4	**40.7**	19.1	19.0	44.2	66.2	**37.1**
	±5.2	±5.2	±4.1	±3.6	**±3.1**	±6.3	±5.3	±6.4	±4.5	**±4.6**
APO	-8.1	-4.5	-2.6	53.8	**9.6**	-1.9	5.1	25.4	55.7	**21.1**
	±8.1	±10.4	±22.5	±7.1	**±9.9**	±10.4	±6.8	±6.4	±6.6	**±6.7**
MK-801	-5.6	15.0	24.9	55.8	**22.5**	-6.4	-8.3	16.2	40.1	**10.4**
	±9.0	±5.7	±6.7	±3.3	**±4.2**	±9.5	±4.8	±5.9	±3.5	**±4.0**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
FEMALE WT 100										
SAL	-4.4	16.7	16.5	39.8	**17.2**	12.0	9.3	22.6	42.6	**21.6**
	±4.9	±6.6	±6.4	±2.7	**±4.3**	±7.1	±3.7	±4.7	±2.4	**±3.0**
APO	-4.0	4.1	14.2	32.4	**11.6**	3.1	-5.3	12.2	43.5	**13.4**
	±5.2	±3.8	±6.4	±5.9	**±3.5**	±3.1	±7.0	±4.4	±4.6	**±3.1**
MK-801	3.2	4.5	12.3	32.3	**13.1**	-10.5	-5.9	6.5	20.7	**2.7**
	±4.5	±10.8	±8.9	±6.7	**±6.5**	±8.0	±6.3	±7.0	±8.5	**±5.9**
FEMALE WT 111										
SAL	-11.9	16.2	22.3	48.6	**18.8**	-4.0	12.2	25.1	47.8	**20.3**
	±8.4	±5.5	±5.3	±3.3	**±3.7**	±5.0	±5.6	±6.3	±4.0	**±3.5**
APO	-18.4	-8.2	9.0	29.5	**3.0**	-1.4	-4.3	19.8	37.9	**13.0**
	±5.6	±9.2	±6.5	±5.7	**±3.8**	±7.0	±7.0	±6.5	±5.4	**±4.5**
MK-801	7.2	1.9	16.6	39.6	**16.3**	9.2	1.3	22.2	35.4	**17.0**
	±5.0	±8.1	±9.4	±7.3	**±6.2**	±6.6	±7.0	±6.6	±9.6	**±5.7**
FEMALE WT 124										
SAL	19.1	28.3	40.8	56.2	**36.1**	10.0	22.2	30.2	53.6	**29.0**
	±6.2	±7.0	±6.0	±7.0	**±5.9**	±5.0	±5.4	±7.8	±6.9	**±4.4**
APO	-18.4	-10.4	14.3	27.3	**3.2**	-6.0	-19.4	15.2	45.7	**8.9**
	±8.2	±11.2	±7.8	±8.2	**±6.0**	±5.7	±14.1	±6.2	±3.8	**±5.1**
MK-801	2.4	7.8	10.3	36.1	**14.1**	0.8	6.6	12.6	30.4	**12.6**
	±10.4	±12.0	±9.0	±7.1	**±6.4**	±6.1	±10.3	±6.6	±6.7	**±5.8**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
FEMALE HET SAL										
SAL	-3.4 ±5.4	26.8 ±4.5	38.6 ±4.2	51.9 ±5.8	**28.5 ±2.7**	16.3 ±4.9	23.4 ±3.8	35.8 ±4.4	54.2 ±5.5	**32.4 ±3.8**
APO	-14.3 ±14.3	-16.9 ±10.1	-8.5 ±9.7	10.6 ±10.0	**-7.3 ±9.3**	-20.0 ±11.0	-17.7 ±9.6	9.4 ±6.0	25.1 ±5.6	**-0.8 ±7.1**
MK-801	9.1 ±6.5	8.7 ±5.3	10.4 ±5.6	37.2 ±5.3	**16.3 ±3.9**	7.9 ±7.8	-3.2 ±8.5	17.7 ±6.1	35.0 ±4.0	**14.4 ±4.9**
FEMALE HET 100										
SAL	-11.9 ±3.6	19.1 ±6.0	33.4 ±4.5	50.2 ±4.8	**22.7 ±3.2**	9.8 ±7.5	17.7 ±2.7	27.7 ±3.6	43.8 ±4.3	**24.7 ±3.1**
APO	-10.8 ±10.1	-9.2 ±10.3	12.0 ±11.0	30.3 ±7.1	**5.5 ±7.1**	2.1 ±6.6	-4.1 ±12.0	12.6 ±7.3	31.2 ±6.1	**10.4 ±6.5**
MK-801	-6.5 ±4.9	-14.0 ±13.6	20.2 ±6.8	41.0 ±6.3	**10.2 ±5.2**	-10.4 ±5.9	0.5 ±6.3	16.5 ±6.9	32.0 ±6.2	**9.6 ±4.3**
FEMALE HET 111										
SAL	0.8 ±6.2	22.1 ±6.4	43.0 ±4.8	57.3 ±5.7	**30.8 ±4.6**	11.6 ±5.5	25.6 ±3.8	39.2 ±5.4	55.2 ±5.4	**32.9 ±3.7**
APO	-7.6 ±6.0	-4.1 ±8.7	13.0 ±6.1	32.8 ±7.3	**8.5 ±4.5**	-1.1 ±4.7	-3.2 ±7.9	13.9 ±6.4	50.2 ±3.1	**14.9 ±3.7**
MK-801	3.9 ±3.7	7.2 ±8.9	23.8 ±6.4	52.2 ±3.9	**21.8 ±4.0**	3.5 ±6.5	-0.9 ±10.4	22.7 ±6.9	42.8 ±5.1	**17.0 ±5.0**

FEMALE HET 124										
SAL	0.9 ±8.3	27.8 ±3.7	35.0 ±5.9	55.3 ±5.0	**29.8 ±4.2**	9.7 ±5.8	25.7 ±5.3	31.3 ±6.7	49.8 ±5.5	**29.1 ±4.8**
APO	-27.7 ±10.3	-14.0 ±9.0	1.1 ±8.5	28.1 ±11.3	**-3.1 ±6.7**	-16.6 ±9.7	-5.1 ±9.6	10.4 ±4.1	37.6 ±6.3	**6.6 ±6.1**
MK-801	16.0 ±6.9	-1.9 ±12.0	15.7 ±10.2	42.0 ±6.8	**18.0 ±7.1**	-0.7 ±8.8	7.9 ±5.3	12.2 ±3.7	33.5 ±7.8	**13.2 ±4.8**

Table 2 BDNF HET/D3KO average PPI at every PP Intensity measured over saline, APO, and MK-801 challenge sessions

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
MALE WT SAL										
SAL	7.3	18.4	41.8	59.4	**31.7**	6.8	21.9	41.2	58.4	**32.1**
	±7.6	±4.4	±5.5	±4.1	±**3.1**	±13.5	±5.9	±3.2	±4.2	±**3.8**
APO	-13.4	-17.4	5.2	28.1	**0.6**	-17.2	3.9	19.7	42.9	**12.3**
	±10.0	±6.7	±8.3	±11.4	±**5.7**	±9.2	±7.1	±8.5	±7.6	±**4.1**
MK-801	-2.1	-7.3	11.0	40.2	**10.4**	2.7	-6.1	11.7	28.4	**9.2**
	±4.8	±5.6	±6.0	±4.5	±**2.8**	±4.7	±3.8	±5.9	±5.4	±**2.6**
MALE WT METH										
SAL	11.2	17.8	38.5	55.4	**30.7**	6.4	24.8	42.2	54.8	**32.0**
	±7.6	±4.0	±4.9	±6.5	±**3.3**	±5.6	±7.3	±4.1	±2.6	±**3.0**
APO	-11.9	-9.1	0.8	31.9	**3.0**	-22.3	4.3	23.9	48.4	**13.6**
	±6.6	±14.4	±7.5	±9.9	±**7.8**	±11.6	±7.8	±6.0	±6.4	±**6.2**
MK-801	10.2	16.9	21.4	39.4	**22.0**	3.3	4.0	8.4	31.1	**11.7**
	±9.6	±5.7	±7.5	±5.6	±**5.3**	±9.4	±6.6	±7.9	±6.7	±**5.9**
MALE D3KO SAL										
SAL	-0.6	27.4	37.6	66.1	**32.6**	9.5	15.6	34.4	52.4	**28.0**
	±12.7	±10.7	±13.7	±6.7	±**8.9**	±8.1	±8.1	±10.0	±7.8	±**6.8**
APO	-17.9	-17.0	8.4	52.8	**6.6**	0.8	-3.3	38.1	58.5	**23.5**
	±9.0	±13.8	±18.0	±8.1	±**8.9**	±6.0	±8.1	±7.1	±4.6	±**4.1**
MK-801	-4.9	7.9	24.5	46.1	**18.4**	-0.7	1.0	1.1	20.3	**5.4**
	±4.8	±10.0	±5.8	±7.9	±**4.7**	±10.7	±5.5	±13.5	±11.9	±**7.7**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
MALE D3KO METH										
SAL	-12.3	19.6	44.2	77.5	**32.3**	18.7	38.2	38.6	71.4	**41.7**
	±9.5	±13.6	±5.5	±2.8	±**6.2**	±6.5	±5.4	±7.0	±3.0	±**3.7**
APO	-32.7	3.9	18.7	52.8	**10.7**	-17.2	-3.3	28.9	55.1	**15.9**
	±7.9	±10.8	±15.0	±11.6	±**9.8**	±7.9	±9.8	±7.9	±7.5	±**6.5**
MK-801	-7.7	-7.0	16.6	45.4	**11.8**	-3.8	3.0	8.0	38.4	**11.4**
	±12.3	±8.9	±7.3	±6.2	±**6.5**	±5.3	±4.7	±7.8	±6.4	±**3.4**
MALE BDNF HET SAL										
SAL	2.6	11.9	29.9	49.4	**23.4**	-2.8	12.2	31.8	53.3	**23.6**
	±6.9	±5.0	±5.5	±5.1	±**4.3**	±5.4	±4.5	±4.3	±4.6	±**2.5**
APO	-8.3	-5.9	-4.2	20.2	**0.4**	-8.3	-4.3	17.7	45.9	**12.8**
	±8.4	±5.3	±5.5	±5.4	±**3.9**	±8.8	±6.3	±3.8	±5.1	±**4.9**
MK-801	-1.7	-12.0	-1.1	32.8	**4.5**	-4.7	-1.2	1.1	24.9	**5.0**
	±3.6	±6.1	±9.2	±5.6	±**3.9**	±4.6	±4.2	±3.2	±5.5	±**3.2**
MALE BDNF HET METH										
SAL	3.9	16.4	20.1	47.6	**22.0**	4.0	20.7	34.3	50.8	**27.4**
	±7.8	±6.3	±6.1	±4.8	±**4.7**	±5.8	±4.4	±2.9	±4.1	±**3.1**
APO	-24.2	-9.8	6.6	30.6	**0.8**	1.1	-2.7	18.7	50.7	**16.9**
	±7.8	±6.4	±7.6	±7.2	±**5.4**	±5.0	±5.2	±5.3	±3.7	±**3.1**
MK-801	7.2	15.0	26.3	49.2	**24.4**	5.4	9.3	23.5	34.7	**18.2**
	±5.6	±4.3	±3.1	±3.3	±**2.2**	±4.1	±5.3	±4.4	±2.4	±**2.6**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
MALE DM SAL										
SAL	0.1	23.3	42.0	54.8	**30.1**	15.3	24.7	39.5	53.8	**33.3**
	±6.8	±5.7	±4.4	±2.7	±**3.7**	±3.4	±3.9	±2.8	±4.1	±**2.4**
APO	-9.5	-6.4	18.1	33.9	**9.0**	3.0	0.8	22.1	58.1	**21.0**
	±6.8	±6.9	±5.2	±7.6	±**5.4**	±3.8	±4.8	±8.8	±6.0	±**4.1**
MK-801	-5.7	-10.5	9.0	45.2	**9.5**	2.8	3.2	16.8	27.9	**12.7**
	±5.4	±8.2	±7.3	±5.6	±**5.4**	±5.1	±7.8	±7.6	±6.1	±**5.3**
MALE DM METH										
SAL	-4.3	19.7	30.0	51.5	**24.2**	11.1	9.2	28.4	46.9	**23.9**
	±8.1	±4.7	±4.3	±6.2	±**4.5**	±6.5	±6.2	±5.2	±4.6	±**4.3**
APO	-3.7	-5.5	14.5	40.0	**11.3**	-2.0	5.0	34.5	51.5	**22.3**
	±6.2	±10.4	±9.7	±7.9	±**7.9**	±5.1	±5.8	±5.5	±5.1	±**3.6**
MK-801	0.0	3.1	22.3	42.9	**17.1**	12.2	13.4	10.4	36.9	**18.2**
	±5.7	±9.0	±7.7	±5.8	±**5.3**	±4.5	±4.4	±9.5	±4.3	±**4.6**
FEMALE WT SAL										
SAL	10.0	26.5	42.0	64.8	**35.8**	15.3	25.3	33.1	56.0	**32.4**
	±3.5	±5.7	±5.3	±4.0	±**2.8**	±4.6	±4.7	±4.6	±3.7	±**3.3**
APO	-16.1	-5.6	3.4	34.8	**4.1**	-4.6	-1.0	31.1	50.9	**19.1**
	±8.3	±11.6	±6.6	±6.4	±**5.3**	±7.9	±7.2	±4.7	±5.0	±**4.3**
MK-801	-9.9	-6.3	14.7	37.4	**9.0**	-9.7	-1.6	8.8	24.2	**5.4**
	±8.7	±6.2	±5.8	±3.8	±**3.4**	±5.9	±5.9	±3.1	±7.1	±**2.5**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
FEMALE WT METH										
SAL	-3.3 ±8.8	24.0 ±4.6	36.4 ±5.9	61.0 ±3.1	**29.5 ±3.1**	10.6 ±6.5	20.2 ±3.5	34.7 ±3.5	54.3 ±3.0	**29.9 ±2.7**
APO	-20.0 ±10.8	-2.1 ±10.1	11.9 ±7.1	35.8 ±5.6	**6.4 ±5.3**	-7.6 ±8.9	11.0 ±4.6	18.8 ±5.6	49.0 ±5.3	**17.8 ±3.6**
MK-801	0.3 ±7.5	2.2 ±5.6	19.9 ±4.8	34.8 ±5.6	**14.3 ±4.0**	0.0 ±5.8	7.2 ±6.7	7.0 ±5.9	23.7 ±7.2	**9.5 ±4.5**
FEMALE D3KO SAL										
SAL	5.6 ±5.2	18.5 ±6.7	35.4 ±3.9	67.5 ±3.1	**31.8 ±3.8**	3.8 ±5.0	27.0 ±7.0	43.7 ±5.6	61.9 ±3.2	**34.1 ±3.7**
APO	-22.2 ±12.5	6.6 ±7.0	13.5 ±6.2	40.0 ±8.1	**9.5 ±6.9**	-13.5 ±7.1	4.5 ±7.1	15.0 ±8.8	50.4 ±4.9	**14.1 ±4.4**
MK-801	-20.6 ±20.2	-8.3 ±14.9	6.8 ±13.0	24.4 ±8.6	**0.6 ±13.3**	-6.5 ±10.6	-3.2 ±8.3	2.7 ±11.3	19.3 ±7.4	**3.1 ±6.9**
FEMALE D3KO METH										
SAL	15.2 ±5.7	26.0 ±7.2	40.5 ±6.1	72.1 ±2.4	**38.4 ±3.5**	15.3 ±5.9	19.6 ±4.2	49.1 ±6.7	61.6 ±5.6	**36.4 ±3.4**
APO	-24.8 ±19.9	-9.4 ±8.4	27.6 ±8.4	49.1 ±9.0	**10.6 ±6.8**	-1.2 ±11.9	11.3 ±11.3	28.5 ±4.4	55.7 ±5.0	**23.6 ±5.2**
MK-801	-4.3 ±18.0	14.4 ±11.5	23.2 ±9.0	39.1 ±11.6	**18.1 ±11.1**	14.5 ±9.5	4.6 ±6.6	18.8 ±10.0	33.5 ±10.9	**17.8 ±7.3**

	30ms ISI					100ms ISI				
	PP2	PP4	PP8	PP16	**Avg.**	PP2	PP4	PP8	PP16	**Avg.**
FEMALE BDNF HET SAL										
SAL	5.6 ±4.8	30.4 ±5.0	33.5 ±3.3	54.9 ±4.3	**31.1 ±2.2**	12.3 ±4.6	15.1 ±3.6	37.5 ±3.6	55.3 ±3.9	**30.1 ±2.6**
APO	-17.8 ±5.9	-5.5 ±8.5	6.8 ±4.8	9.9 ±13.3	**-1.7 ±5.7**	6.4 ±4.3	-6.1 ±5.5	14.5 ±4.4	46.4 ±4.2	**15.3 ±3.0**
MK-801	-5.3 ±6.8	6.0 ±6.2	14.5 ±7.1	39.2 ±4.3	**13.6 ±4.8**	-13.6 ±9.6	-13.6 ±9.1	10.0 ±6.4	28.8 ±4.3	**2.9 ±5.8**
FEMALE BDNF HET METH										
SAL	9.0 ±8.3	17.9 ±8.0	31.4 ±7.5	57.1 ±6.4	**28.9 ±6.4**	6.6 ±5.3	25.8 ±5.6	38.9 ±5.5	60.1 ±4.1	**32.8 ±4.0**
APO	-18.4 ±15.3	-14.7 ±11.6	8.5 ±10.6	22.9 ±13.3	**-0.4 ±10.8**	-3.6 ±9.8	-5.3 ±8.7	17.3 ±8.6	41.4 ±8.3	**12.4 ±7.0**
MK-801	-6.5 ±14.0	-1.4 ±8.8	14.0 ±9.6	40.4 ±7.3	**11.6 ±7.7**	0.0 ±6.7	6.8 ±10.3	22.4 ±6.3	37.2 ±7.5	**16.6 ±5.5**
FEMALE DM SAL										
SAL	-1.3 ±6.1	8.8 ±5.9	35.5 ±4.4	46.9 ±3.0	**22.5 ±3.6**	3.4 ±3.5	13.4 ±3.0	33.2 ±2.3	48.6 ±3.6	**24.6 ±1.7**
APO	-21.1 ±8.7	-17.0 ±9.0	7.3 ±10.7	29.8 ±5.7	**-0.2 ±6.6**	-1.4 ±6.6	2.3 ±5.5	16.4 ±6.6	48.0 ±3.6	**16.3 ±3.8**
MK-801	-1.9 ±5.6	-2.0 ±5.6	18.2 ±5.8	38.6 ±5.1	**13.2 ±3.9**	2.1 ±4.0	-5.1 ±6.0	7.1 ±5.7	28.2 ±4.4	**8.1 ±3.9**

FEMALE DM METH										
SAL	6.7 ±4.5	32.1 ±5.0	33.5 ±4.6	56.0 ±4.2	**32.1** ±**2.9**	11.8 ±4.6	25.1 ±5.1	36.3 ±3.8	59.8 ±2.4	**33.2** ±**2.2**
APO	-12.2 ±7.0	-8.6 ±7.5	13.4 ±6.6	22.7 ±8.6	**3.8** ±**5.8**	-4.1 ±4.8	-1.6 ±7.2	19.3 ±6.8	42.2 ±3.0	**14.0** ±**3.2**
MK-801	-1.1 ±6.2	-7.3 ±8.8	14.9 ±7.2	38.8 ±7.5	**11.3** ±**6.3**	5.2 ±7.4	-2.5 ±6.9	15.1 ±4.3	24.9 ±6.6	**10.7** ±**3.6**