



MONASH University

**Investigating the interaction between BDNF-
TrkB signaling, inhibitory interneurons and
cognition with relevance to schizophrenia**

Adrienne Mary Grech

Bachelor of Behavioural Neuroscience (Honours)

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Abstract

Cognitive symptoms of schizophrenia are a key symptom category that occur before and persist in the absence of other symptoms categories, including psychiatric symptoms. They are predictive of functional and social outcomes, offering a possible preventative target before the onset of schizophrenia. To target this symptom category, it is necessary to understand the molecular pathophysiology. Key systems implicated in the pathophysiology of cognitive symptoms of schizophrenia include the BDNF-TrkB signaling pathway and the inhibitory system. For this thesis, three different models of altered BDNF-TrkB signaling were used to understand how this signaling pathway interacts with the inhibitory system for cognitive disruptions to arise. Sex and stress were investigated alongside as moderators of these systems.

Neurodevelopmental disorders, including schizophrenia, are thought to be caused by a combination of adverse genetic and environmental insults. The “two-hit” hypothesis suggests that an early first “hit” primes the developing brain to be vulnerable to a second “hit” during adolescence, an epoch when the brain is undergoing extensive restructuring, which triggers behavioural dysfunction. This contributes to the peak onset of schizophrenia occurring at late adolescence and early adulthood. Key findings from a two-hit model (BDNF heterozygosity and chronic corticosterone treatment) investigating the potential of environmental enrichment (EE) as a preventative found that the EE protocol was protective for spatial memory only for female two-hit mice. To investigate specific pathways by which each of these negative and positive ‘hits’ influence spatial memory performance, the first results chapter (chapter 3) of this thesis describes a comprehensive examination of hippocampal proteins pertaining to neurotrophic signalling, and both inhibitory and excitatory signalling pathways. Protein expression results indicated that glutamatergic receptors were particularly vulnerable to the two negative ‘hits’, while the

neurotrophin signalling pathway was altered by EE. Interestingly, some subtle changes in the GABAergic inhibitory subtype marker, parvalbumin were noted in two hit animals, which warranted further, more refined exploration.

Parvalbumin-expressing inhibitory interneurons (PV-IN) have been implicated in schizophrenia and cognition and are thought to rely on BDNF signalling for healthy development. Chapter 4 of this thesis sought to specifically investigate the relationship between BDNF-TrkB signaling and PV-IN. Here cre-lox recombination was used to alter expression of TrkB receptors on PV-IN. A PV-Cre mouse was crossed with a TrkB heterozygote floxed mouse, to generate PV-Cre:Fl +/- offspring. These animals underwent a battery of behavioural and affect tests. Here, male specific changes to spatial memory were observed in PV-Cre:Fl +/- mice including short-term spatial memory impairments and differences in perseverance and novelty seeking.

To further develop a more clinically relevant understanding of the relationship between sex, BDNF-TrkB signaling and GABAergic IN a mouse model of the common BDNF Val66Met polymorphism was utilised in chapter 5 of this thesis. The Val66Met mutation involves the substitution of a valine to a methionine at codon 66 in the BDNF gene and results in decreased activity-dependent secretion of BDNF. It has been implicated as a moderator of psychiatric and cognitive phenotype. This final results chapter was a histological study investigating the effects of the BDNF val66met polymorphism with and without exposure to chronic corticosterone on hippocampal inhibitory interneuron cell density. Key results included that hBDNF^{Met/Met} mice had lower somatostatin (SST) density, and that irrespective of genotype females had higher SST density. Strikingly, PV was unchanged. SST has a role in cognition and schizophrenia

pathophysiology and the final results chapter of this thesis demonstrates for the first time that the BDNF val66met polymorphism alters SST cell density within the hippocampus. .

Ultimately, this work provides support for these systems in the pathophysiology of cognitive symptoms of schizophrenia, with sex and stress moderating the dynamics of these systems and subsequent phenotype. In light of the very convincing evidence this thesis provides of the role of BDNF in maintaining excitatory: inhibitory balance within the hippocampus and subsequent cognitive ability, future studies arising as a result of these findings should investigate the potential of targeting BDNF signalling pathways during early prodromal periods to dampen or even prevent the devastating cognitive impairment associated with schizophrenia.

Publications during enrolment

Grech, A.M., Notaras, M.J., Sepulveda, M, van den Buuse, M and Hill, R.A. *Brain-Derived Neurotrophic Factor Val66Met polymorphism is associated with selective reduction of somatostatin interneuron density in the dorsal hippocampus of mice*, J Neurochem, 2019, Under Review.

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Du, X., Serena, K., Hwang, W.J., **Grech, AM**, Wu, Y.C., Schroeder, A. and Hill, RA. *Prefrontal cortical parvalbumin and somatostatin expression and cell density increase during*

adolescence and are modified by BDNF and sex. Mol Cell Neurosci. 2018 Apr;88:177-188. doi:
10.1016/j.mcn.2018.02.001.

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Grech, A.M., Ratnayake, U., Hill, R.A. and Van den Buuse, M. *Environmental Enrichment in a 'two-hit' model of developmental stress: changes in TrkB Signaling and learning and memory.* The Students of Brain Research (SOBR) Symposium, Swinburne University, 23 November 2015, Hawthorn, Victoria, Australia. Oral Presentation.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer reviewed journals and 1 submitted publications. The core theme of the thesis is the molecular foundations of cognitive symptoms of schizophrenia. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Clinical Sciences under the supervision of Dr. Rachel Hill and Dr. Xin Du. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision, submitted)	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
3	Sex-Dependent Effects of Environmental Enrichment on Spatial Memory and Brain-Derived Neurotrophic Factor (BDNF) Signaling in a Developmental "Two-Hit" Mouse Model Combining BDNF Haploinsufficiency and Chronic Glucocorticoid Stimulation.	Published	70% - AMG performed all Western blot analysis and data analysis and wrote the first draft of the paper.	5% - UR performed the behavioral testing and its analysis. 5% - AH advised on the original project and on data analysis and interpretation. 10% - MvdB designed the project, oversaw behavioral testing, performed data analysis, and edited the manuscript.	No to all co-authors

				10% - RAH co-designed the project, oversaw Western blot analysis, performed data analysis, and edited the manuscript.	
4	Sex-specific deficits in spatial memory and cognitive flexibility in mice with a conditional TrkB deletion on parvalbumin interneurons	Published	70% - AMG performed all behaviour and immunohistochemistry, data analysis and wrote the first draft of the paper.	<p>10% - XD co-designed the project, oversaw behavioural testing and edited the manuscript.</p> <p>10% -RAH co-designed the project, oversaw behavioural testing and immunohistochemistry and edited the manuscript.</p> <p>5% - SSM and JX imported the TrkB heterozygote floxed (Ntrk2tm1Lfp) mice and kindly donated these for use in this study.</p>	No to all co-authors
5	Sex and genotype differentially influence hippocampal inhibitory-marker cell density in hBDNFVal66Met mice	Submitted	60% - AMG performed all immunohistochemistry, data analysis and wrote the first draft of the paper.	<p>5% - MJN performed the animal perfusions</p> <p>5% - MS cut all free-floating sections.</p>	No to all co-authors

				<p>2% - JAG donated the mice.</p> <p>10% - MvdB co-designed the project and edited the manuscript.</p> <p>18% - RAH co-designed the project, oversaw confocal microscopy experiments and edited the manuscript.</p>	
Appendix A	Prefrontal cortical parvalbumin and somatostatin expression and cell density increase during adolescence and are modified by BDNF and sex	Published	5% - AMG performed immunohistochemistry experiments to measure somatostatin density.	<p>50% - XD performed Western blots, data analysis and wrote the first draft of the paper.</p> <p>10% - KS performed immunohistochemistry experiments for parvalbumin and calretinin.</p> <p>10% - WJH performed Western blot experiments.</p> <p>5% - YCW performed Western Blot experiments.</p> <p>5% - AS performed Western blot experiments.</p> <p>15% - RAH co-designed the project, oversaw experiments and edited the manuscript.</p>	No to all

Appendix B	The importance of distinguishing allocentric and egocentric search strategies in rodent hippocampal-dependent spatial memory paradigms - getting more out of your data.	Published	40% - AMG determined the scope of the review, reviewed the literature, co-wrote the chapter and created figures.	40% - JPN determined the scope of the review, reviewed the literature, co-wrote the chapter and created figures. 20% - RAH determined the scope of the review and edited the chapter.	Yes - JPN
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I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:**

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Abbreviations

AMPA	α -Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
ANOVA	Analysis of Variance
ASRB	Australian Schizophrenia Research Bank
BDNF	Brain Derived Neurotrophic Factor
BDNF HET	Brain Derived Neurotrophic Factor Heterozygous
CA	Cornus ammonis
CA1, CA2, CA3	CA sub-regions of the hippocampus
CAL	Calretinin
CB	Calbindin
Cg	Cingulate Cortex
CGE	Caudal Ganglionic Eminence
CNS	Central Nervous System
CORT	Corticosterone
DG	Dentate Gyrus
DHP	Dorsal Hippocampus
DLPFC	Dorsolateral Prefrontal Cortex
EE	Environmental Enrichment
E/I	Excitatory/Inhibitory
ERK1/2	Extracellular signal-regulated kinases 1/2

GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
glucocorticoids	Glucocorticoids
GluNR	Metabotropic Glutamate Receptors
GR	Glucocorticoid Receptor
GWAS	Genome-wide association studies
HPA	Hypothalamic Pituitary Adrenal
IN	Inhibitory Interneuron
IPSCs	Inhibitory Postsynaptic Currents
IrL	Infralimbic Cortex
LMS	Lateral Migratory Stream
LTP	Long Term Potentiation
MAPK	Mitogen-Activated Protein Kinase
mBDNF	Mature Brain Derived Neurotrophic Factor
MGE	Medial Ganglionic Emience
MMS	Medial Migratory Stream
mPFC	Medial Prefrontal Cortex
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
MWM	Morris Water Maze
NeuN	Neuronal Nuclear Protein
NGF	Nerve Growth Factor
NMDAR	N-methyl-D-aspartate receptor

NORT	Novel Object Recognition Task
NPY	Neuropeptide Y
NRG1	Neuregulin-1
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
p75^{NTR}	p75 Neurotrophin Receptor
PB	Phosphate Buffer
PBS	Phosphate-Buffered Saline
pERK1/2	Phosphorylated Extracellular Signal-Regulated Kinases 1/2
PFC	Prefrontal Cortex
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PLC-γ	Phospholipase C- γ
POA	Preoptic Area
PPI	Pre-Pulse Inhibition
PrL	Prelimbic Cortex
pTrkB	Phosphorylated Tropomyosin-related kinase B
PTSD	Post-Traumatic Stress Disorder
PV	Parvalbumin
SEM	Standard Error Of The Mean
SH	Standard Housed
SNP	Single Nucleotide Polymorphisms

SST	Somatostatin
TrkB	Tropomyosin-related kinase B
TrkB^{TK+}	Full-length TrkB receptor
Val66Met	Valine66Methionine
VHP	Ventral Hippocampus
WT	Wildtype-like
Y515	Tyrosine residue 515
Y705	Tyrosine residue 705
Y816	Tyrosine residue 816

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Chapter 1. Introduction

1.1 An overview of schizophrenia

Schizophrenia is a neurodevelopmental disorder that has a peak onset during late adolescence/early adulthood. It affects roughly 1% of the population with incidence reports of up to 3% (Reichenberg et al., 2010) and has devastating consequences upon normal functioning (Kalache et al., 2015; Solanki et al., 2008) due to the significant disruptions across multiple domains of normal cognition and behaviour. The disorder was originally identified as a ‘dementia praecox’ (premature dementia) by Emil Kraepelin in the 1880s and renamed ‘schizophrenia’ in 1911 by Eugen Bleuler (Lake and Hurwitz, 2007). The name change reflects the current state of the literature and dynamic definition of the syndrome due to ongoing and evolving understanding of the disorder. Despite being identified for over 150 years, its exact causes are still unknown, making it hard to treat and impossible to cure. This is due to its highly heterogeneous nature, with individuals precipitating the disorder in distinctive ways due to their own highly unique profile.

Schizophrenia is a leading cause of disability-adjusted life year (DALY) worldwide despite affecting only a tiny percentage of the population (Rössler et al., 2005). DALY quantifies the burden of disease by combining the mortality and disability impacts, which represent years of life lost due to premature death and years lived with disability respectively. In this way, one DALY is equivalent to one year of healthy life lost (Rössler et al., 2005). There are not only costs to the individual; schizophrenia has a major, negative economic impact to society. According to a report by The Royal Australian and New Zealand College of Psychiatrists (RANZCP) (Sweeney and Shui, 2016), in 2014 estimated figures of A\$3.9 billion and A\$6.2 billion were incurred by the Australian government and individuals respectively, due to psychosis. Similarly in the USA, schizophrenia cost the government US \$155 billion in 2013, equivalent to double the US Health budget for 2013 (Cloutier et al., 2016). A systematic review by Chong et al. (Chong et al., 2016)

reported that schizophrenia contributes an economic burden in the range of 0.02%-1.65% of GDP. Much of this economic burden is due to indirect costs such as loss of productivity, unemployment and income assistance (Chong et al., 2016). Cognitive deficits of schizophrenia are the main predictor of functional and social outcomes (Green et al., 2004; Seidman et al., 2010). They are intimately linked with the ability to hold long term employment and self-sufficiency (Bowie and Harvey, 2006). There is a pervasive breadth and depth to the impact of this illness and clearly it is a problem desperate to be addressed.

1.1.1 Symptomology of schizophrenia

Schizophrenia is a heterogeneous group of disorders (Pillai, 2008) but hallmarks of the disease include positive, negative and cognitive symptoms (Saha et al., 2005). Positive symptoms are named as such since they “add” perceptions and experiences to the affected individual’s reality. Typical positive symptoms include hallucinations, delusions and disordered thinking. Negative symptoms describe the “removal” of experience from the affected individual, and are primarily driven by two main factors: decreased emotionality and decreased enjoyment and drive (Carbon and Correll, 2014). This category is characterised by anhedonia, asociality (Carbon and Correll, 2014) and apathy (Lee, 2013). The main symptom category of interest for this thesis due to its potential as a therapeutic target is cognitive symptoms.

1.1.2 The social and economic impact of cognitive symptoms of schizophrenia

Cognitive decline during adolescence that precedes the onset of positive and negative symptoms of schizophrenia was first identified in 1893 by Kraepelin (Sakurai et al., 2015). Schizophrenia can be associated with major disruptions to memory, attention, cognitive speed and executive functions (Lewis, 2012; Zhang et al., 2012). Cognitive deficits are the result of genetic and

environmental disruptions during critical periods of development (Falkai et al., 2015; Jiang et al., 2013; Wu et al., 2013). These cognitive impairments occur in up to 80% of patients (Heinrichs and Zakzanis, 1998) and are highly predictive of future cognitive and social functioning (Green, 1996; Green et al., 2004; Lewis, 2012; Seidman et al., 2010). Cognitive patient clusters have recently emerged in the literature (Wells et al., 2015). Figure 1.1 illustrates research that found in an Australian Schizophrenia Research Bank (ASRB) sample, 29% of patients were ‘preserved’ in their cognitive functioning, performing within one standard deviation (s.d.) of control in all cognitive domains tested (Wells et al., 2015). A second cluster of 44% of patients performed more than one s.d. below control means in the majority of cognitive domains tested and were deemed ‘deteriorated’. A final group were labelled ‘compromised’ and consisted of 26% of the patient sample. This group was greatly impaired, performing almost 2 s.d. from the mean, across all tested domains. Comparison of a premorbid IQ estimate found that the ‘deteriorated group’ experienced a decrease in cognitive abilities as their schizophrenia progressed while the ‘compromised’ group had been severely impaired across development. This research supports literature suggesting that the majority of the schizophrenia patient population has impaired cognition, which has the greatest impact potentially on an individual’s day to day functioning (Green et al., 2000).

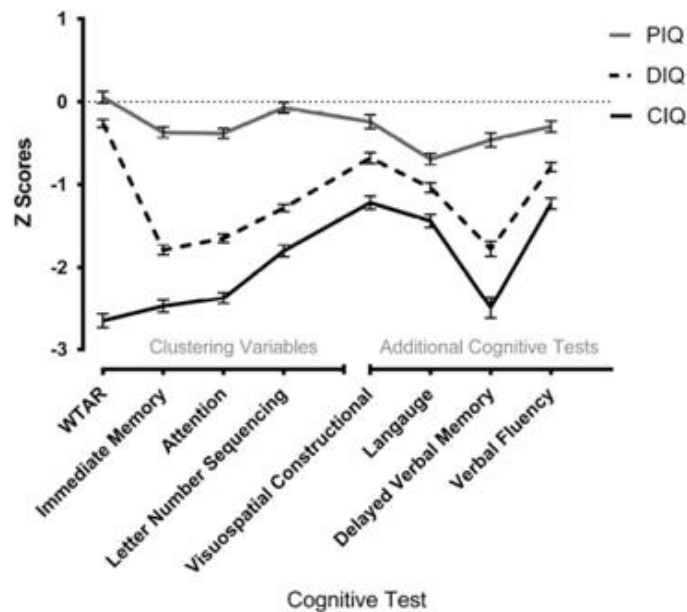


Figure 1.1 - Cognitive clusters of schizophrenia

In an Australian Schizophrenia Research Bank (ASRB) sample three patient clusters emerged using the empirical clustering method: preserved (PIQ), deteriorated (DIQ) and compromised (CIQ) as determined by a range of cognitive tests including Wechsler test of adult reading (WTAR).. Source (Wells et al., 2015).

The ability to marshal and manage various cognitive processes involving multiple brain regions is disrupted in this disease (Cho et al., 2006; Uhlhaas and Singer, 2015), affecting an individual's abilities to participate in the community (Nieto et al., 2013). It is well established that impaired cognition is detected long before positive symptoms of schizophrenia arise (Meier et al., 2014), and persists in the absence or even improvement of positive symptoms (Lewis, 2012; Volk et al., 2012; Zhang et al., 2012). A New Zealand study investigated the prodromal stage of schizophrenia, tracking a representative cohort of over 1,000 subjects from age 3 to 32 years of age (Reichenberg et al., 2010). Prodromal refers to the initial stage of changes within an individual, before they develop characteristic symptoms of schizophrenia (Yung and McGorry,

1996). In this New Zealand sample, disrupted cognitive features in subjects who went on to develop adult schizophrenia could be detected before puberty and schizophrenia diagnosis. Additionally, affected individuals progressively lagged developmentally in comparison to their peers. A North American study similarly reported that at-risk individuals who develop psychosis have cognitive impairments at baseline (Seidman et al., 2010). More recently, Meier et al. (Meier et al., 2014) reported a decrease in IQ from childhood to adulthood that is close to mild cognitive impairment in schizophrenia patients (Figure 1.2). Comparatively, it has been reported that 16-45% of a patient sample displayed no cognitive deficits, however this range was dependent upon the criteria and tests used (Reichenberg et al., 2010). Working memory impairments in individuals with schizophrenia have been demonstrated by many studies (Chen et al., 2014; Cho et al., 2006). Unequivocally, impaired working memory and other cognitive deficits can be devastating for an individual's quality of life. The cognitive deficits of this illness can impact the individual's autonomy, interpersonal relationships and participation in work and society (Falkai et al., 2015). Working memory deficits have been most related to functional disability (Zaragoza Domingo et al., 2015) . Despite this, there is currently no treatment available for cognitive symptoms.

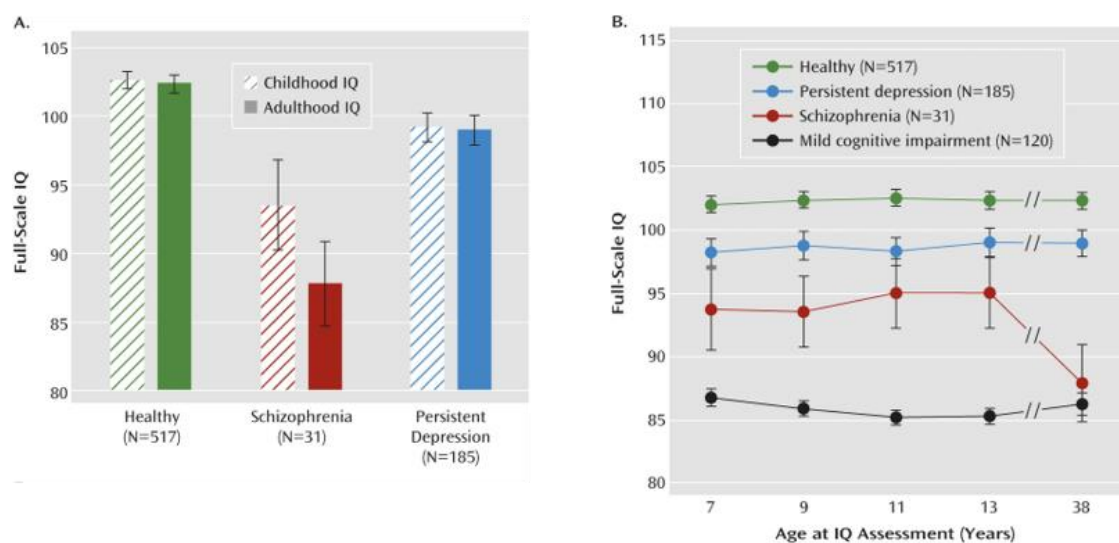


Figure 1.2 - Cognitive impairment in schizophrenia patients across the lifetime

Cognitive impairments in schizophrenia are evident from childhood (panel A) and cognitive decline continues into adulthood (panel B). Source (Meier et al., 2014).

1.1.3 A need for treatments for cognitive symptoms of schizophrenia

Current pharmaceutical treatments for schizophrenia only treat the positive symptoms, and do not ameliorate learning and memory deficits (Gonzalez-Burgos et al., 2010). Antipsychotics come with a range of side effects including weight gain, slowed thoughts and emotional numbing (Jameson and Longo, 2015; Stroup and Gray, 2018). Side effects occur due to the antipsychotics acting at multiple sites, and can impact adherence with 74-90% of patients discontinuing medication within 12-24 months of hospital discharge and clinical trial completion (Wade et al., 2017). Atypical antipsychotics tend to have better adherence rates (Ascher-Svanum et al., 2008) and less cognitive side effects (Hill et al., 2010), although en masse both benefits are modest. Future pharmaceutical approaches need to specifically target key biological pathways to avoid similar side effect issues. Given the significant heterogeneity of schizophrenia, precision medicine

is likely to be the most successful strategy for patients. It takes into account the personal experience, needs and biological profile of an individual patient (Jameson and Longo, 2015). However, the heterogeneity in disease pathophysiology precludes any prediction for which treatment is appropriate for a patient (Breen et al., 2016; Sakurai and Gamo, 2019). Recently, the Brainstorm Consortium (Brainstorm et al., 2018) compared GWAS data across various psychiatric diseases and found common genetic heritability risk variants. Part of this work includes designing algorithms that will use symptoms, rather than diagnosis, to suggest the best treatments. Further understanding of the neurobiology that underlies each symptom will then allow more directed, patient specific, treatment strategies

The lack of specific approaches to cognitive impairments is a major gap in the treatment approach to schizophrenia, since as mentioned above, cognitive symptoms are major directives of the illness trajectory. It is imperative to develop effective approaches that will reduce the burden of this highly disabling disease. Current preventative methods are broadly aimed at mental health issues, and provide a generalised approach, rather than a targeted preventative process for individuals at risk of schizophrenia (Arango et al., 2018). Therefore, there is a need to firstly understand the biological foundations of these cognitive deficits to develop targeted treatment strategies.

1.1.4 Sex differences are evident in schizophrenia

Despite the methodological challenges in sampling and diagnostic criteria, the general consensus within the literature is that sex differences exist in the prevalence, onset, course and presentation of schizophrenia. The ratio for male:female in the schizophrenia population has been reported to be 1.4:1 (McGrath et al., 2008). In addition, men tend to have more severe negative and cognitive symptoms, possibly due to experiencing an earlier onset of schizophrenia. Whereas women generally need more risk factors to catalyse the onset of schizophrenia (Ochoa et al., 2012). For women, the peak age of onset has been found to fall between 20-29 years old whilst in men this

peak is 15-24 years of age (Markham, 2012). Other studies have reported the peak age of onset for women to be 25-35 years of age and men at 18 – 25 years of age (Ochoa et al., 2012). Importantly, the roughly 3-4 year difference in peak onset-age seems to be consistent in most studies (Häfner, 2003). Peri-menopausal women have a second peak in incidence (Hafner and Heiden, 1997; Wu et al., 2014) coinciding with the substantial loss of estrogen (Gurvich et al., 2018; Searles et al., 2017). This parallels the closeness of the peak onset of schizophrenia with that of puberty, when sex hormone levels radically increase. The differences in onset between the sexes could be attributable to fluctuations in sex hormones during this period (Begemann et al., 2012; Kulkarni, 2009). The earlier onset of puberty in females compared to males and the neuroprotective properties of estrogen may explain both the favourable epidemiological pattern for females as well as the second peak of risk around perimenopause (Figure 1.3).

Sex differences in the human presentation of schizophrenia indicate that this should be a variable for investigation in preclinical studies, as it may indicate differences in the underlying pathophysiology and potentially inform divergence in treatment approaches between the sexes.

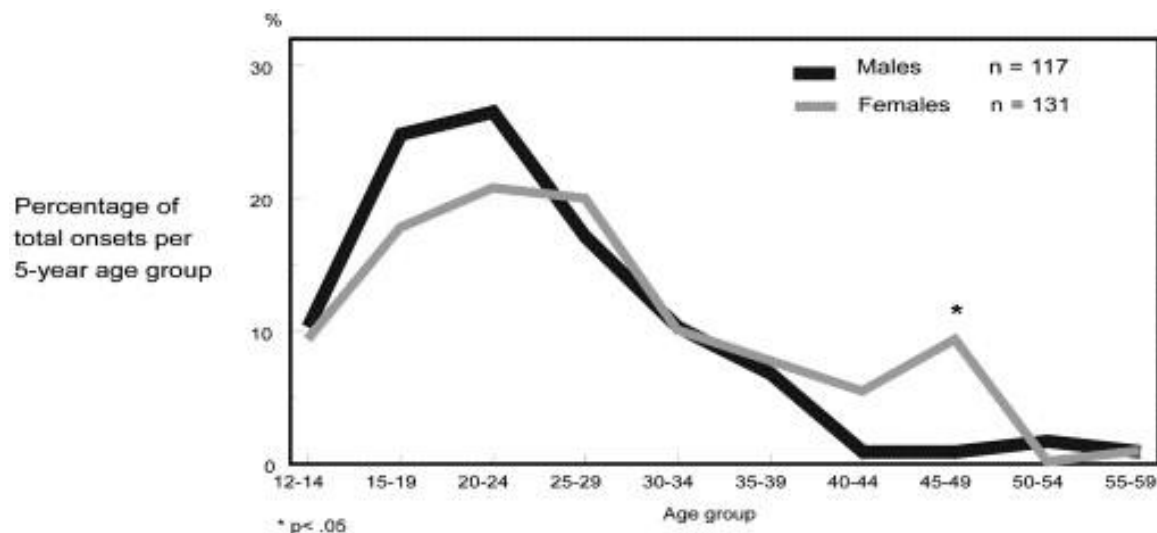


Figure 1.3 - Sex differences in the onset of schizophrenia

*Sex differences are evident in schizophrenia across the lifetime. Males tend to have an earlier onset while females have a second peak of onset around peri-menopausal (indicated by the asterisk *), indicating a potential protective effect of estrogen. Source (Häfner, 2003).*

1.2 Aetiology of schizophrenia

1.2.1 Genetics of schizophrenia

Schizophrenia is a complicated disorder, partly due to the complexity of the genetic blueprint. Twin studies strengthen a strong role for genetics in schizophrenia. Figure 1.4 deftly illustrates the increased risk for developing schizophrenia from 1% in the general population to 9% for a sibling relationship. However, for fraternal twins this risk increases to 17-28% and increases yet again for identical twins to 41-65 % risk (Henriksen et al., 2017). Familial and twin studies demonstrate that while genetics can contribute largely to the emergence of the disorder, it is not the only factor. This is supported by adoption studies. Adopted children with biological parents with schizophrenia are more likely to develop schizophrenia compared to adopted children with

non-mentally ill parents (Heston, 1966) but environment was likely to play a role here too (Tienari et al., 2004).

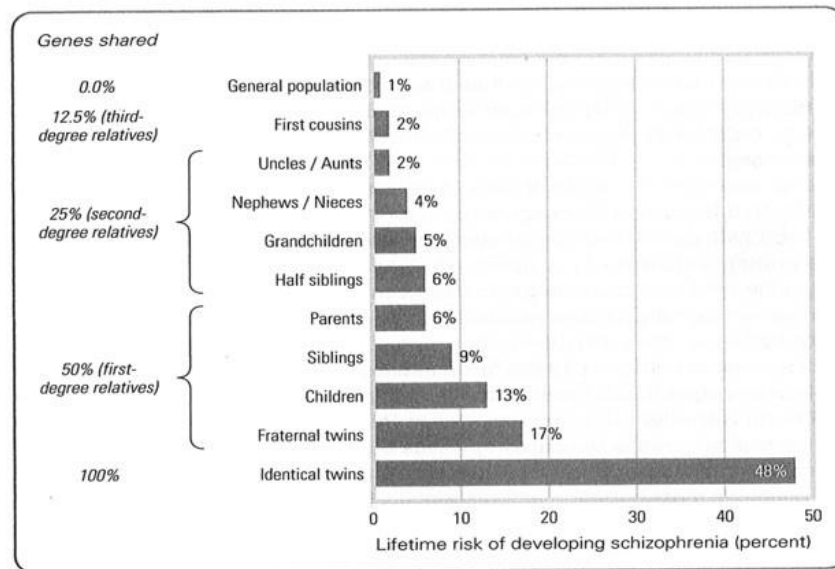


Figure 1.4 - Lifetime genetic risk of developing schizophrenia

The lifetime risk of developing schizophrenia increases as the percentage of genetics shared with a relative with schizophrenia increases. Source (Lambert and Kinsley, 2005).

Schizophrenia is currently associated with 138 risk loci across the genome (Li et al., 2017; Schizophrenia Working Group of the Psychiatric Genomics, 2014) and a multitude of haplotypes (Salvoro et al., 2018; Weickert et al., 2012). A major risk gene is neuregulin-1 (*NRG1*) (Harrison and Law, 2006), which gives rise to multiple functionally distinct isoforms of which type IV and type I NRG are particularly associated with schizophrenia (Harrison and Law, 2006; Law et al., 2006). A recent meta-analysis concluded that genetic variation at both the 5' and 3' ends of *NRG1* were associated with schizophrenia, emphasising the need for further research into this gene (Mostaid et al., 2017). In the general population the major histocompatibility complex (MHC)

locus, located on chromosome 6, has arguably the strongest genetic association with schizophrenia (Coelewij and Curtis, 2018). Located within the MHC locus is a gene that has been directly linked to schizophrenia pathophysiology. Named *C4*, it is the gene responsible for coding of complement component 4 (Coelewij and Curtis, 2018). The higher the expression of *C4A* produced by each common *C4* allele, the greater the association with schizophrenia (Sekar et al., 2016). The human *C4* protein is found at multiple neuronal sites including synapses and dendrites, with animal work indicating that excessive *C4* destroys synapses (Sekar et al., 2016). These are examples of the most common genetic risks for schizophrenia, but there is broad heterogeneity to the genetic blueprint of schizophrenia.

A mini-review by Coelewij and Curtis (Coelewij and Curtis, 2018) reported that the risk for schizophrenia was increased by very rare disruptions to the *SETD1A*, *RBM12* or *NRXN1* genes. Indeed, there is a broad list of genes associated with schizophrenia that span a range of areas including calcium signaling (Berridge, 2014; Xu et al., 2017), synaptic plasticity (Hayashi-Takagi, 2017; Leber et al., 2017), neurotrophic and growth factors (Mei and Nave, 2014; Misiak et al., 2018; Zhang et al., 2016), glutamatergic (Yasuda et al., 2017) and dopaminergic signaling pathways (Howes et al., 2015; Ripke et al., 2014). The main point here is that there is a broad range and number of genes associated with schizophrenia, which can also have variations in contribution to risk. Genome-wide association studies (GWAS) provide an overview of common risk genes, which support the ‘common disease – common variants’ hypothesis’ and suggest schizophrenia is associated primarily with common genetic variants (Hayashi-Takagi, 2017; Pritchard and Cox, 2002). However, schizophrenia is a highly heterogeneous disorder in both pathophysiology and phenotype. Here, the ‘common disease – rare variants hypothesis’ suggests that rare de novo single nucleotide polymorphisms (SNP) or copy number variances (CNV) with relatively high penetrance are partially attributable to the pathophysiology of schizophrenia (McClellan et al., 2007). GWAS studies can fail to reveal key pathophysiological factors, for

example Brain Derived Neurotrophic Factor (BDNF). BDNF is strongly associated with the pathophysiology of schizophrenia (Reinhart et al., 2015), and in some cases this is via a single nucleotide polymorphism known as Val66Met. It involves the substitution of a valine (Val) to methionine (Met) at codon 66 (nucleotide 196) in the 5' pro-region of BDNF (Notaras et al., 2015b). This mutation has not surfaced in GWAS studies, but is found to interact with childhood trauma to precipitate schizophrenia (Bi et al., 2018). There is mixed literature on whether this genotype is associated with schizophrenia (Notaras et al., 2015a). In human studies, the majority of literature suggests that the BDNF val66met polymorphism is not a standalone risk factor associated with schizophrenia (Kawashima et al., 2009). However, a few case studies have found direct associations (Neves-Pereira et al., 2005; Rosa et al., 2006) but this is dependent upon the haplotypic background of the carrier (Neves-Pereira et al., 2005) and may instead only contribute to susceptibility to schizophrenia (Rosa et al., 2006; Zakharyan et al., 2011). In humans, the BDNF Val66Met SNP has been found to confer a vulnerability to psychiatric expression after exposure to stress (Verhagen et al., 2010), such as childhood trauma (Veras et al., 2019). It is generally considered that the Val66Met genotype is a moderator of psychiatric phenotype, with changes to brain structure including reduced temporal and occipital gray matter volumes (Ho et al., 2006) and hippocampal volume (Szeszko et al., 2005; Takahashi et al., 2008), impaired cognitive function (Dincheva et al., 2012; Egan et al., 2003) and psychiatric symptoms (Numata et al., 2006; Zhai et al., 2013). However, there are multiple meta-analyses that do not find any association between the BDNF Val66Met polymorphism and schizophrenia (Kanazawa et al., 2007; Naoe et al., 2007; Zintzaras, 2007). Additionally, this polymorphism has been found to have no impact on cognitive functioning (Zhai et al., 2013) but this study did not take into account other contributing variables such as childhood trauma (Veras et al., 2019).

Animal studies such as (Ninan et al., 2010a) and (Pattwell et al., 2012) have reported that the BDNF Met/Met genotype, with subsequent decreased activity-dependent secretion, disrupts the

NMDA receptor-dependent LTP and LTD. This aligns with the glutamatergic hypothesis of schizophrenia, that glutamate hypofunction produces positive and negative symptoms. Animal behavioural studies have found cognitive impairments that mimic those of psychiatric disorders including the Prepulse Inhibition paradigm (Notaras et al., 2017d), spatial memory (Notaras et al., 2016a) and extinction learning (Soliman et al., 2010).

In summary, while the BDNF Val66Met polymorphism is not a direct genetic risk for schizophrenia, it is likely to be associated with modifying psychiatric phenotype such as clozapine response (Hong et al., 2003) and, of particular interest to this thesis - cognition (Lu et al., 2012). It has not yet been investigated whether this polymorphism interacts with other systems to produce cognitive deficits, such as the inhibitory system (discussed in section 1.8).

1.2.2 Environmental factors of mental illness

The environment/s children are raised in and exposed to greatly contribute to the development of the adult brain (O'Mahony et al., 2017). Maternal immunity challenges during pregnancy (Svrakic et al., 2013), living standards, access to proper healthcare and nutrition all play a role in neurodevelopment (Arango et al., 2018). Social disadvantage throughout life has been identified as a major environmental risk factor for the development of schizophrenia (Bulla et al., 2017). This can include poverty (Arango et al., 2018), immigration status (Brown, 2011) and urban living (Colodro-Conde et al., 2018). Specific stressors during adolescence that are associated with the onset of schizophrenia include social stress (Arango et al., 2018) and substance abuse and trauma (Brown, 2011). Even the immune system is implicated in the onset and modulation of schizophrenia (Erhardt et al., 2017). However, exposure to a single or multiple adverse events does not lead to mental illness in all individuals (Uher and Zwickler, 2017). Therefore, it would

appear that it is not just environmental factors that initiate the cascade which precipitates schizophrenia, but a combination of genetics and environment is required.

1.2.3 Gene × environment interactions: the two-hit hypothesis

Schizophrenia is a neurodevelopmental disorder, which means there is opportunity for multiple ‘disruptions’ at key time-points along the developmental trajectory. One theory of schizophrenia pathophysiology is the ‘two-hit hypothesis’, which postulates that the combination of genetic predisposition and environmental insults during critical periods of development can culminate in significant behavioural disruption in adulthood (Klug et al., 2012; Maynard et al., 2001). The “first hit”, such as genetic risk or maternal infection, during development creates a vulnerable brain, and when coupled with the “second hit” (environmental factor) in adolescence, triggers the onset of mental illness in early adulthood (Figure 1.5) (Bayer et al., 1999). Studies have found that individuals who develop schizophrenia experience delays in developmental milestones (Sørensen et al., 2010) and cognitive domains including memory, attention, cognitive speed and executive functions (Lewis and Glausier, 2016; Sakurai et al., 2015). This concept is supported by Tienari et al. (Tienari et al., 1985; Tienari et al., 2004) adoption studies, which demonstrated that adopted *children* with a preexisting risk for schizophrenia, assumed by having a parent with schizophrenia, were more likely to develop schizophrenia when placed into an adoptive home that was determined to be “dysfunctional” (Tienari et al., 1985; Tienari et al., 2004). Environment × environment interactions (van Os et al., 2010) have also been found for the development of psychosis including: childhood sexual abuse interacting with early adolescent cannabis use (under 16 years) (Houston et al., 2008) and a cumulative interaction between childhood trauma, cannabis and urbanicity for higher 3-year persistence rates of psychotic experience (Cougnard et al., 2007).

The two-hit hypothesis can be modelled in animals, which is used to model phenotypes and molecular correlates associated with schizophrenia. There are variations including the use of the BDNF heterozygote mice as a genetic risk (“first hit”) and the addition of the stress hormone corticosterone (CORT) during adolescence (“second hit”).

Other two-hit models include the maternal immune activation model (Meyer et al., 2006; Monte et al., 2017; Zuckerman et al., 2003) or maternal separation as a “first hit” (Choy et al., 2008; Hill et al., 2014) and sub-chronic unpredictable stress during peripubertal maturation as the “second hit” (Giovanoli et al., 2016; Isgor et al., 2004). These animal studies support human observations, with cognitive disruptions in adulthood (Isgor et al., 2004; Monte et al., 2017), modelling of positive symptoms through disruption to pre-pulse inhibition (PPI) of the startle reflex (Choy et al., 2009; Giovanoli et al., 2016) and negative symptoms as measured by a social impairment (Monte et al., 2017; Schroeder et al., 2015). For humans this has been extended upon in recent years, with discussion arising that the two-hit hypothesis is too simple and binary a theory to explain the heterogeneity of the schizophrenia disorder (Davis et al., 2016). Instead it is suggested that multiple vulnerability factors are cumulative at key neurodevelopmental time points and precipitate this pervasive disorder (Davis et al., 2016). This has been explored in a proof-of-concept study, which showed that an aggregate environmental risk score (the polyenviromic score (PERS)) was significantly correlated to psychotic conversion in familial high risk individuals (Padmanabhan et al., 2017). The risk factors and aetiology of schizophrenia that have been described above, contribute to cognitive symptoms of schizophrenia through the alteration of key structures and molecular signaling, which are explored in the sections below.

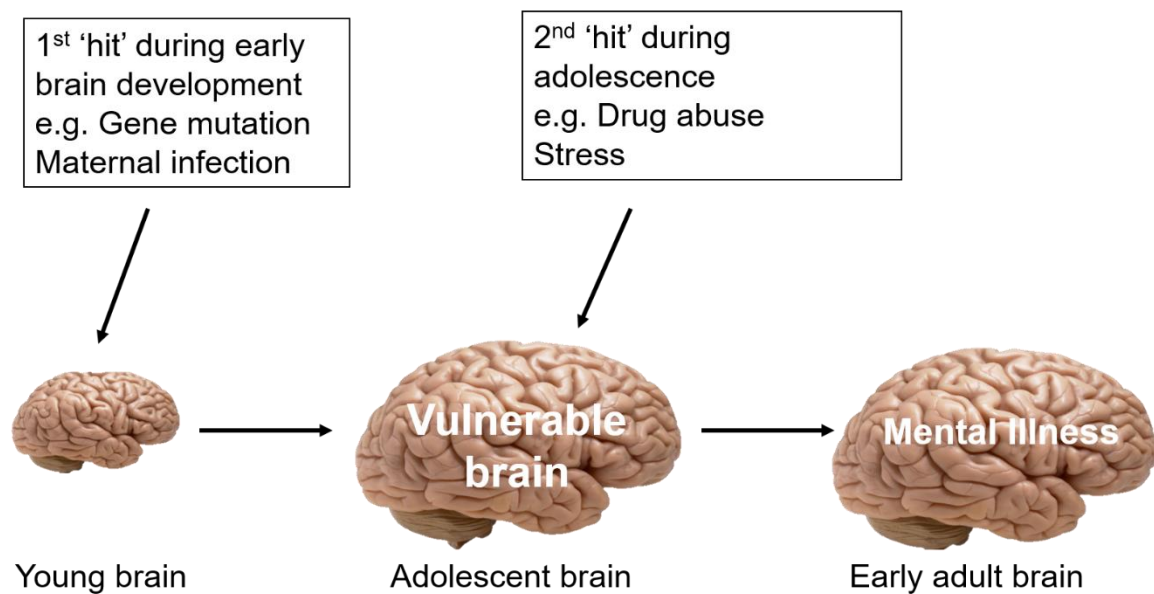


Figure 1.5 - The two-hit hypothesis

A theory for the development of schizophrenia is the two-hit hypothesis, which suggests that 'stressors' at key neurodevelopmental milestones can compound each other and precipitate mental illness in young adulthood.

1.3 The Prefrontal Cortex

The prefrontal cortex is a highly evolved part of the brain, and its development and complexity are thought to distinguish humans from other animals. It is one of the last regions of the brain to fully develop, and therefore is vulnerable to developmental insults such as those described above. Subsequently, it is a focus brain area for a range of mental health disorders including anxiety (Mah et al., 2016), depression (Palazidou, 2012), post-traumatic stress disorder (PTSD) and schizophrenia (Gamo and Arnsten, 2011).

1.3.1 Key functions of the prefrontal cortex

The prefrontal cortex (PFC) is a key region of the frontal lobe, which is densely connected to the other major lobes of the brain (Sakurai et al., 2015). Through its dense networks with other brain regions, the PFC is imperative for many higher order functions such as inhibitory control, working memory and planning (Sakurai et al., 2015). The PFC undergoes extensive maturation during adolescence (Sakurai et al., 2015) and disruption to normal development of this brain region is associated with neurodevelopmental disorders such as schizophrenia (Gamo and Arnsten, 2011).

1.3.2 Anatomical regions of the prefrontal cortex

The PFC can be separated into orbital, medial and dorsolateral regions that have specific processes (Senkowski and Gallinat, 2015). Three key areas in the medial regions of PFC (mPFC) includes prelimbic (PrL), anterior cingulate (Cg) and infralimbic (IrL) cortices, which have been associated with altered cognition in schizophrenia (Schubert et al., 2014). These are specialized regions, with individual connections to other brain areas that underlie the broad capability of higher order functions for which the mPFC is responsible (Schubert et al., 2014). The rodent PFC is much less specialised than humans and monkeys. Rodents do not have a lateral PFC that is associated with higher order cognitive abilities (Laubach et al., 2018). There are similarities across human and rodent medial PFC in for cytoarchitecture (Laubach et al., 2018) and executive function, such as cognitive flexibility (Bizon et al., 2012).

1.3.3 The prefrontal cortex and cognition in schizophrenia

Dysfunction of the PFC has been implicated as part of the pathophysiology of schizophrenia (Sakurai et al., 2015; Schubert et al., 2014). Patients with schizophrenia experience changes to their PFC networks, leading to changes in cognition (Sakurai et al., 2015). Post-mortem studies

have found reductions in grey matter volume (Fornito et al., 2009), both at first-episode (Ohtani et al., 2018) and chronically, which has been associated with cognitive decline (Davies et al., 2018; McEwen et al., 2015; Weinberg et al., 2016). Patient studies have found that exercise can increase PFC grey matter (McEwen et al., 2015) and can improve PFC cognitive processes (Firth et al., 2016). All major neurotransmitter systems are disrupted in this brain region including GABAergic and glutamatergic (Wang et al., 2016), dopaminergic (van Winkel et al., 2008) and acetylcholinergic (Carruthers et al., 2015). These systems are linked to positive and cognitive symptoms of schizophrenia. GABAergic and glutamatergic neurotransmitter systems undergo dynamic changes during adolescence and thus are vulnerable to developmental insults (Fung et al., 2010; Kasanetz and Manzoni, 2009; Wu et al., 2014).

1.4 The Hippocampus

The hippocampus is a major research interest for schizophrenia researcher (Figure 1.6). It is a junction of interconnections for key emotional centres and higher executive thinking regions (Cembrowski and Spruston, 2019). Consequently, the hippocampus is implicated in a range of neurological and psychiatric disorders and has been the focus for a range of memory research (Frick, 2013; Oishi et al., 2019; Sherrill et al., 2013). It is a dynamic brain region, being responsive to changes of environment (Tanti et al., 2013) and experience (Maguire et al., 2000).

1.4.1 Key functions of the hippocampus

Due to its numerous and varied connections to other regions of the brain, the hippocampus has a large range of roles, including spatial processing (Burgess et al., 2002; Cembrowski and Spruston, 2019), learning (Tian et al., 2017), episodic memory (Thoma et al., 2009; Vargha-Khadem et al., 1997) and higher order processes such as imagination and mind-wandering (Dalton et al., 2019).

Through its network connections to the amygdala, the hippocampus is also associated with affective processes including fear, stress and anxiety (Aisenberg et al., 2017; McEwen et al., 2016).

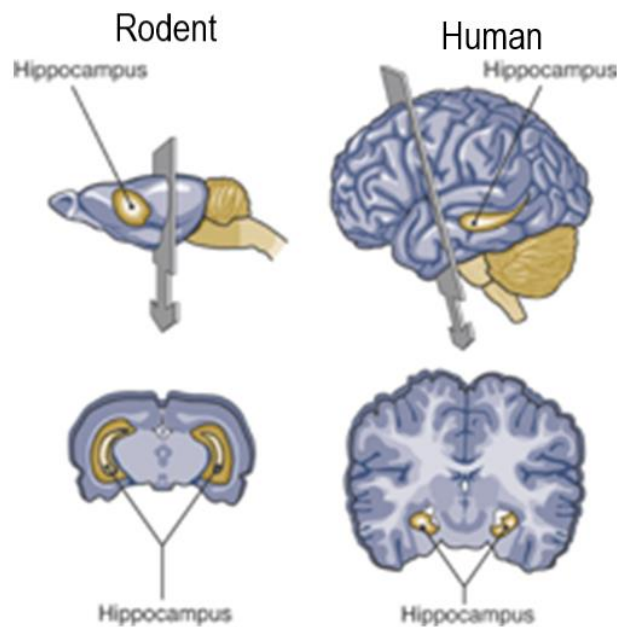


Figure 1.6 - Human and rodent hippocampus

Location and comparison of the hippocampus in the rodent and human brains. The hippocampus is located below the neocortex in the rat brain (left) and human brain (right). Adapted from (Hiller-Sturmhöfel and Swartzwelder, 2004).

An important hippocampal function is neurogenesis. Neurogenesis is the production of new neurons from neural progenitors and stem cells. This process is important for synaptic plasticity and memory (Le Strat et al., 2009). Synaptic plasticity is a key process of learning and memory. It is the strengthening or weakening of synaptic connections through their use or disuse (Mizuno and Giese, 2010; Poo, 2001). Studies have shown the dentate gyrus of the hippocampus to be a

site for neurogenesis in adult mice (Clelland et al., 2009) and in humans (Eriksson et al., 1998). Although there is much support for neurogenesis in the adult mammalian hippocampus (Yagi and Galea, 2019), there is still debate about whether neurogenesis occurs in adult humans. A recent letter in *Nature* by Sorrells et al. (Sorrells et al., 2018) failed to identify neurogenesis occurring in adult human hippocampal tissue. However, there is evidence that neurogenesis is reduced in schizophrenia and this may contribute to hippocampal dysfunction (Allen et al., 2016). Interestingly, sex differences in neurogenesis in the adult rodent brain have been observed (Duarte-Guterman et al., 2015). Neurogenesis in female rats has been observed during a high estradiol phase (Gould and Tanapat, 1999) and estrogen has been found to facilitate neurogenesis in rabbits (Tibrewal et al., 2018). This potentially contributes to the underlying physiology of sex differences in learning, memory and disease states of the hippocampus (Duarte-Guterman et al., 2015). The variety of functions assigned to the hippocampus can in be part be attributed to its rich connections to other brain regions, as well as the density of intra-hippocampal connectivity. Anatomically the hippocampus can be grossly divided into dorsal and ventral subregions, with each subregion possessing individual connections and functions (Fanselow and Dong, 2010).

1.4.2 Anatomical segregation of the hippocampus

1.4.2.1 The Dorsal Hippocampus

The rodent dorsal hippocampus (DHP) is analogous to the posterior hippocampus in humans (Fanselow and Dong, 2010). The DHP receives inputs from the neocortex (Fanselow et al., 2008) and projects to brain regions that are involved in spatial navigation (Fanselow and Dong, 2010). In rodent research spatial memory is often used to measure changes in the hippocampus, due to its relevance and ability to be measured in the laboratory. Since spatial memory has been directly linked to the hippocampus (explored in depth below) and changes incurred there can subsequently

be directly measured in animal research. The role of the DHP in spatial memory has been investigated in both human and animal studies. Spatial memory is the encoding and utilisation of landmarks to successfully navigate the environment (Gagnon et al., 2018). Spatial memory encompasses two distinct but related reference frames, egocentric and allocentric. Egocentric navigation is based on direction (left-right) responses and actions independent of environmental cues. Comparatively, allocentric navigation utilises external cues or landmarks in relation to each other to navigate and is independent of self (Ekstrom and Isham, 2017). In schizophrenia patients, spatial memory has been found to be impaired (Park et al., 1999; Saperstein et al., 2006) and the hippocampus-dependent allocentric strategy has been specifically identified as impaired in schizophrenia patients (Weniger and Irle, 2008; Wilkins et al., 2017). In human taxi drivers, recalling complex routes through a city activated the right posterior hippocampus (Maguire et al., 1997). This study was followed up by demonstrating a strong association between activation of the right hippocampus and accurate navigation through a virtual town (Maguire et al., 1998). This is further supported by impaired navigation in unilateral right temporal lobectomy patients (Spiers et al., 2001b) and an interesting case study of an individual with perinatal anoxia induced bilateral hippocampal pathology (Spiers et al., 2001a). The importance of the DHP has also been demonstrated in rats (Clark et al., 2005), with Klur and colleagues showing that inactivation of the right DHP in rats disrupts spatial memory retrieval (Klur et al., 2009). Additionally, a study by Greicius et al. (Greicius et al., 2003) showed that when participants recalled verbal material the left posterior hippocampus was activated. Evidently the dorsal hippocampus has a role in a variety of memory processes.

1.4.2.2 The Ventral Hippocampus

The ventral hippocampus (VHP), analogous to the anterior hippocampus in humans (Fanselow and Dong, 2010), has direct reciprocal connections with the amygdala, hypothalamus, nucleus accumbens and medial prefrontal cortex, supporting a role in emotional processing for the VHP (Bonne et al., 2008; Hill et al., 2013; Wu et al., 2012). It acts as a conduit for information to be communicated between the mPFC and amygdala (Camp et al., 2012; Gruber and McDonald, 2012), as well as other autonomic and neuroendocrine systems (Arszovszki et al., 2014). Patients experiencing PTSD have been observed to have smaller posterior hippocampal volumes (Wu et al., 2012). Reduced hippocampal volumes is also observed in major depressive disorder patients (Campbell and MacQueen, 2004). In mice, reduced fear expression has been observed after the VHP had been lesioned (Kjelstrup et al., 2002). An impressive optogenetic study demonstrated that inhibiting inputs from the basolateral nucleus of the amygdala to the VHP had an anxiolytic effect upon mice (Felix-Ortiz et al., 2013). In rodents, the VHP has been found to be involved in some memory processes including habit biases (Barfield et al., 2017) and goal encoding (Burton et al., 2009). A recent paper identified spatial specializations across the dorsal-ventral orientation of the posterior cingulate cortex, with ventral portions associated with spatial encoding and dorsal associated with spatial recall (Burles et al., 2018). Contributing to compartmentalisation of function may be underlying differences in cytoarchitecture, with divergence in GABA receptor subtypes and subsequent differences in pharmacological properties one example (Sarantis et al., 2008). Despite evidence of compartmentalisation, the neural circuitry throughout both the dorsal and ventral hippocampi are similarly based upon a trisynaptic circuit, and communication between the two zones does exist (Fanselow and Dong, 2010). This is demonstrated by impairments in functions supposedly unrelated to the damaged hippocampal zone (Felix-Ortiz et al., 2013).

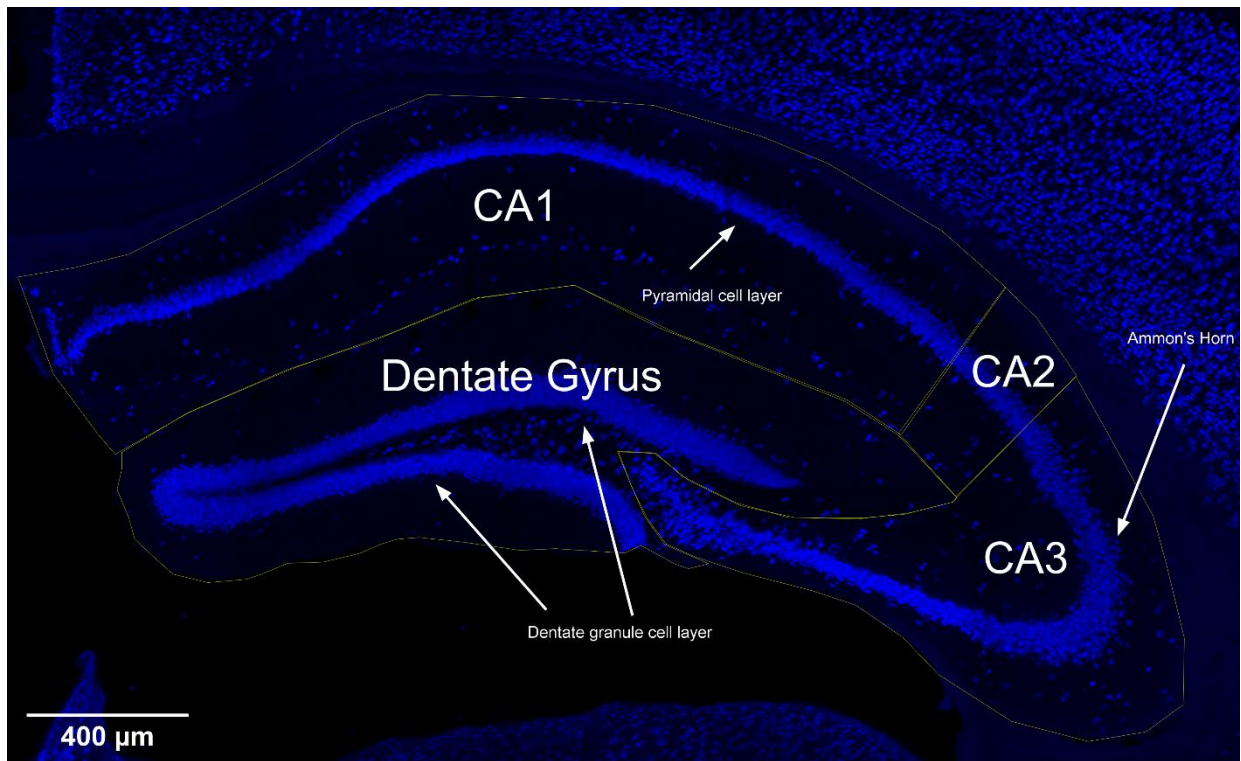


Figure 1.7 - Subregions of the hippocampus

NeuN staining of cell bodies in the mouse dorsal hippocampus at bregma -1.82. The divisions of the subregions of the hippocampus are labelled. CA = cornu ammonis DG= dentate gyrus. Image author's own.

1.4.2.3 Subregions of the hippocampus

The hippocampus can be subdivided into 4 main hippocampal regions, the cornu ammonis (CA) 1-3 and the dentate gyrus (DG) (Figure 1.7). It's intra- and extra-connections are dependent on the hippocampal subregion, with complex circuitry evident (Dalton et al., 2019). Data was acquired using both high resolution structural MR imaging and high-resolution Resting State Function MRI to investigate functional connectivity (temporal correlation in BOLD signal between voxels (Greene et al., 2016)) between hippocampal subregions (Dalton et al., 2019). As illustrated in Figure 1.8 the human DG has significant reciprocal functional connectivity to both

CA3/2 and CA1, while CA3/2 is most significantly functionally connected to the pre/parasubiculum and the CA1 to subiculum, uncus, CA3/2 and posterior parahippocampal cortex (Dalton et al., 2019). Hippocampal structures and functions are well conserved across mammal species, including between human and rodents (Allen and Fortin, 2013). The subregions of the hippocampus have been reported to have a broad degree of specialisation, with the DG the site of neurogenesis (Christie and Cameron, 2006), and the CA1 highly implicated in spatial memory (Blum et al., 1999; Yu et al., 2018). However, even for these subregions there is further division between dorsal and ventral functionality, for example dorsal CA1 is often linked to spatial memory (Murray et al., 2011; Yu et al., 2018) while the ventral has been associated with information routing (Ciocchi et al., 2015). The above literature demonstrates the complexity of these brain regions, and their integral role for higher order cognitive processes.

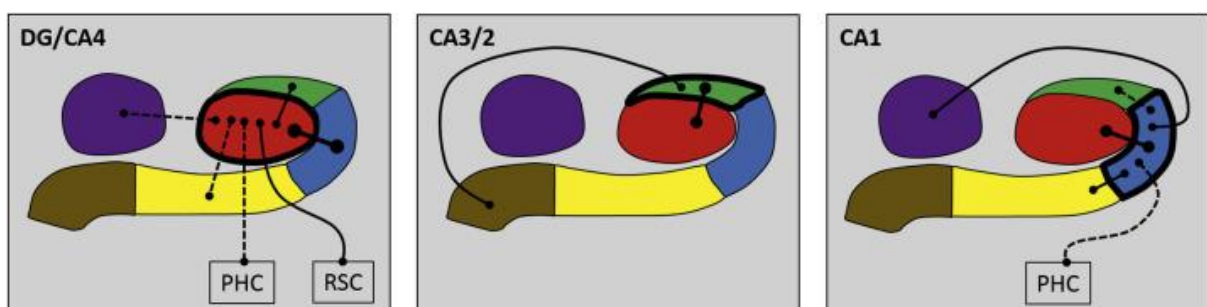


Figure 1.8 - Functional connectivity analysis of young human adult hippocampi

The region of interest is delineated by a thick black outline. Strength of significant correlations of activity between areas is illustrated by line type between two regions. Thick unbroken lines = $t > 10$; thin unbroken lines = $t > 5$; thin broken lines = $t < 5$. DG/CA4 (red), CA3/2 (green), CA1 (blue), subiculum (yellow), pre/parasubiculum (brown), uncus (purple); ENT = entorhinal cortex, PRC = perirhinal cortex, PHC = posterior parahippocampal cortex, RSC = retrosplenial cortex. Source (Dalton et al., 2019).

1.4.3 The hippocampus is part of the pathophysiology of schizophrenia

The hippocampus has been implicated in the pathophysiology of schizophrenia. A wide range of evidence from human studies involving patients with schizophrenia supports this, including disruptions to episodic memory (Weinberger, 1999) and correlations between psychotic symptoms and hippocampal metabolic activity (Medoff et al., 2001; Tamminga et al., 1992). Studies as early as the 1900s have found alterations to the temporal lobe in individuals with schizophrenia (Southard, 1910; Southard, 1915). Following this, studies by Bogerts et al. also found smaller hippocampal volumes in post-mortem individuals with chronic schizophrenia (Bogerts et al., 1990; Bogerts et al., 1985). Results from recent studies have found a decrease in the hippocampal volume of people with schizophrenia, using more advanced neuroimaging techniques (Sim et al., 2006; Weiss et al., 2005). These reductions in hippocampal volume have been associated with a range of cognitive impairments in individuals with schizophrenia (Herold et al., 2015; Ledoux et al., 2014; Schobel et al., 2009; Thoma et al., 2009). Animal models support this. For example, a significant neurodevelopmental model of schizophrenia is the neonatal ventral hippocampal lesion model (NVHL) (Lipska et al., 1995), which induces excitotoxic damage during early development resulting in an adult phenotype that replicates schizophrenia symptoms (Brady et al., 2010).

Other brain regions are implicated in the pathophysiology of schizophrenia, such as the striatum (Kenk et al., 2015). However, this thesis focuses on the PFC and hippocampus as these regions are more heavily implicated in the cognitive symptoms of schizophrenia. Not only are there gross changes to these key brain structures, but also molecular changes that are thought to contribute to the cognitive symptoms of schizophrenia. Normal development of the key brain regions mPFC

and hippocampus require the involvement of neurotrophins, and aberrant neurotrophin signaling has been implicated in schizophrenia.

1.5 Neurotrophins and their receptors

Neurotrophins are a group of proteins that have a vital role in the development, function and survival of the central nervous system (Le Strat et al., 2009; Reichardt, 2006). This development includes the establishment of neural networks through the specification of axons and dendrites, neurogenesis and synaptogenesis, amongst other processes (Libman-Sokolowska et al., 2015; Luzuriaga et al., 2019; Nieto et al., 2013). Mammals express four neurotrophins including nerve growth factor (NGF), Brain Derived Neurotrophic Factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). There is approximately 50% overlap in amino acid sequence of these proteins, with all 4 containing a signal peptide and prodomain with the capability of undergoing glycosylation within the N-terminus (Cardenas-Aguayo et al., 2013). Neurotrophins are primarily differentiated by the prodomain (Chen et al., 2005), with this likely determining differences in processing (Chen et al., 2005) and functionality at the synapse (Notaras et al., 2015b). Figure 1.9 illustrates the divergence in receptor affinities between neurotrophins. BDNF and NT-4 both bind the tyrosine kinase B (TrkB) receptor, however their binding leads to different downstream effects. For this thesis, there is a focus on the BDNF-TrkB signaling pathway due to the strong association of BDNF with a broad range of mental illness pathophysiology (Hill et al., 2012b; Polyakova et al., 2015; Xu et al., 2018a). The investigation of other neurotrophins is required to account for any compensatory action for disrupted BDNF functionality, however this is largely outside the purview of this thesis. In the following section, there is a focus on BDNF structure and function due to its strong association with schizophrenia (Reinhart et al., 2015; Rizos et al., 2011) and cognitive processes (Leal et al., 2017).

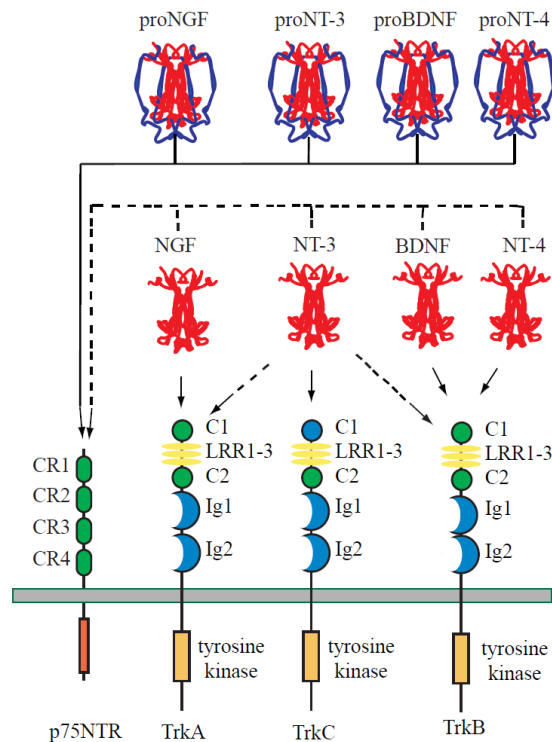


Figure 1.9 - Binding relationships between neurotrophins and neurotrophic receptors

This schematic illustrates the binding relationship of both the proneurotrophins and neurotrophins to the Trk receptor family and the p75NTR receptor. Notably, all proneurotrophins and neurotrophins bind the p75NTR receptor but there is some specificity for the Trk receptor subtypes. NGF = Nerve Growth Factor, NT-3 = Neurotrophic -3, BDNF = Brain Derived Neurotrophic Factor, NT-4 = Neurotrophin-4, CR = cysteine rich, C =cysteine, LRR = leucine-rich repeat, Ig = immunoglobulin-like, p75NTR = p75 neurotrophin receptor, Trk = tropomyosin-related kinase. Source (Reichardt, 2006).

1.5.1 Brain-derived neurotrophic factor (BDNF)

BDNF mediates a range of pathways important for the structural and functional integrity of the central nervous system. It is commonly expressed in most brain cell types and a range of brain structures, including the hippocampus (Pillai, 2008). Indeed, the loss of BDNF disrupts the

development of striatal neurons (cells of the striatum which is a convergence point for the cortex), thalamus and midbrain (Kreitzer, 2009). Hippocampal atrophy is also associated with decreased BDNF (Erickson et al., 2011). BDNF knock-out is lethal in mice, which commonly die during the second postnatal week (Ernfors et al., 1994). Important to the theme of this thesis, BDNF mRNA and protein expression has been consistently reported to be reduced in schizophrenia patients specifically in key brain regions the mPFC (Hashimoto et al., 2005a; Reinhart et al., 2015; Weickert et al., 2003) and hippocampus (Thompson Ray et al., 2011), as well as in serum of first episode (Jindal et al., 2010) and chronic patients (Rizos et al., 2010; Toyooka et al., 2002).

1.5.1.1 BDNF gene structure

The BDNF structure was first reported by Barde in 1990, purified from adult pig brain (Barde, 1990), characterised in the rat by Timmusk et al in 1993 (Timmusk et al., 1993) and eventually located in the human brain at chromosome 11 band p13-14 (Hanson et al., 1992). It is quite complex, and there are differences between the respective rodent and human *BDNF* genes. The human *BDNF* gene has 11 exons that can be alternatively spliced for 17 transcripts, 9 of which are within the 5' region, where the regulation of gene transcription occurs (Pruunsild et al., 2007). There are 9 functional promoters that are tissue and brain-region specific (Pruunsild et al., 2007). Comparatively, the rodent *Bdnf* gene has 9 exons (Aid et al., 2007) and 11 transcripts, of which the alternative splicing of *Bdnf* exon II is uniquely rodent (Aid et al., 2007). Rodents also lack BDNF-antisense transcripts (Aid et al., 2007; Liu et al., 2006), which are present in humans (Liu et al., 2005).

1.5.1.2 BDNF synthesis and isoforms

BDNF is synthesized as the precursor prepro-BDNF (32 kDa), which is proteolytically cleaved within the endoplasmic reticulum to pro-BDNF (28 kDa). While still not clearly understood, the pro-BDNF isoform is biologically active and once secreted binds to the p75 neurotrophin receptor (p75^{NTR}) (Chao and Bothwell, 2002; Lee et al., 2001; Pang et al., 2004; Teng et al., 2005). Pro-BDNF is cleaved to form mature BDNF (mBDNF) (13.5 kDa), which can bind to the p75^{NTR} but has its most significant activity at the TrkB receptor (Minichiello, 2009). BDNF expression levels can be influenced through a variety of factors, such as sex and positive environment. Positive environment in humans relates to a ‘healthy lifestyle’, that includes diet and exercise. Both of these factors have been associated with increased BDNF from mouse to man (Hung et al., 2018; Molteni et al., 2002; Okudan and Belviranli, 2017; Sánchez-Villegas et al., 2011; Szuhany et al., 2015). In humans, sex has been identified as a moderator of the effect of exercise on BDNF, with females demonstrating smaller increases in BDNF compared to males (Szuhany et al., 2015). Indeed, animal studies have revealed that removal of estrogen has been found to decrease hippocampal BDNF expression across development (Solum and Handa, 2002) into adulthood (Singh et al., 1995) and that estrogen can specifically increase BDNF expression (Scharfman and MacLusky, 2005). BDNF and estrogen regulate each other and converge on shared signaling pathways (Scharfman and MacLusky, 2005), presenting a possible paradigm for sex differences in schizophrenia phenotype. BDNF signaling through its cognate receptor TrkB is explored in the next section.

1.5.1.3 BDNF receptors and signaling

BDNF has two endogenous receptors: tyrosine kinase B (TrkB) and p75 neurotrophin receptor (p75^{NTR}). The full length TrkB receptor possesses the catalytic tyrosine kinase domain, which is

necessary for the binding of BDNF to the TrkB receptor and signal transduction (Carim-Todd et al., 2009; McCarty and Feinstein, 1998). BDNF-TrkB signaling activates the phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and phospholipase C- γ (PLC- γ) pathways (Reichardt, 2006). These pathways promote neuronal growth, differentiation, and survival (Li et al., 2012). In contrast, the truncated TrkB receptor sequesters BDNF to inhibit BDNF signaling (Hill et al., 2014). The mBDNF isoform has low affinity for the p75^{NTR} receptor but binding here also promotes survival signaling pathways. However, the pro-BDNF isoform has high affinity for the p75^{NTR} receptor which instead promotes long term depression and apoptosis (Buckley et al., 2007; Reichardt, 2006).

1.5.2 Tyrosine kinase B (TrkB) Receptor

TrkB is a member of the type III receptor tyrosine kinases, a family of receptors important for initiating intracellular signaling cascades that contribute to the proper maintenance and function of the central nervous system (CNS). It is encoded by the *NTRK2* gene, with three isoforms for both human and rodent. The full-length TrkB receptor (TrkB^{TK+}) contains all of the typical tyrosine kinase receptor identifiers including "...signal peptide, two cysteine clusters, a leucine-rich motif, two immunoglobulin-like C2-type motifs, a transmembrane region, a tyrosine kinase domain and a short carboxy-terminal tail of 15 amino acids that includes a Tyr residue" (Minichiello, 2009). The full-length TrkB receptor is alternatively spliced in humans to give TrkB-T1 and TrkB-T2 (aka TrkB-TK⁻ and TrkB-Shc), that are identical to TrkB^{TK+} except that the catalytic kinase domain is removed (Minichiello, 2009; Wong and Garner, 2012). They differ from each other by their individual C-terminus amino acid sequence and their expression profiles across the brain (Wong and Garner, 2012). It is thought that the TrkB-Shc isoform is a negative regulator of TrkB signaling in the CNS (Stoilov et al., 2002). In rodents they are referred to as

tr.TrkB¹ and t.TrkB² (Gupta et al., 2013). Mature neurotrophins that can bind directly to TrkB include BDNF and NT4 (see Figure 1.9), but they have discrete functions due to differences in endocytic sorting (Proenca et al., 2016) . When neurotrophins dock at the binding site this causes TrkB receptor dimerization and transphosphorylation of tyrosines located in the activation loop of the receptor.

TrkB is a membrane-bound receptor, with extracellular ligand binding activating multiple intracellular signaling cascades (Haapasalo et al., 2002). This leads to phosphorylation of other tyrosine residues within the cytoplasmic domain of the receptor, which are important scaffolding sites for multiple proteins to be recruited to and activated for a variety of intracellular signaling pathways (Atwal et al., 2000; Reichardt, 2006). These are explored in further detail below. TrkB can be activated through both neurotrophin dependent and independent means. Transactivation involves activation of a receptor by a ligand indirectly interacting with it. Other factors including zinc, epidermal growth factor and GPCRs that can transactivate TrkB (Helgager et al., 2014).

1.5.2.1 TrkB residues and signaling

Neurotrophin dependent activation of TrkB occurs through the autophosphorylation of tyrosine residues. The intracellular structure of the receptor consists of multiple tyrosine sites. The tyrosine 705 (Y705) residue is located in the auto-regulatory loop of the kinase domain of the TrkB receptor (Huang and Reichardt, 2001). The autophosphorylation of the Y705 residue is a critical preliminary step for TrkB receptor activation (Furmaga et al., 2012). Indeed Y705 has been called the initiator of receptor autophosphorylation (Benmansour et al., 2016) with the extent of phosphorylation of this residue correlating with tyrosine kinase activity levels (Huang and McNamara, 2010). Y705 contributes to increased activation of the TrkB receptor (Carbone and Handa, 2013), and moderates the phosphorylation and activation of other tyrosine residues including Y515 and Y816. Figure 1.10 shows the tyrosine residue 515 (Y515) as the Shc adapter protein docking site (Ambjørn et al., 2013; Benmansour et al., 2016), which catalyzes multiple signaling cascades. Shc has been shown to be important for the survival and growth of neuronal structures (Atwal et al., 2000), which is primarily due to being a crucial step for multiple signaling pathways including extracellular signal-regulated kinases 1/2 (ERK 1/2) and PLC γ 1 pathways

(see Figure 1.10). The ERK 1/2 signaling pathway is part of the MAPK family, which are responsible for converting extracellular stimuli into intracellular signaling cascades (Cargnello and Roux, 2011). ERK 1/2 are 44 and 42 kDa respectively, with an 86% similarity between their amino acid sequences (Cargnello and Roux, 2011). Upon activation, ERK 1/2 is able to phosphorylate multiple substrates that are important for a broad range of cellular processes including cell proliferation (Cargnello and Roux, 2011), which may contribute to its involvement in learning and memory (Yang et al., 2011). The third main residue is tyrosine 816 (Y816). This residue is linked to the phospholipase C- γ 1 (PLC γ 1) pathway. When Y816 is phosphorylated, PLC γ 1 is recruited and activated to hydrolyze phosphatidylinositol 4,5-bisphosphate (Yoshii and Constantine-Paton, 2010), which subsequently produces diacylglycerol (DAG) and inositol trisphosphate (IP3). Protein kinase C (PKC) is activated by DAG, which can have downstream effects for synaptic plasticity (Minichiello et al., 2002). Alongside this, IP3 causes the release of intracellular calcium. The above cascade has a role in synaptic plasticity, minor role in cell survival and axon elongation (Atwal et al., 2000; Ming et al., 1999; Minichiello, 2009). Phosphorylation of Y816 can also contribute to ERK activation (Ambjørn et al., 2013), and distinguishing which residue, Y515 or Y816, contributes to production of ERK is not possible.

These three residues have been reported to have differing levels of response based on ligand, including antidepressant treatments (Benmansour et al., 2016), transactivation by glucocorticoids, adenosine (Ambjørn et al., 2013) and zinc (Helgager et al., 2014). This highlights their discrete roles and contribution to the function of the CNS, and as such should be individually considered. The TrkB receptor is of primary interest to this thesis due to its established association with schizophrenia (Ray et al., 2014; Reinhart et al., 2015) and cognitive processes (Minichiello, 2009; Minichiello et al., 2002).

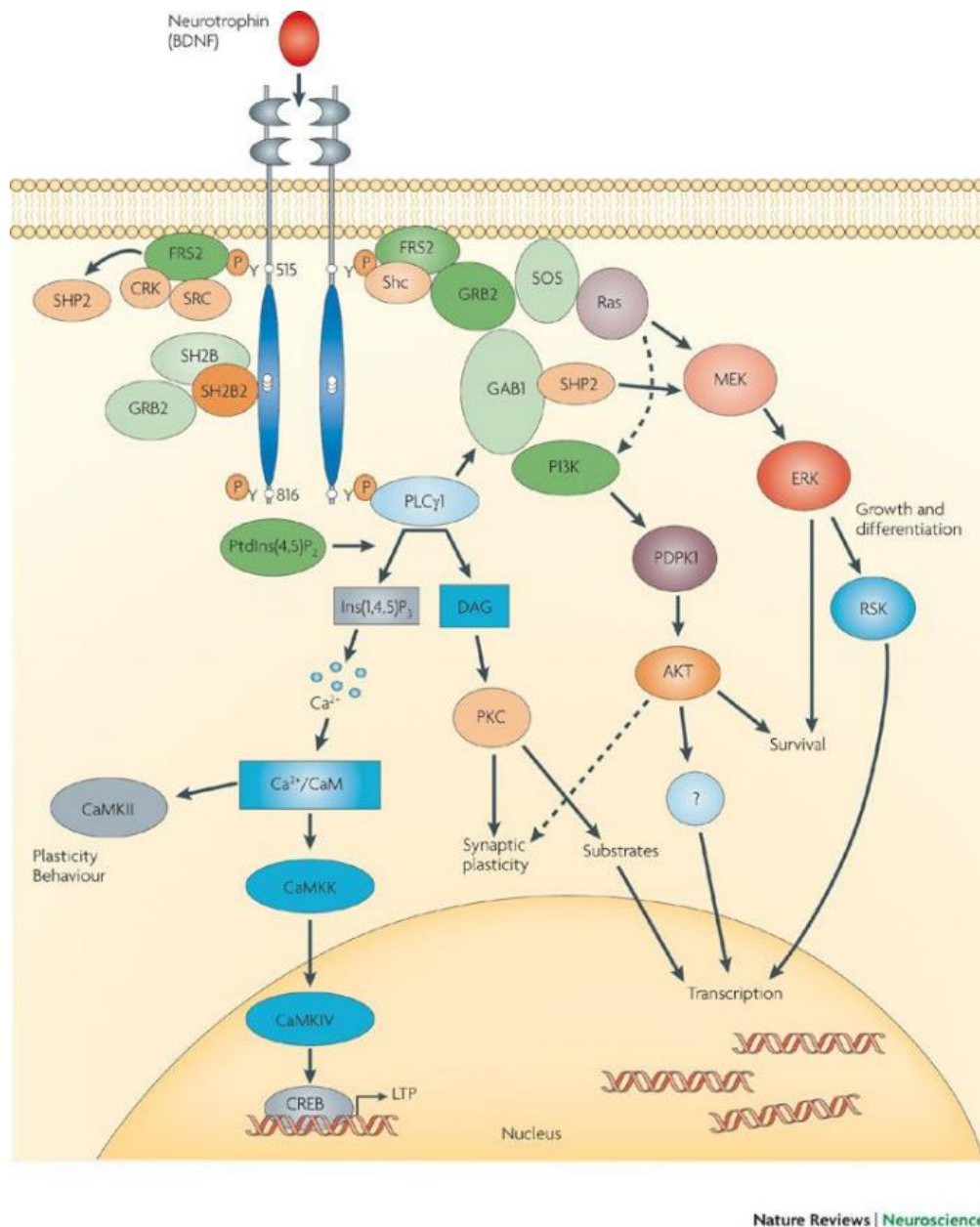


Figure 1.10 - Downstream signaling pathways of BDNF-TrkB binding

Binding of neurotrophins to the receptor tyrosine kinase TrkB initiates autophosphorylation of tyrosine residues, that activate the three main signaling cascades of extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K) cascades via Y515 and phospholipase Cγ1 (PLCγ1) via Y816. These signaling cascades regulate gene transcription, long-term potentiation (LTP) (Reichardt, 2006) and synaptic plasticity (Minichiello et al., 2002). Source (Minichiello, 2009).

1.5.3 BDNF-TrkB Signaling

1.5.3.1 BDNF-TrkB signaling has a role in learning and memory

Importantly for this thesis, the literature indicates that BDNF-TrkB signaling has an important role in learning and memory (Hill et al., 2014; Mu et al., 1999; Yamada and Nabeshima, 2003). BDNF immunoreactivity is concentrated at the soma, dendrites and fibres, locations at which plasticity is orchestrated (Pillai, 2008). LTP is a form of synaptic plasticity through which memories are formed and reinforced through repeated stimulation of a neuronal connection (Bliss and Lomo, 1973; Vierk et al., 2014). An important aspect of LTP is BDNF-TrkB signaling (Hill et al., 2014; Poo, 2001). Studies using BDNF heterozygous (BDNF HET) rodents, which express ~50% of normal levels of BDNF, have demonstrated deficits in learning and memory (Geist et al., 2017). Using a viral gene transfer, Korte and colleagues (Korte et al., 1996) deftly illustrated the pivotal role BDNF plays in LTP in the CA1 region of the hippocampus. BDNF HET mice had an impaired LTP response to tetanic stimulation. When injected with the adenovirus Ad-CMV-BDNF, which recovers gene expression of BDNF, they subsequently expressed normal LTP responses. Disruption of acute TrkB signaling impaired presynaptic generation of LTP at mossy fibre synapses in hippocampal brain slices (Schildt et al., 2013).

BDNF-KIV mice do not express promoter-IV dependent BDNF and have a selective impairment of BDNF-dependent LTP, as well as deficits in memory reversal and extinction tasks (Sakata et al., 2009). Selective decrease of BDNF in the dorsal hippocampus of male mice, through the injection of a lentiviral vector, results in deficits in spatial memory and a reduction in extinction of conditioned fear (Heldt et al., 2007). The same mouse model has impairments in contextual fear learning (Liu et al., 2004) but enhanced contextual fear learning has been observed in early-onset forebrain-restricted BDNF mutant mice (Emx-BDNF^{KO}) (Gorski et al., 2003).

Comparatively, studies have found that the BDNF HET memory impairment is exacerbated by using a “second hit” such as chronic CORT treatment (Klug et al., 2012) and this was associated with changes to multiple CNS systems, including hippocampal glutamatergic receptor subunit expression (Klug et al., 2012). Hippocampal analysis has found that sex interacts with BDNF genotype for protein expression of key systems including glutamatergic markers (Klug et al., 2012; McCarthy et al., 2018) and serotonergic markers (Wu et al., 2012). All the above studies strongly support an integral role for BDNF-TrkB signaling in learning and memory.

1.5.3.2 BDNF-TrkB signaling in schizophrenia

Post-mortem studies have reported reduced levels of BDNF and TrkB in the PFC and hippocampus of individuals with schizophrenia, highlighting the role of BDNF-TrkB signaling in cognitive processes (Reinhart et al., 2015). Significant reductions in BDNF mRNA have been found in the dorsolateral PFC (DLPFC) and PFC of patients with schizophrenia (Hashimoto et al., 2005a; Ray et al., 2014; Weickert et al., 2003). A post-mortem immunohistochemical study found more immunoreactive BDNF staining in the hippocampal neurons of schizophrenia patients (Iritani et al., 2003), implying BDNF secretion dysfunction.

Concordantly, in individuals with schizophrenia, several BDNF transcripts are significantly decreased in the hippocampus (Reinhart et al., 2015; Wong et al., 2010). Conversely, an increase in BDNF in the hippocampus (excluding the entorhinal cortex) but not the frontal cortex has been reported (Takahashi et al., 2000), in disagreement with the studies discussed above. However, this study did not include the surrounding entorhinal and parahippocampal areas in the tissue sample. Importantly, significantly decreased BDNF serum levels have been found in first-episode schizophrenia patients, which was positively correlated with smaller hippocampal volume (Rizos

et al., 2011). This was consistent with other studies examining BDNF in schizophrenia patients (Akyol et al., 2015; Jindal et al., 2010; Xiu et al., 2009). However, a recent study has found that females with schizophrenia had significantly increased plasma BDNF compared to healthy female controls and males with schizophrenia (Weickert et al., 2019). The proposed caveat was that this was a young sample that was not chronically ill, and in fact these results are somewhat expected based on sex differences in schizophrenia aetiology (Weickert et al., 2019).

Similarly, in the prefrontal cortex TrkB mRNA and protein was decreased (Takahashi et al., 2000; Weickert et al., 2005). This is relevant as the hippocampus and PFC communicate frequently for a range of cognitive processes (Spellman et al., 2015). Significant decreases in TrkB protein expression (Takahashi et al., 2000) and TrkB-immunoreactive products in the hippocampus have been observed in schizophrenia patients (Iritani et al., 2003). Significant reductions of both BDNF and TrkB mRNA have been compartmentalised in the hippocampus (Thompson Ray et al., 2011), with BDNF mRNA specifically decreased in the DG, and TrkB mRNA reduced in both DG and the entorhinal cortex. Discordant results can be attributed to different protocols and sample type. For example, studies using enzyme-linked immunosorbent assay (ELISA) do not differentiate between the different isoforms of BDNF, whereas Western Blot analysis does. Results are influenced by illness state (chronic vs acute), sample type (serum or brain tissue) and antipsychotic drug prescription, with clozapine increasing BDNF more so than other antipsychotics (Huang, 2013). A limitation of many studies is that they use exclusively male animals, but as discussed above, there are sex differences in both development and manifestation of symptoms. Additionally, there are sex differences in the adolescent trajectory of BDNF and TrkB protein expression in both the prefrontal cortex and hippocampus of mice (Hill et al., 2012b). The

collective evidence strongly suggests that BDNF-TrkB signaling is altered as part of the pathophysiology of schizophrenia.

1.6 The BDNF^{Val66Met} Single Nucleotide Polymorphism

The human *BDNF* gene has been reported to have hundreds of SNPs, most of which occur within non-coding *BDNF* gene regions. However, the functional consequences of the majority of SNPs are yet to be determined. The functional BDNF Val66Met polymorphism has been associated with a range of symptom categories and psychiatric illnesses (Chen et al., 2006; Notaras et al., 2015a) and is considered a modifier of mental illness phenotype (Chao et al., 2008a). This mutation involves the change from a valine (Val) to methionine (Met) at codon 66 (nucleotide 196) in the 5' pro-region of BDNF (Notaras et al., 2015b). The frequency of this polymorphism varies by ethnicities. It is present in 30% of Caucasians but up to 72% of Asians (Petryshen et al., 2009).

1.6.1 Molecular Biology of the BDNF^{Val66Met} Polymorphism

The Val66met substitution occurs within a sortilin interaction region that disrupts the sorting of BDNF^{Val66Met} to the activity-dependent release pathway (Chen et al., 2005), while constitutive BDNF release is unchanged (Egan et al., 2003). The molecule translin controls BDNF mRNA trafficking and the BDNF^{Val66Met} polymorphism disrupts the binding of translin (Chiaruttini et al., 2009). Subsequently, there is a disruption to targeting of dendritic BDNF mRNA (Chiaruttini et al., 2009). The sum effect of this mutation is decreased activity-dependent secretion of BDNF into the synapse (Chen et al., 2006; Notaras et al., 2015b). This has neuronal and structural consequences (Dincheva et al., 2012) including: smaller hippocampal architecture (Takahashi et al., 2008) and reduction in frontal gray matter volume (Ho et al., 2007), which may contribute to the pathophysiology of cognitive deficits associated with this mutation due to altered hippocampal

structure (Notaras et al., 2015b). Functionally, this SNP disrupts N-methyl-D-aspartate receptor (NMDA) plasticity at key brain regions including the hippocampus (Bath et al., 2012b; Ninan et al., 2010a) and mPFC (Pattwell et al., 2012). This is due to the decreased secretion of BDNF. Ninan et al. observed this through a decrease in NMDA receptor-dependent LTP and LTD (Ninan et al., 2010a).. More recently Afonso et al. (Afonso et al., 2019) reported that BDNF increases NMDAR-dependent LTP through an increase in Pyk2. In turn, this causes an accumulation of NR2B containing receptors in the hippocampal neurons to increase mEPSCs (Afonso et al., 2019). Therefore, BDNF influences the plasticity of NMDA plasticity at key brain regions for cognition.

1.6.2 The BDNF^{Val66Met} Polymorphism, Cognition and animal models

The Val66Met polymorphism has been associated with poorer episodic memory and altered hippocampal functioning in humans (Egan et al., 2003). The first BDNF^{Val66Met} mouse model was developed by Prof. Francis Lee's group; a transgenic knock in mouse that was designed to have a BDNF_{Met} knock-in allele (Chen et al., 2006). The transcription of BDNF_{Met} in these mice was regulated by endogenous mouse promoters. An alternative model, used in the present study and generated by Prof. Joseph Gogos (Cao et al., 2007), uses a genetic construct that expresses human BDNF (hBDNF) *in vivo* and does not modify endogenous mouse promoters. In this knock-in mouse model, LTP and long-term depression (LTD) are disrupted in the hippocampus of BDNF^{Met/Met} mice compared to BDNF^{Val/Val} control mice (Bath et al., 2012b; Ninan et al., 2010a). hBDNF^{Met/Met} mice have been found to have disruptions to spatial memory (Notaras et al., 2016a) and exhibit deficits in extinction learning (Dincheva et al., 2012) and show a depression-like phenotype (Notaras et al., 2017b). Interestingly, Met/Met mice have been shown to have better cognitive flexibility (Vandenberg et al., 2018), supporting the hypothesis that Met/Met carriers are more responsive to environmental factors (Notaras et al., 2016a; Vandenberg et al., 2018).

There is limited evidence that links the BDNF^{Val66Met} SNP to risk of developing schizophrenia (Kanazawa et al., 2007; Kawashima et al., 2009; Naoe et al., 2007; Qian et al., 2007; Zintzaras, 2007) but as detailed above, there is strong evidence that the BDNF^{Val66Met} SNP modulates cognitive symptoms associated with schizophrenia. This includes episodic memory (Tan et al., 2005), working memory (Lu et al., 2012), visuospatial processing (Ho et al., 2006) and attention (Xiu and Zhang, 2010).

1.7 Stress plays a role in cognition and schizophrenia

As mentioned in section 1.2.3, one theory for the onset of psychiatric illness like schizophrenia is the two-hit hypothesis. While the ‘first hit’ is postulated to occur in early development, the ‘second hit’ focuses on external stressors that compound with the initial hit to precipitate the development of mental illness. There is a well-established literature on the effect of stress on the brain, including disturbances to cognition (Colciago et al., 2015; Jayatissa et al., 2008; Mirescu and Gould, 2006; Rainer et al., 2012) and the pathophysiology of schizophrenia model (Brown, 2011; Holtzman et al., 2013; Magariños et al., 2018; van Os et al., 2010).

1.7.1 The Hypothalamic-Pituitary Axis

The hypothalamic pituitary adrenal (HPA) axis is the well-conserved control centre for the body’s stress response. It is a negative feedback loop comprising of key central and peripheral tissues. Briefly, the paraventricular nucleus of the hypothalamus releases corticotrophin releasing hormone (CRH) that initiates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the bloodstream (Charmandari et al., 2004). The ACTH is destined for the adrenal glands, whereby it stimulates production and release of glucocorticoids into the bloodstream (Kalman and Spencer, 2002). In humans this is cortisol and the rodent equivalent is

corticosterone (Papadimitriou and Priftis, 2009). Glucocorticoids cross the blood-brain barrier to bind glucocorticoid (GR) and mineralocorticoid (MR) receptors (Kalman and Spencer, 2002). Upon binding to GR, the CORT-GR complex is transported to the nucleus and dissociated for binding to glucocorticoids responsive elements present in DNA of glucocorticoid-responsive genes (Bamberger et al., 1996; Charmandari et al., 2004), which may occur within exon IV promoter of *Bdnf* (Adzic et al., 2019; Chen et al., 2017a).

1.7.2 Stress, cognition and schizophrenia

The ‘inverted-U’ response to stress (Sapolsky, 2015), refers to the positive effects on cognition that small amounts of stress provide, while chronic stress can lead to cognitive deficits (LePine et al., 2004). The HPA axis moderates the stress response, but it can cause damage through prolonged release of glucocorticoids (Du and Pang, 2015). Dysregulation of this loop can have a range of negative effects upon cognition. In the hippocampus there is a dense expression of GR, and it is thought that the excess activity of glucocorticoids here could be contributing to the cognitive deficits associated with chronic stress (Jayatissa et al., 2008; Mirescu and Gould, 2006; Rainer et al., 2012). In rodent studies, excess activation of GR disrupts both structure and function of the hippocampus. Structurally, chronic stress can lead to altered synaptic terminal assembly (Magariños et al., 1997), loss of dendrites (Vyas et al., 2002), apoptosis (Arimoto-Matsuzaki et al., 2016) and reduced hippocampal volume (Lee et al., 2009).

Functionally, chronic stress disrupts LTP (Pavlidis et al., 1993), reduces neurogenesis (Gould and Tanapat, 1999) and alters neurochemical signaling (Luine et al., 1993). These alterations to structure and function result in disrupted cognitive performance including spatial memory (Conrad et al., 1996) and recognition memory (Elizalde et al., 2008). Stress has been recognized

as a major environmental risk factor in the pathophysiology of schizophrenia (Brown, 2011; van Os et al., 2010). Stress is considered to be a common factor for the onset of related mental disorders such as anxiety and depression (Binder and Nemeroff, 2010) and bipolar disorder (Dienes et al., 2006), potentially affecting common biological pathways such as the BDNF-TrkB signalling pathway (Nuernberg et al., 2016).

Modelling stress that can precipitate mental illness in humans is difficult. Environmental stress paradigms can produce different effects across studies (Magariños and McEwen, 1995; Roth et al., 1982). Other stress paradigms such as restraint stress are broader in their subsequent molecular changes, which makes interrogation of results more complex. The application of chronic CORT through drinking water mimics the physiological parameters of chronic stress so that a focus on GR-mediated effects can be achieved. A consideration of the physiological stress paradigm that we chose to use is whether it is unlikely to be mimicking psychosocial and other complex stressors that precipitate schizophrenia in humans. However, as a physiological paradigm it directly replicates specific biological changes that we wanted to investigate and provides an accurate way to investigate interactions between glucocorticoids, excitatory/inhibitory markers and BDNF-TrkB signalling.

1.7.3 Relationship between glucocorticoids and BDNF-TrkB signaling

It has been postulated that BDNF may be a molecular conduit for the broad range of effects that stress can have on both structure and function of the CNS. As explored earlier in the chapter, BDNF is a vital neurotrophin that moderates a variety of activities that underpin cognition including synaptic activity (Tyler and Pozzo-Miller, 2001), morphology (Horch and Katz, 2002; McAllister et al., 1997) and neurogenesis (Rossi et al., 2006; Scharfman et al., 2005). There is a range of evidence to show that glucocorticoids and BDNF-TrkB systems interact in the

hippocampus. Hippocampal neurons co-express TrkB with GR and MR, indicating that these two signaling systems can interact (Daskalakis et al., 2015; Jeanneteau et al., 2012). BDNF has been found to phosphorylate GR *in vitro* (Lambert et al., 2013), and conversely glucocorticoids via their genomic mechanism can increase phosphorylation of TrkB (Jeanneteau et al., 2008) (Figure 1.11). Additionally, GR activity on TrkB can dampen downstream PLC γ signaling and subsequently decreased glutamate release (Numakawa et al., 2009). This can have cognitive consequences, with convergence upon the ERK1/2 pathway by BDNF-TrkB signaling and glucocorticoids activation reported to enhance fear memory (Revest et al., 2014). This convergence can go either way, with BDNF initiated ERK signaling also dampened by GR-TrkB interaction (Kumamaru et al., 2011). Stress has been shown to negatively impact BDNF-TrkB signaling (Buckley et al., 2007). Chronic CORT has been shown to decrease levels of BDNF mRNA and protein, as well as intracellular BDNF content (Nitta et al., 1999). However this is not a one-way relationship, with evidence available that BDNF can moderate HPA-axis regulation via CRH expression (Jeanneteau et al., 2012) and influence the glucocorticoids transcriptome (Lambert et al., 2013). BDNF treatment has been shown to phosphorylate specific residues on GR, serines 155 and 287, and of these 287 has been directly linked to stress signaling (Lambert et al., 2013). BDNF treatment is able to do this via TrkB signalling directing key intracellular pathways such as CREB which can facilitate the recruitment of GR cofactors at selected promoters. (Lambert et al., 2013). In summary, there is strong evidence that not only do both these systems contribute to the pathophysiology of cognitive symptoms of schizophrenia, but that there is a functional relationship between these two systems. Subsequently they should be investigated alongside each other for further elucidation of their combined role in the cognitive symptoms of schizophrenia.

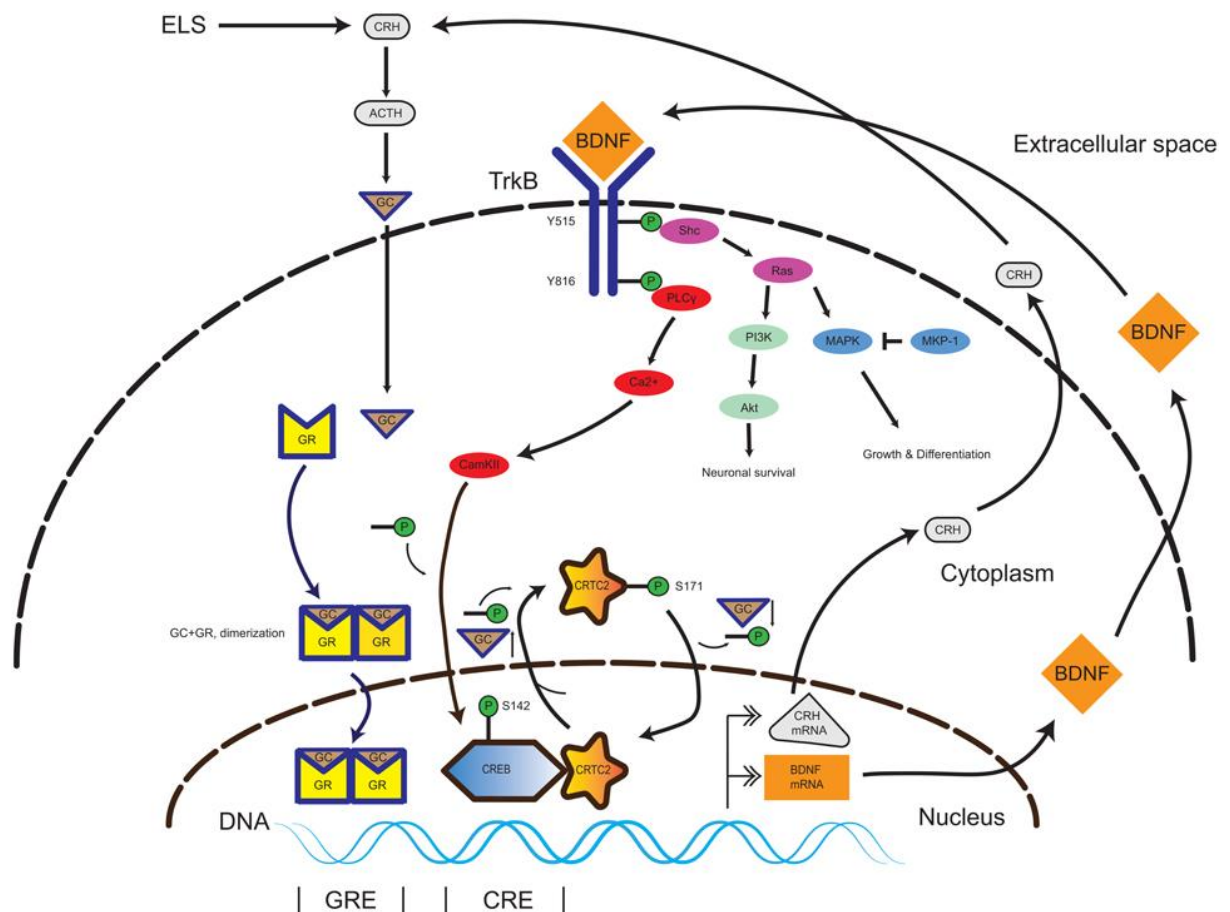


Figure 1.11 - Overview of the interaction between TrkB and GR signaling pathways in the brain

Exposure to stress such as early life stress (ELS) activates the HPA axis and results in increased glucocorticoids. Glucocorticoids bind to GR (glucocorticoids-GR complex) and glucocorticoids-GR complex acts at the BDNF promotor, thereby increasing basal BDNF. Source (Daskalakis et al., 2015)

1.8 Excitatory and Inhibitory Systems of the CNS

The two main neuronal communication pathways are the excitatory and the inhibitory systems. Afferent and efferent information is communicated through the excitatory system, and its activity is moderated by the inhibitory system, which can block, or inhibit, excitatory signaling.

Dysfunction of the inhibitory circuits and consequently the tilting of the excitatory/inhibitory balance towards over-excitation is a major contributor to cognitive deficits present in schizophrenia (Daskalakis et al., 2002; Heckers and Konradi, 2014; Yizhar et al., 2011).

1.8.1 Excitatory Network in the CNS

The primary neurons of the excitatory system are pyramidal cells (Figure 1.12A) that can chemically and electrically communicate between brain regions (Cembrowski and Spruston, 2019). Glutamate is the main neurotransmitter of the excitatory system, making it integral for neuronal communications. It binds to a range of receptors including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), kainite and metabotropic glutamate receptors (mGluR) (Parpura et al., 2017). Of particular interest is the glutamate-binding ionotropic NMDA receptors (NMDAR; also referred to as GluN), due to their well-characterized role in cognitive processes and schizophrenia (Balu, 2016). NMDAR are constructed as heteromeric tetramers by different combinations of NMDAR subunits. Usual combinations include one GluN1 subunit and at least one or more of the GluN2(A-D) or GluN3(A,B) subunits (Paoletti et al., 2013).

The GluN1 subunit can be alternatively spliced, providing further scope for heterogeneity of the receptor (Horak and Wenthold, 2009; Traynelis et al., 1995). These subunits create a pore, which at resting membrane potential is blocked by extracellular magnesium. The activation of NMDAR requires postsynaptic depolarization to remove the magnesium block that occurs simultaneously with the binding of glutamate to NMDAR, and finally, the binding of glycine or D-serine at the GluN1 subunit (Sanz-Clemente et al., 2013). These subunits can be functionally discrete (Cull-Candy and Leszkiewicz, 2004; Henson et al., 2010) and are expressed differentially across brain

regions (Farrant et al., 1994; Laurie and Seeburg, 1994). NMDAR are located both at the pre- and post-synaptic sites, positioning them well to play vital roles in LTP and plasticity (Corlew et al., 2008; Paoletti et al., 2013). Both processes are highly implicated as fundamental foundations of cognitive processes such as learning and memory (Nithianantharajah and Hannan, 2006; Vierk et al., 2014). The GluN2A and 2B subunits are highly expressed in the hippocampus and play an important role in synaptic plasticity (Sanhueza et al., 2011). The ratio of GluN2A/2B contributes to learning and memory. If this ratio has a high GluN2A content, this is better for learning and memory. The theory is that there is less opportunity for inappropriate synaptic plasticity due to a small window of synaptic response time, thereby increasing the threshold for LTP induction and making potentiation harder to reach (Sanz-Clemente et al., 2013). Conversely, over-activation of NMDAR can lead to excitotoxicity. Excitotoxicity is characterised by hyper extracellular concentrations of glutamate, which over activate NMDAR and allow an excess of calcium influx into the NMDAR. The hyper-levels of calcium activate a range of enzymes that cause cell damage, with subsequent cell death and a variety of detrimental neuronal and cognitive consequences.

The glutamatergic system has been implicated in the pathophysiology of schizophrenia. Variants in genes responsible for AMPA receptor trafficking have been identified as risk factors for schizophrenia (Kos et al., 2016), with reduced expression of NMDAR subunits in key areas mPFC and hippocampus (Balu, 2016). Comparatively, while mGluRs are a target for schizophrenia treatment (Ellaithy et al., 2015), and have been identified in a range of GWAS studies they are not convincingly implicated in the pathophysiology of schizophrenia (Ellaithy et al., 2015)

In summary, the NMDAR are key molecular markers for cognitive processes, and the interaction between NMDAR subunits, BDNF TrkB signalling and stress should be investigated in mouse models of schizophrenia.

1.8.2 The Inhibitory Network in the CNS

The inhibitory network moderates the activity of the excitatory pyramidal networks (Otsuka and Kawaguchi, 2009). The main neurotransmitter for the inhibitory system is gamma-aminobutyric acid (GABA), which mainly moderates neuronal excitation. GABA is synthesized by the enzyme glutamate decarboxylase (GAD). Glutamate is decarboxylized to GABA and CO₂ by GAD and the co-factor pyridoxal phosphate. There are two isoforms of GAD, with molecular weights of 65 and 67 kDa, denoted as GAD65 and GAD67. These are encoded by the *Gad1* and *Gad2* genes, respectively (Pinal and Tobin, 1998b). These are encoded by the *Gad1* and *Gad2* genes, respectively (Pinal and Tobin, 1998b).

GAD has been found to be altered in schizophrenia. GAD mRNA has been found to be both decreased in post-mortem brains of schizophrenia in the hippocampus (Thompson Ray et al., 2011) and PFC (Akbarian et al., 1995; Curley et al., 2011; Volk et al., 2012). GAD67 has been found to be increased in post-mortem schizophrenia brains when measured by optical densitometry for axon terminals (Mabry et al., 2019) and Western Blot for protein (Guidotti et al., 2000; Schoonover et al., 2017).

This thesis is primarily interested in GAD67 because it produces greater than 90% of basal GABA (Asada et al., 1997; Kash et al., 1997). Subsequently GAD67 is commonly used as a GABAergic marker (Engel et al., 2001a).

1.8.2.1 Characteristics of Inhibitory Neurons

The inhibitory circuit is modulated by GABAergic inhibitory neurons in the mammalian CNS. Inhibitory neurons (IN) comprise ~15% of rodent hippocampal cells (Pelkey et al., 2017) and surround excitatory pyramidal neurons through extensive arborisation to orchestrate their activity (Beierlein et al., 2000; Fuchs et al., 2007). IN serve vital roles in brain function, as this extensive

arborisation allows each IN to synapse on many pyramidal cells to coordinate local network firing and prevent over-excitation for proper circuit functioning (Hu and Vervaeke, 2018). IN can be differentiated by their morphology, electrophysiological and chemical properties (Batista-Brito and Fishell, 2009). There are over 20 different subtypes of GABAergic IN in the hippocampus, which are classified based on their unique morphology, specific targets (e.g. pyramidal cell or other interneuron), innervation and electrophysiological output as well as specific molecular markers such as calcium binding proteins or peptide expression profiles (Kelsom and Lu, 2013b). Fourteen molecularly unique mouse interneuron classes were determined by single-cell RNA sequencing (Zeisel et al., 2015), deftly illustrating the variety of interneurons present in the brain. This creates a dynamic system, as the IN heterogeneity creates the capability of inhibiting pyramidal and other interneurons at different points along the cell body, and subsequently at different hierarchical levels of cell to cell communication as an action potential moves down the cell.

For this thesis, IN are identified by the calcium binding protein or peptide expressed. Specifically, parvalbumin (PV), somatostatin (SST) and calretinin (CAL) are a focus. In the mouse cortex, PV-expressing interneurons constitute ~40% of GABAergic interneurons while SST and CAL each constitute ~25%, albeit with significant overlap (Miyoshi et al., 2007; Xu et al., 2006). PV and SST were selected due to their role in the pathophysiology of schizophrenia (discussed in detail in section 1.8.3.1 and 1.8.4.1) (Fung et al., 2014; Morris et al., 2008) and their reliance upon functional BDNF-TrkB signaling during development (Du et al., 2018; Hashimoto et al., 2005a), while CAL was chosen as a control to contrast with PV and SST. CAL-expressing interneurons appear to be relatively spared in schizophrenia (Brisch et al., 2015).

Studies have demonstrated protracted development of inhibitory GABAergic interneurons during adolescence in regions of the brain pertinent to schizophrenia, namely the prefrontal cortex and hippocampus (Donato et al., 2015; Wu et al., 2014). Concordantly, IN have been found to be key regulators of multiple cognitive processes including working memory (Kim et al., 2016a). The different IN exert their inhibitory action differentially, targeting different architecture of pyramidal neurons and each other (Gulyás et al., 1999; Tatti et al., 2017; Yavorska and Wehr, 2016), as well as expressing and functioning through different GABA receptors (Booker et al., 2017; Schulz et al., 2018). Distinct homeostatic regulatory pathways have been observed for individual IN (Horn and Nicoll, 2018), indicative of discrete functionality between the subtypes. While there is evolution conservation of IN between primates and humans (Sherwood et al., 2010), they are not necessarily conserved from mouse to man (Raghanti et al., 2010) but are very similar (Mayer et al., 2018), and supportive of the evolution of the mammalian cerebral cortex (Raghanti et al., 2010).

1.8.2.2 *Inhibitory Interneurons and the BDNF-TrkB signaling system*

BDNF is critical for inhibitory network development and function (Fiorentino et al., 2009; Sakata et al., 2009). BDNF-TrkB signaling directs inhibitory synapse assembly (Chen et al., 2011; Rico et al., 2002), moderates GABAergic synaptic plasticity (Park and Poo, 2013) and neurotransmission (Jovanovic et al., 2004a). BDNF heterozygosity alters inhibitory cell density and protein expression in the PFC of mice (Du et al., 2018) and disruptions to BDNF-TrkB signaling at IN can lead to cognitive dysfunction in mice (Grech et al., 2019b; Lucas et al., 2014; Xenos et al., 2017b). Male BDNF^{Met/Met} mice have been found to have reduced GABAergic inputs onto pyramidal neurons in the hippocampal CA1 region (Chen et al., 2017c). However, the different IN have differing degrees of relation to the BDNF-TrkB signaling pathway. Up to 80%

of PV-positive cells and 50% of SST-positive cells co-express the TrkB receptor but less than 20% of CAL neurons have been found to co-express with TrkB (Gorba and Wahle, 1999). PV genotypic expression requires both BDNF and TrkB, while SST only needs BDNF (Glorioso et al., 2006b). BDNF has been found to promote the differentiation of hypothalamic SST neurons (Loudes et al., 2000) and the maturation and development of CAL neurites (Iwasaki et al., 1998). In summary, there is encouraging evidence that there is a two-way relationship between the BDNF-TrkB signalling pathway and inhibitory interneurons. However, the way they intersect to precipitate cognitive deficits in mental illness is not completely understood and this limits research's capacity to target this intersection for development of targeted treatments.

1.8.2.3 *Inhibitory interneurons, cognition and schizophrenia*

Across the research pipeline, evidence suggests that INs may act as the conduit between pathophysiology and cognitive symptoms of schizophrenia or psychiatric disorders. Psychiatric human post-mortem tissue studies found altered inhibitory cell density and protein expression in brain regions highly implicated in cognitive functioning (Fung et al., 2014; Hashimoto et al., 2005a; Konradi et al., 2011). Animal studies have found that disruptions to IN development lead to cognitive disruptions in adulthood (Cho et al., 2015) and *in vivo* optogenetic silencing of INs disrupted higher order functioning (Murray et al., 2015b). Key INs and their relationship to BDNF-TrkB signaling, cognition and schizophrenia are explored in more depth below.

1.8.3 Parvalbumin

PV is a calcium binding protein and PV-expressing interneurons (PV-IN) represents 30-50% of INs (Pelkey et al., 2017). It is most commonly found in cortical layers 2-6 and targets the soma and proximal dendrites of pyramidal neurons (Yavorska and Wehr, 2016) and of nearby

minicolumns (Tatti et al., 2017) – excitatory pyramidal cells that are structurally organised into vertical columns that transverse the cortical layers of the brain. PV-IN are fast-spiking and generate rhythmic and repetitive neural firings of electrical impulses at a high frequency (30-80Hz), termed gamma oscillations (Gulyas et al., 2010; Kuki et al., 2015; Sohal et al., 2009). These oscillations synchronise neuronal activity with great precision in local neural networks. PV-IN can elicit this behaviour due to three criteria: 1) depolarizing and repolarizing rapidly and accurately 2) fast, precise and efficient GABA neurotransmission 3) exploitation of gap junctions between PV neurons to amplify gamma oscillation magnitude (Nakazawa et al., 2012). PV-IN are phase-locked to gamma oscillations (Jonas et al., 2004; Nakazawa et al., 2012; Pelkey et al., 2017), which are thought to be critically necessary for higher order brain processes, including attention and memory (Howard, 2003; Jensen et al., 2007; Uhlhaas and Singer, 2010). They are expressed in the hippocampus (Pelkey et al., 2017) and mPFC (Ueno et al., 2018), as well as other CNS sites including the amygdala (Lucas et al., 2016) and hypothalamus (Siemian et al., 2019). PV protein is also expressed at heart and muscle peripheral sites {Asp, 2016 #2585}, which makes it a broadly active protein. PV-IN largely target the soma and perisomatic dendrites, as well as the axon initial segment of PNs where action potentials are generated (Pelkey et al., 2017). PV-IN can somatically inhibit themselves (Scheyltjens and Arckens, 2016) and innervate each other in the perisomatic region (Gulyás et al., 1999). Compared to CAL-IN, PV-IN have the largest dendritic tree, thickest dendrites and lowest ratio of inhibitory inputs, and highest density of excitatory inputs (Gulyás et al., 1999). Morphologically PV-IN can be divided into three main types: axo-axonic (chandelier), basket and bistratified (Booker and Vida, 2018; Pelkey et al., 2017) (see Figure 1.12B-D). Basket cells (BC) (Figure 1.12B) are the most common PV-expressing cell in the CA1, and are characterised by aspiny and bitufted dendritic trees (Pelkey et al., 2017) with a low membrane resistance that allows them to be fast spiking (Booker and Vida, 2018). One PV-BC can moderate up to 2,500 pyramidal cells and innervates the perisomatic regions of excitatory cells (Pelkey et

al., 2017). PV-BC can contact other PV-BC and form dendro-dendritic synapses with other PV-BC (Booker and Vida, 2018). Axo-axonic cells (AACs) (Figure 1.12C) are characterised by aspiny, radially orientated dendrites that can span all cell layers or horizontally spanning dendrites that specifically target the axon initial segment of excitatory cells (Pelkey et al., 2017). These inhibitory cells are very well connected, with one AAC able to moderate the activity of up to 1300 pyramidal cells (Booker and Vida, 2018). The final morphological group is the bistratified cells (BiStr) (Figure 1.12D). These cells have vertically orientated dendrites that synapse with pyramidal cells via fast inhibitory postsynaptic currents (IPSCs) or horizontally orientated dendrites that co-express SST. The latter type produces sustained inhibition due to an absence of short-term depression (Booker and Vida, 2018). PV-BiStr can additionally co-express neuropeptide Y (NPY) (Pelkey et al., 2017), further diversifying this subtype.

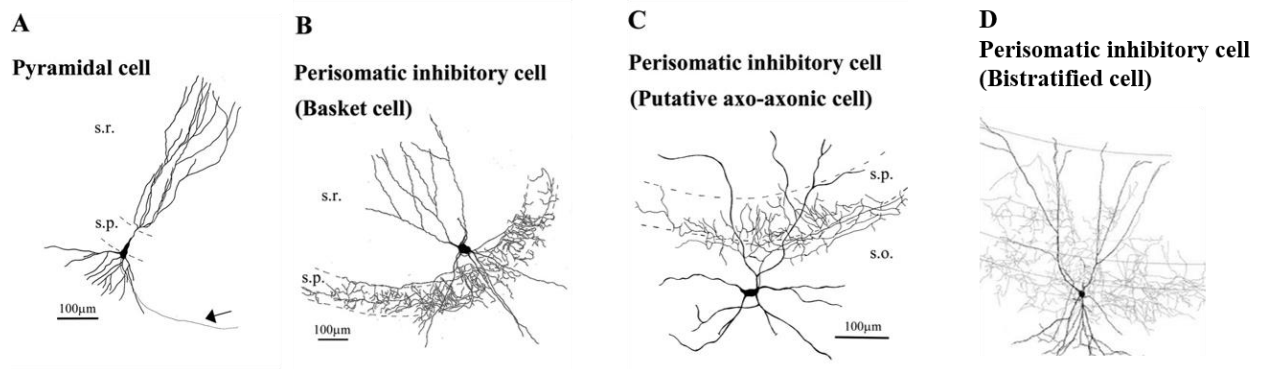


Figure 1.12 - Comparison between excitatory and inhibitory cell types

Comparison between excitatory pyramidal cell (panel A) and the various morphological types of parvalbumin interneurons. The basket cell (panel B) is characterised by aspiny and bitufted dendritic trees. The axo-axonic cell aka chandelier cell (panel C) is characterised by aspiny, radially orientated dendrites. The bistratified cell (panel D) is characterised by vertically orientated dendrites. S.R. = stratum radiatum and S.P. = stratum pyramidale. Panels A-C source (Hájos et al., 2004) and panel D source (Booker and Vida, 2018).

1.8.3.1 Parvalbumin, cognition and schizophrenia

PV-IN have a very important role in hippocampal function (Zhang and van Praag, 2015). Human studies have found cognitive deficits to be associated with disrupted gamma oscillations (Chen et al., 2014; Lewis et al., 2012; Minzenberg et al., 2010). Individuals with schizophrenia have disorganised gamma oscillations when performing cognitive tasks (Cho et al., 2006), or an absence of gamma modulation in response to changes in cognitive load (Basar-Eroglu et al., 2007). Working memory impairments are associated with disrupted gamma oscillations (Basar-Eroglu et al., 2007; Haenschel et al., 2009), potentially through dysregulation of BDNF-TrkB signaling at PV-IN in the PFC (Sakata et al., 2013). Post-mortem studies found specific reductions in the number of PV-IN in the PFC (Fung et al., 2014; Hashimoto et al., 2005a) and hippocampus

(Konradi et al., 2011; Wang et al., 2011; Zhang and Reynolds, 2002) of individuals with schizophrenia. In mice the disruption of PV-IN molecular function leads to spatial and recognition memory deficits (Fuchs et al., 2007; Murray et al., 2011). Rodent studies have found PV expression and density significantly increased during adolescence in the hippocampus of female, but not male mice, in correlation with serum 17 β -estradiol levels (Wu et al., 2014). In this study, the DHP was particularly responsive to estradiol manipulation. This is behaviourally pertinent as generally the DHP is responsible for modulating memory functions, whereas VHP is responsible for mediating anxiety-related behaviours (Fanselow and Dong, 2010). Sex differences in schizophrenia (onset and symptom severity) have been discussed in section 1.1.4 and sex-dimorphic behavioural phenotypes can arise from disrupted BDNF-TrkB signaling at PV-IN (Lucas et al., 2014). This is interesting for this thesis, as it indicates that sex interacts with both BDNF-TrkB signalling and inhibitory systems providing mechanistic insight into why there are sex differences in the age of onset and cognitive symptoms of schizophrenia. Further elucidating these interactions may inform sex-specific treatment approaches to schizophrenia.

1.8.4 Somatostatin

SST is a neuropeptide (Cammalleri et al., 2019; Sibille, 2017) that is expressed by 30% of INs (Scheyltjens and Arckens, 2016). Typically, SST-IN target PV-IN and the more distal excitatory dendrites, and it is here that intracortical inputs converge (Gentet et al., 2012; Scheyltjens and Arckens, 2016). SST-IN do not inhibit the activity of other SST-IN (Scheyltjens and Arckens, 2016). SST-IN generate low threshold, regular spiking and can be depolarised by single presynaptic pyramidal neurons (Scheyltjens and Arckens, 2016).

SST-IN are a very diverse group that can be divided morphologically (Yavorska and Wehr, 2016). The most common type is defined as Martinotti cells, comprising 50% of layer I SST-IN. Their ovoid-shaped cell bodies are found in layers II/III and V but less so in layer VI. Martinotti cells are further characterized by local bi-tufted dendritic arborization and long, translaminal ascending axon collaterals (Scheyltjens and Arckens, 2016). SST Martinotti cells are part of the disynaptic feedback inhibitory pathway of pyramidal neurons in layer V, meaning they are initiated by pyramidal cells exciting each other. Martinotti cells provide dense inhibition onto layer I dendrites [84, 85], via fast GABA_A receptor-mediated synaptic input [27, 86].

Three subsets of SST-expressing cells have been identified using transgenic mouse lines: GINs-, X98-, and X94-mouse strains (Figure 1.13) (Scheyltjens and Arckens, 2016). These SST-subtypes are distinguished by their morphology, molecular and electrophysiological features. The X98- and GINs-IN are similar to the Martinotti cells, with the X94-IN being most different (Scheyltjens and Arckens, 2016). X98- and GINs-IN target the dendrites of pyramidal cells, exhibit low-threshold spiking and can co-localize with calbindin (CB) and NPY. All of these are features of Martinotti cells. Comparatively, X94-IN inhibit PV-IN and dendrites of pyramidal cells, exhibit an electrophysiological profile similar to that of fast-spiking IN and do not co-localize with other calcium-binding proteins or neuropeptides (Scheyltjens and Arckens, 2016). It has been reported that up to 50% of SST Martinotti cells co-express with CB, NPY, or cholecystokinin (Scheyltjens and Arckens, 2016). In mouse, SST and CAL can co-express in a cell-layer specific manner (Urban-Ciecko and Barth, 2016).

In summary, this is a diverse cell type that is yet to be fully characterised but current literature indicates that it has a large role to play in the excitatory/inhibitory balance that impacts healthy cognition.

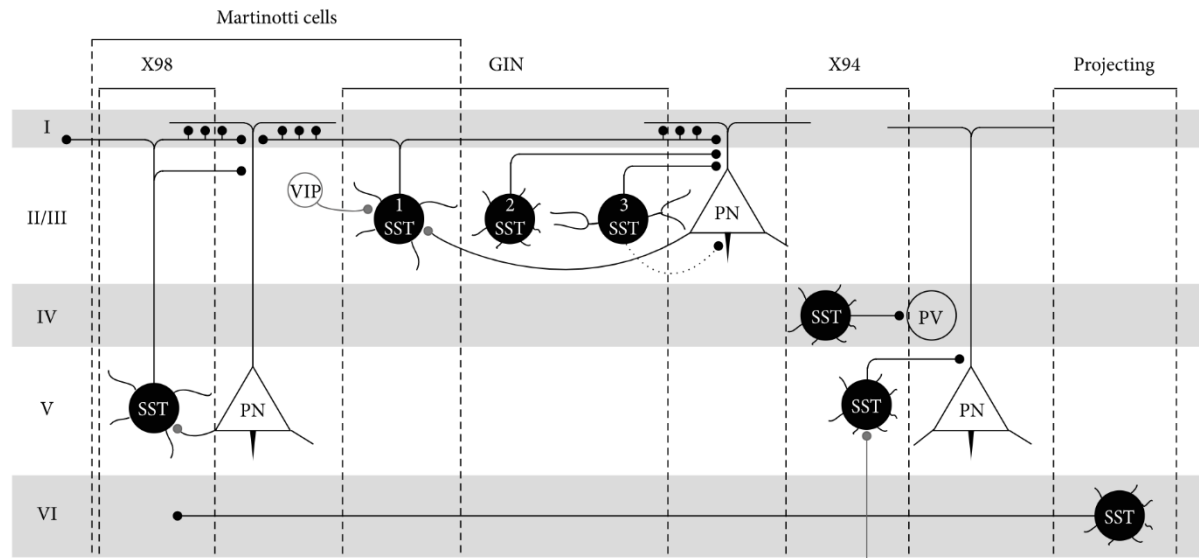


Figure 1.13 - Morphologically differentiated somatostatin interneurons

The different kinds of somatostatin-expressing interneurons. These cells have been identified through transgenic mice and are distinguished by their morphology, molecular and electrophysiological features. Cell layers I-VI shown. Source (Scheyltjens and Arckens, 2016).

1.8.4.1 Somatostatin, cognition and schizophrenia

SST has several roles in the hippocampus, moderating both hyperpolarization and inhibiting the release of glutamate from excitatory cells (Yavorska and Wehr, 2016). SST cells release GABA for fast inhibition via fast synaptic GABA_A receptors and slow inhibition via metabotropic GABA_B receptors (Urban-Ciecko and Barth, 2016). SST-IN have a role in synaptic plasticity (Scheyltjens and Arckens, 2016), due to their ability to control the activity of pyramidal cells through dendritic inhibition (Booker and Vida, 2018). They are important for integration of sensory information in cortical circuits (Sibille, 2017) through the regulation of LTP (Kluge et al., 2008; Schmid et al., 2016). There is evidence that through the dual inhibition at soma and dendrite sites that SST-IN can enhance gamma oscillation synchrony in the visual cortex (Veit et al., 2017). Indeed, they are important for hippocampal synchrony from early development (Flossmann et al.,

2019). This continues into adulthood, with mPFC SST-IN important for synchrony between the hippocampus and mPFC (Abbas et al., 2018a).

Additionally, changes to SST expression in the rat hippocampus has been found to disrupt spatial memory performance (Cao et al., 2005). Reduced SST mRNA and SST-IN are found post-mortem in the amygdala, hippocampus and DLPFC of schizophrenia patients, implicating SST dysfunction across schizophrenia symptom categories (Alherz et al., 2017; Konradi et al., 2011; Morris et al., 2008; Pantazopoulos et al., 2017). Disinhibition of SST-IN, an electroencephalographic biomarker of positive symptoms of schizophrenia, has been associated with mismatch negativity in mouse (Hamm and Yuste, 2016) and man (Javitt et al., 2017) (Hamm and Yuste, 2016). There is a correlation between NMDAR hypofunction at SST-IN and cognitive symptoms associated with schizophrenia that is evident across mouse and man (Alherz et al., 2017). Decreased SST serum levels have been found in cognitively impaired schizophrenia patients (Reinikainen et al., 1990), while disrupted theta oscillations have been suggested as a neurophysiological marker for neuroleptic-naïve schizophrenia patients (Won et al., 2018). There is strong evidence across the research pipeline for SST to be part of the underlying pathophysiology of cognitive symptoms of schizophrenia.

1.8.5 Calretinin

CAL is a calcium binding protein (Rogers, 1987) representing between 10-30% of INs (Cauli et al., 2014; Xu, 2003). This is an especially heterogeneous group with multiple morphological, molecular and electrophysiological possibilities. In rodents, CAL-IN can be morphologically characterised by cell body size (Garas et al., 2018) and by their cytoarchitecture. CAL-IN have been reported to have bipolar, multiple polar and double-bouquet morphology (Cauli et al., 2014; Liu et al., 1996; Tatti et al., 2017) (Figure 1.14). CAL-IN have the smallest dendritic tree and

thinnest shafts but have more GABAergic inputs than PV-IN (Gulyás et al., 1999). Molecularly, CAL-IN can co-express with a range of other neuropeptides (Cauli et al., 2014) and specifically in mouse with SST (Gonchar et al., 2008; Xu et al., 2006). CAL-IN have been characterised by transcription factor, and this has led to the emergence of three CAL-IN subtypes in the rodent dorsal striatum (Garas et al., 2018). These subtypes are defined by structural and topographical profiles. They are identifiable by selective co-expression combinations of CAL with the calcium-binding protein secretagoin (Scgn) and transcription factor specificity protein 8 (SP8), or CAL and the transcription factor LIM homeobox protein 7 (Lhx7). SP8 is a marker of newborn cells while Lhx7 is a marker for cholinergic interneuron development. Additionally, these subtypes are divided by somatic diameter (Garas et al., 2018). In the hippocampus CAL-IN appear to be influenced by multiple signaling pathways and factors including cholinergic dysfunction (Ahn et al., 2018), melatonin (Ramírez-Rodríguez et al., 2014) and to the A β peptide (Verdaguer et al., 2015). Turning to the electrophysiological properties of CAL-IN, there is a range due to the diversity of their molecular and architecture profiles. Bipolar CAL/VIP neurons have a high input resistance (Cauli et al., 2014), while striatal CAL-IN can have unpredictable firing that ranges from spike bursts at peaks of slow oscillations to sporadic single spike firing (Garas et al., 2018). Interestingly, CAL may play a role in human intrauterine cerebellar development by moderating excitatory activity (Pibiri et al., 2017).

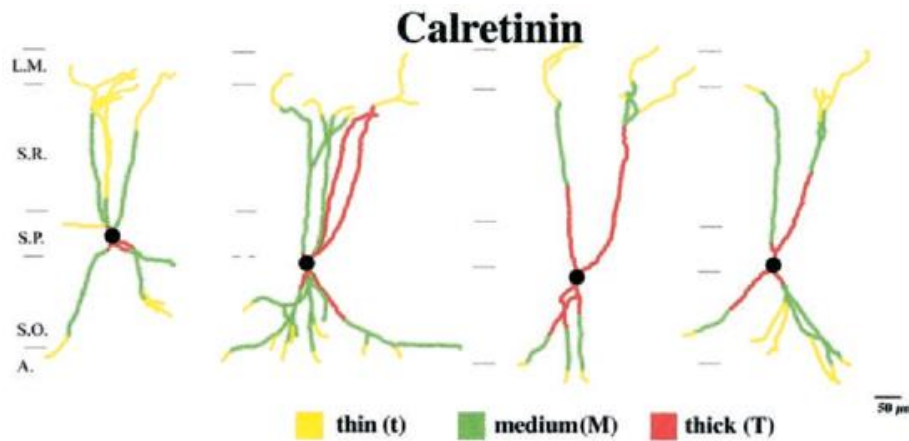


Figure 1.14 - Different examples of calretinin interneurons (CAL-IN) in rodent hippocampus

CAL-IN have a narrow dendritic tree. L.M., Stratum lacunosum-moleculare; S.R., stratum radiatum; S.P., stratum pyramidale; S.O., stratum oriens. Source (Gulyás et al., 1999).

1.8.5.1 Calretinin, cognition and schizophrenia

CAL-IN can inhibit excitatory pyramidal cells and other IN (Booker and Vida, 2018), positioning themselves to integrate inputs (Cauli et al., 2014) and provide disinhibitory effects for neuronal circuits (Booker and Vida, 2018). This is true across rodents to humans (Urbán et al., 2002). These functions ensure appropriate firing of the circuits for healthy cognitive functioning. Additionally, CAL-IN in the dentate gyrus are linked to neurogenesis (Schwaller, 2014; Todkar et al., 2012). While CAL-IN evidently have an important role to play in the proper functioning of neuronal circuits, they are not strongly implicated in the pathophysiology of schizophrenia (Banasr et al., 2017; Knable et al., 2004) and are generally spared in schizophrenia (Brisch et al., 2015; Eyles et al., 2002). Potentially, this may be due to their weaker relationship with the BDNF-TrkB signaling pathway compared to PV- and SST-IN, with ~20% of CAL-IN co-expressing TrkB receptor

compared to ~80% of PV-IN and ~50% of SST-IN (Gorba and Wahle, 1999). It is therefore a good control for analysis of the relationship between BDNF-TrkB signaling pathways and IN.

1.9 Mouse Models of Schizophrenia

1.9.1 Validity criteria of mouse models of schizophrenia

Schizophrenia is a heterogeneous disorder with complex pathophysiology and variability in the presentation of symptoms. No animal model and recapitulate the full complexities of a human psychiatric disorder, however, we can model risk factors associated with the disorder to understand how these risk factors, either on their own, or in combination, impact brain development and behaviour (Jones et al., 2011). Generally, the validity of any animal model depends on the following criteria (Jones et al., 2011; Willner, 1986):

- Face Validity: whether the model has a similar presentation of symptoms to the human illness
- Construct Validity: whether the experimental tests that model undergoes test what they claim to test and can be objectively analysed
- Predictive Validity: whether the model can be used to make predictions for the human population

Additional validity criteria includes (Sjoberg, 2017):

- Internal Validity: whether the model is well controlled for third variable influences
- External Validity: whether the model's outcomes can be generalized to other populations
- Convergent Validity: whether predicted associations between parameters occur
- Discriminant Validity: whether predicted disassociations between parameters occur

Other validity considerations include:

- Mechanistic validity: whether the underlying pathophysiology and subsequent phenotype is the same between model and human

Mouse models can be limited and not meet all validity criteria, which is an important consideration for interpretation of experimental results and their translation to the human condition.

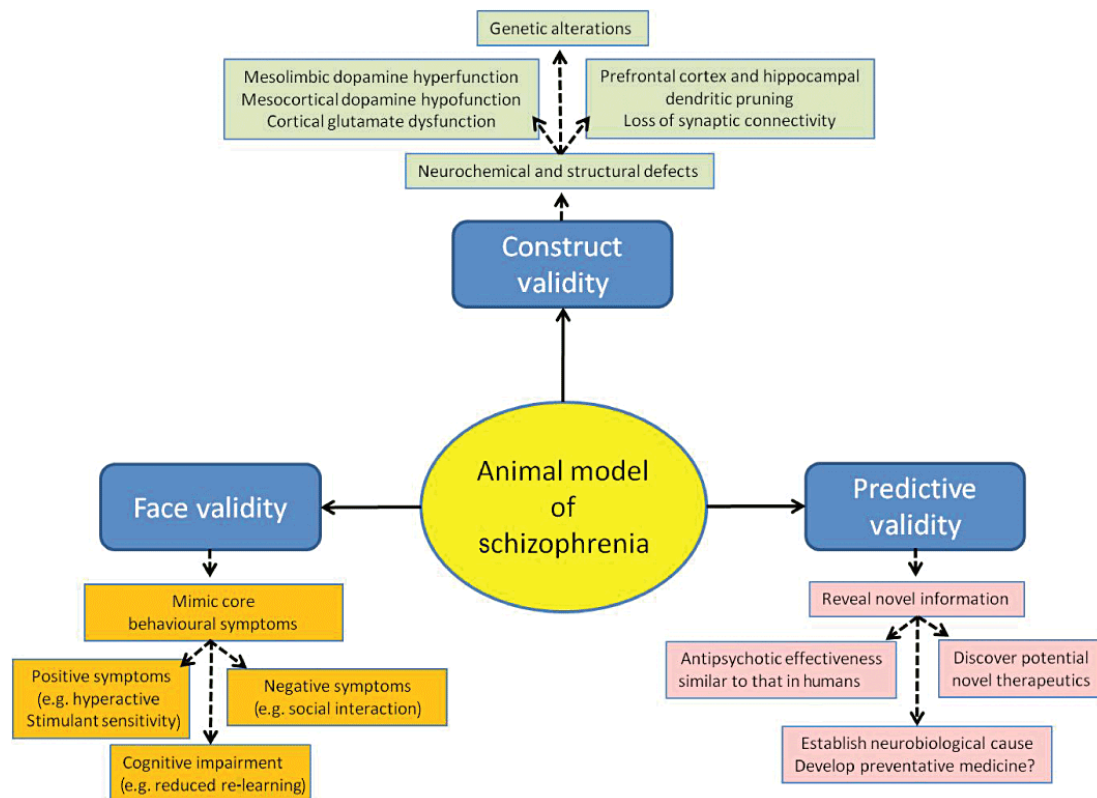


Figure 1.15 - Validity considerations for mouse models of schizophrenia

Mouse models of schizophrenia should express the above parameters in order to be translational for understanding the core symptom categories of schizophrenia. Source (Jones et al., 2011).

1.9.2 Mouse models of schizophrenia

Mouse models can be either holistic or reductionist in nature. A holistic model has the capacity to exhibit most symptom categories and common pathophysiology (Sjoberg, 2017). Comparatively, a reductionist model will have a much narrower focus and may only represent one symptom or pathophysiological feature (Sjoberg, 2017). There are many mouse models of schizophrenia that

mimic the core symptom categories of schizophrenia, and these are induced through a range of models including: developmental, drug-induced, lesion or genetic manipulation (Jones et al., 2011). A key developmental model is the NVHL model previously described in section 1.4.3, which has strong face validity as it exhibits prodromal-like dysfunction and adult-onset prefrontal cortical dysfunction (Cabungcal et al., 2014). A gold standard drug-induced mouse model is the Phencyclidine (PCP) model. Here, the administration of PCP induces schizophrenia-like behaviours across all the core symptom categories (Mouri et al., 2007). Lesions in key brain regions associated with the pathophysiology of schizophrenia, including the PFC and hippocampus, lead to schizophrenia-like symptoms including hyperactivity and cognitive disruptions (Flores et al., 2016). Genetic mouse models of schizophrenia encompass a range of chemical systems including NR1 knock out (KO) animals (glutamatergic), synaptic proteins (dysbindin and DISC1) and GABAergic dysfunction (reelin KO) (Flores et al., 2016). The variety of genetic models reflects the complexity and heterogeneity of schizophrenia, with one genetic manipulation unable to truly reflect the pathophysiology of the disorder (Flores et al., 2016). However, these models provide insight into one branch of the pathophysiology and symptom presentation, providing foundational knowledge on which to build research.

1.9.2 Cognitive Domains assessed in mouse models of schizophrenia

Mouse models provide the opportunity to investigate whether the neuronal systems being experimented upon are involved in cognitive disruptions experienced in schizophrenia. There are six key cognitive domains that are tested in animal models to reflect the human condition including: working memory (e.g. Y-Maze, T-Maze), attention/vigilance (e.g. Prepulse Inhibition), Visual learning and memory (e.g. Novel Object Recognition Task), speed of processing (e.g. 5-Choice Serial Reaction Time Task), reasoning and problem solving (e.g. attentional set shifting)

and social cognition (e.g. Social Interaction) (Hagan and Jones, 2005). The seventh cognitive domain verbal learning and memory is, obviously, unable to be tested for in rodents (Hagan and Jones, 2005). Cognition is a broad term that covers a range of domains, as listed above. Multiple cognitive domains should be characterised when investigating a mouse model, to determine the extent of disruption caused by an experimental condition.

1.10 Aims and Overview of thesis

The overall aim of this thesis is to investigate the interaction between BDNF-TrkB signaling and INs with relevance to the cognitive symptoms of schizophrenia. To do so, three different mouse models of altered BDNF-TrkB signaling were used. The first aim of this thesis was to investigate the interaction between BDNF heterozygosity and different environments, and the effects this had on spatial memory and the excitatory/inhibitory (E/I) balance of the hippocampus. Specifically, whether a “positive environment” could reverse the spatial memory deficit in our “two-hit” model of BDNF haploinsufficiency and CORT treatment, and how this would compare to environmental enrichment (EE) effects in wildtype-like (WT) controls. Subsequently, what were the molecular changes to the BDNF-TrkB signaling pathway and the NMDAR system in the DHP, and if these were modulated differentially by EE according to BDNF genotype, sex, and CORT treatment. The published manuscript that arose from this work is presented in Chapter 3, with unpublished Western Blot data presented in the accompanying supplementary chapter.

The second aim of this thesis was to investigate the consequences of disrupted BDNF signaling on PV-IN upon spatial memory and cognitive flexibility. We crossed a PV-Cre parent with a TrkB heterozygous floxed parent to generate $PV^{cre} \times TrkB^{floxed}$ ($PV-Cre:Fl^{+/-}$) offspring and performed a battery of affective and behavioural tests. We hypothesised that spatial memory and cognitive

flexibility would be impaired due to disrupted BDNF-TrkB signaling at PV-IN. There are sex differences in the onset and manifestation of schizophrenia in humans, with males developing schizophrenia earlier and with more severe negative and cognitive symptoms [33]. Therefore, a second hypothesis was that there would be sex differences in spatial memory performance in response to disrupted BDNF-TrkB signaling at PV-IN. The accepted manuscript that arose from this work is presented in Chapter 4, with unpublished behaviour and immunohistochemistry data presented in the accompanying supplementary chapter.

The third and final aim of this thesis was to investigate IN density in the hippocampus of hBDNF^{Val66Met} mice that have been exposed to chronic CORT treatment. Here, I firstly investigated whether hippocampal inhibitory marker density differed between hBDNF^{Val66Met} genotypes and whether adolescent CORT exposure and sex modified this genotype effect. The submitted manuscript is presented in Chapter 5, with additional immunohistochemistry data and analysis presented in the accompanying supplementary chapter. The following chapter will provide a general overview of materials and methods used for all presented experiments, with Chapter 6 providing a general discussion of all projects, caveats and future directions.

Chapter 2. General Methods

2.1 Animals

All surgical and experimental procedures were conducted according to the guidelines in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia, 8th edition 2013) and approved by the Animal Ethics Committee of the Florey Institute of Neuroscience and Mental Health (Parkville, Victoria, Australia). Three cohorts of mice were used for the studies in this thesis. All three cohorts were bred and behaviourally tested at the Florey Institute of Neuroscience and Mental Health. Behavioural experiments for all three cohorts and 50% of the molecular experiments for cohort 1 were conducted at the Florey Institute of Neuroscience and Mental Health (Parkville, VIC, Australia). Molecular experiments for cohort 2 and 3 were performed at the School of Clinical Sciences, Monash Health (Clayton, VIC, Australia) following the move of the Behavioural Neuroscience Laboratory.

Cohort 1 aided the investigation of the interaction between environmental enrichment and a two-hit neurodevelopmental model on molecular expression of BDNF-TrkB signaling, inhibitory and excitatory protein targets (chapter 3). Cohort 2 included the generation and behavioural testing of PV-Cre \times TrkB floxed mice that were used to investigate the dynamic relationship between PV-IN and the BDNF-TrkB signaling pathway (chapter 4). Cohort 3 included a novel humanized BDNF val66met transgenic mouse strain, which was used to investigate further the relationship between IN and the BDNF-TrkB signaling pathway (chapter 5).

All three cohorts were obtained from respective breeding colonies at the Florey Institute of Neuroscience and Mental health, Melbourne, Australia. All mice had *ad libitum* access to food and water in a temperature-controlled room maintained at $\sim 22^{\circ}\text{C}$ and were on a 12/12 h, 7am/7pm light/dark cycle. For all three cohorts, males and females were housed separately in individually

ventilated cages (IVC, $39.1 \times 19.9 \times 16$ cm, Tecniplast GM500 model, Tecniplast, Italy) unless otherwise described. For cohort 1 there was an average of 3 mice per cage, for cohort 2 there were 2-7 mice per cage and for cohort 3 there were 2-6 mice per cage. A power analysis was run using the G*Power program for cohort 2. A compromise was made between the greatest power (ideal total of 612 mice) and to practise Reduce, Refine and Reuse. This is how the 12-14 mice/group (up to 56 mice) was decided.

2.2 Mouse models and manipulations

2.2.1 Cohort 1 – Developmental “Two-Hit” Mouse Model Combining BDNF Haploinsufficiency and Chronic Glucocorticoid Stimulation

Male and female BDNF HET mice (Ernfors et al., 1994) and WT littermate controls were on a C57BL/6 background and breeders were originally obtained from The Jackson Laboratory (USA). Offspring were randomized into 8 experimental groups: (1) Group-housed males, water and standard housed (SH) (WT n = 7, HET n = 7), (2) Group housed males, CORT, and SH (WT n = 11, HET n = 10), (3) Group-housed males, water, and EE (WT n = 9, HET n = 9), (4) Group-housed males, CORT, and EE (WT n = 11, HET n = 12), (5) Group-housed females, water, and SH (WT n = 15, HET n = 11), (6) Group-housed females, CORT, and SH (WT n = 8, HET n = 10), (7) Group-housed females, water, and EE (WT n = 10, HET n = 10), and (8) Group-housed females, CORT, and EE (WT n = 10, HET n = 8). CORT treatment was administered in the drinking water from week 6-8, while EE was provided for an overlapping period from week 7-9. Mice were given a 2 week wash-out period from CORT exposure before behavioural testing at 11 weeks of age. Figure 2.1 below shows a timeline of experimental conditions for cohort 1.

	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 16
Mice: Male/Female WT/HET	Water or CORT treatment (50 mg/L in drinking water)				Adapt to IVC cages	Y-Maze	Animals euthanized and brain tissue collected
		Standard Housed or Environmental Enrichment					

Figure 2.1 - Timeline of the experiments for cohort 1

Male and female WT and BDNF HET mice were used to investigate whether EE could ameliorate an established spatial memory deficit in a neurodevelopmental “two hit” model. BDNF heterozygosity was the first hit and the second hit was chronic CORT administered in the drinking water. Environmental enrichment was administered during and after chronic CORT treatment.

2.2.2 Cohort 2 – TrkB deletion on parvalbumin-expressing interneurons using cre-lox recombination

Cre recombination involves the removal of a gene that is flanked by a loxP site on either side. Cre recombinase binds at these sites to catalyze recombination. The result is the gene is removed and thus will not be expressed. Cre-lox recombination was used to knock down TrkB receptors on PV-expressing interneurons (Figure 2.2).

The following method was performed to generate mice that have a knockdown of TrkB receptors on PV-expressing cells. PV-Cre (B6;129P2-*Pvalb*^{tm1(cre)Arbr/J}) mice were crossed with TrkB heterozygote floxed (*Ntrk2*^{tm1Lfp}) mice on a C57BL/6 background for a PV-Cre × TrkB heterozygote floxed mouse line. This breeding was based on Lucas et al. (Lucas et al., 2014), who reported that homozygote, but not heterozygote, knockout of TrkB lead to locomotor dysfunction. PV-Cre mice were originally generated by Hippenmeyer et al. (Hippenmeyer et al., 2005). PV-Cre mice (Jackson Laboratories, USA) contain a knock-in allele containing the endogenous

parvalbumin (*Pvalb*) promotor/enhancer elements. This guides Cre recombinase expression specifically to *Pvalb*-expressing cells, and was achieved through the insertion of an IRES-cre-pA cassette into the 3' UTR of exon 5 (Hippenmeyer et al., 2005). This IRES-cre-pA cassette contained the cre coding sequence, internal ribosome entry site (IRES), and polyadenylation site sequence. Subsequently, the construct was electroporated into 129P2/OlaHsd-derived E14 embryonic stem (ES) cells. Recombinant clones were injected into recipient blastocysts to generate chimeric founder animals that were crossed with C57BL/6 mice (Hippenmeyer et al., 2005).

TrkB heterozygote floxed mice (Fl^{+/-}) were kindly donated by Dr. Simon Murray and Dr Junhua Xiao. The original TrkB knockout mouse that lacked both full-length and truncated TrkB receptor isoforms was generated by Luikart et al. (Luikart et al., 2003). To do so, the kinase domain was deleted, which generates an allele expressing only truncated TrkB. This included the removal of 3-kb of genomic DNA, which included the two initiation of transcription sites and the first coding exon of the *trkB* gene. This completely removed *TrkB* gene transcription in mutant allele and subsequently the absence of expression of all TrkB isoforms (Luikart et al., 2003). Through breeding, heterozygous mouse were attained. For Cre-lox recombination, two loxP sites flanked the two transcriptional start sites and first coding exon region of the TrkB gene (Luikart et al., 2005). For PV-Cre:Fl^{+/-} mice the cre recombinase, driven by the *Pvalb* promotor, recognised the loxP sites surrounding the TrkB gene, and removed expression of the TrkB gene in PV-expressing cells. Fl^{+/-} mice were backcrossed three time (N Gen 3) and the PV-Cre mice were N Gen 4.

Mice used in this these were generated from two main breeder pairs: 1) female TrkB Fl ^{+/-} and male PV-Cre/+ and 2) female PV-Cre/+ and male TrkB Fl ^{+/-}. For genotyping, tail tissue samples were sent to Transnetyx (Cordova, TN, USA). For the PV-cre, the forward primer was:

TTAATCCATATTGGCAGAACGAAAACG. The reverse primer was: CAGGCTAAGTGCCTTCTCTACA. For wild-type PV, the forward primer was: TCTCCACTCTGGTGTGAA. The reverse primer was: TCTCCACTCTGGTGGCTGAA. For the TrkB-fl, the forward primer was: CTTGGTCTGATCTCACCTTGCT. The reverse primer was: CAAATCAAGGGTATCAGAGGAAAGCT. For the wild-type TrkB, the forward primer was: CTTGGTCTGATCTCACCTTGCT. The reverse primer was: CAAATCAAGGGTATCAGAGGAAAGCT.

Genotyping determined whether each mouse was a TrkB-floxed genotype and/or PV-Cre genotype. Breeding between two breeding pairs produced the following genotypes: PV-Cre/+;Fl+/-, PV-Cre/+;TrkB+/+, PV-Cre/PV-Cre:Fl+/-, Pv-Cre/PV-Cre:TrkB+/+ and +/+;Fl+/-.

For the first pair of breeders, 48% of offspring were determined to have TrkB floxed genotype (PV-Cre:Fl+/-) and 52% were determined to be PV-Cre:WT. For the second pair of breeders, 42% were determined to have TrkB floxed genotype (PV-Cre:Fl+/-) and 51% were determined to be PV-Cre:WT. The remaining 7% were genotyped as +/+;Fl+/-.

Mice had *ad libitum* access to food and water, except for the duration of the Cheeseboard Maze where they were food restricted to 85% of their body weight. Mice were group housed 2–7 per box and the 4 experimental groups were: 1) male PV-Cre (n = 14) 2) male PV-Cre:TrkB+/- (n= 13) 3) female PV-Cre (n=14) and 4) female PV-Cre:TrkB+/- (n=14).

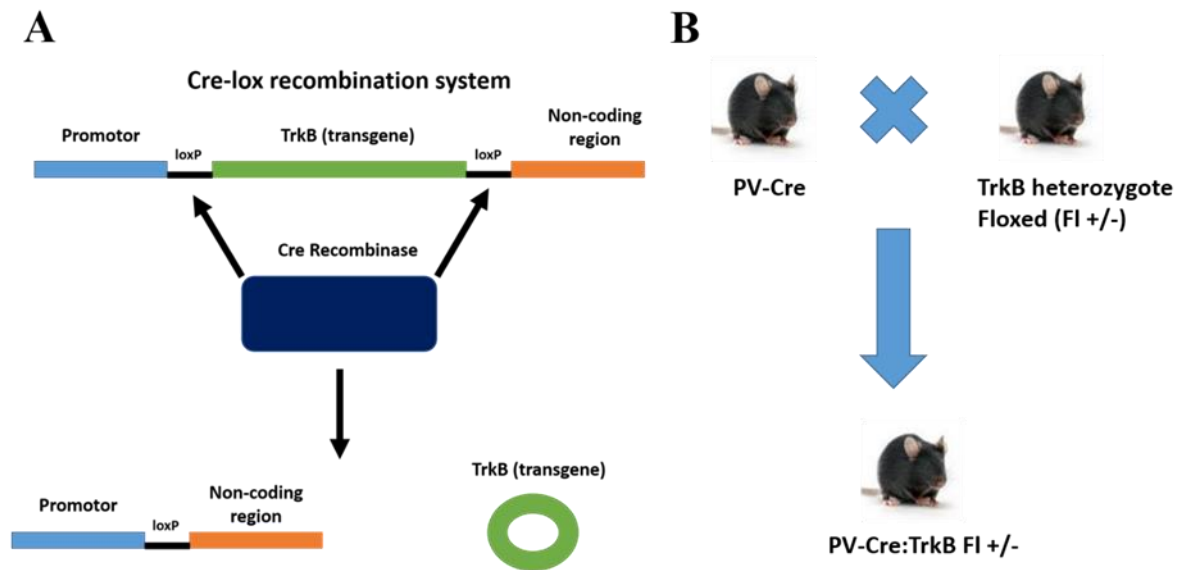


Figure 2.2 – Using cre-lox recombination for a mouse model of global disrupted BDNF-TrkB signaling at parvalbumin-positive cells.

Panel **A** illustrates the cre-lox recombination system. Cre-lox recombination involves the targeting of a specific sequence of DNA and splicing it with the help of an enzyme called cre-recombinase. A target of interest is flanked by loxP sites, in this case the TrkB gene (*Ntrk2*). Cre recombinase binds at these sites to catalyze recombination to remove the TrkB gene, and subsequently it is not expressed *in vivo*. PV-Cre (*B6;129P2-Pvalbtm1(cre)Arbr/J*) mice possess the knock-in allele that contains the endogenous parvalbumin (*Pvalb*) promoter/enhancer elements. These elements confine Cre recombinase expression to *Pvalb*-expressing cells. Panel **B** illustrates the breeding process that used the cre-lox recombination system. PV-Cre mice were crossed with TrkB heterozygote floxed (*Ntrk2tm1Lfp*) mice to specifically ablate the TrkB receptor at parvalbumin-expressing interneurons.

2.2.3 Cohort 3 – hBDNF^{Val66Met} mice exposed to chronic corticosterone stimulation

The hBDNF^{Val66Met} mice used in this study were obtained from the laboratory of Prof. Joseph Gogos at Columbia University. This mouse model expresses human BDNF *in vivo* with no

modifications to endogenous mouse promoters. To generate these animals, a 274 base pair (bp) region that includes a portion of the coding region and 5' untranslated region of the human BDNF gene inclusive of the Val66Met substitution, was amplified from a BDNF^{Val/Val} and a BDNF^{Met/Met} human donor. This was subsequently inserted into the mouse *Bdnf* gene to replace the corresponding mouse sequence and thereby 'humanize' the mouse BDNF peptide amino acid sequence (Cao et al., 2007). Following this, the sequences was cloned into a self-excisable neo cassette, then electroporated into 129Sv/EV embryonic stem cells and assayed for recombination and germline transmission to yield hBDNF^{Val66Met} mice (Cao et al., 2007). Mice were transported to The Florey Institute of Neuroscience and Mental Health and were rederived to establish a breeding colony of hBDNF^{Val66Met} mice. Male and female hBDNF^{Val66Met} mice were generated from hBDNF^{Val/Met} × hBDNF^{Val/Met} breeding pairs on a C57BL/6 genetic background (Notaras et al., 2017d). For the work conducted as part of this thesis, the following samples size of offspring experimental groups were used: (1) Group-housed males, water (Val/Val n = 5, Met/Met n = 4), (2) Group housed males, CORT (Val/Val n = 5, Met/Met n = 4), (3) Group-housed females, water (Val/Val n = 5, Met/Met n = 4), and (4) Group-housed females, CORT (Val/Val n = 5, Met/Met n = 4). E

2.2.4 Chronic corticosterone protocol

In cohort 1 and cohort 3, adolescent/young adult mice were treated with CORT in the drinking water from 6 to 8 weeks of age (see Figure 2.1 and Figure 2.3). These time points were based upon previous studies by our laboratory that show sexual maturation occurs during this period (Hill et al., 2012b). Specifically, that a rapid rise in seminal vesicle weight, serum testosterone and uterine weight is observed around 6–9 weeks of age (Hill et al., 2012b). CORT treatment was chosen over other stress paradigms to mimic the physiological parameters of chronic stress so that

a focus on the interactions between glucocorticoid effects, BDNF-TrkB signaling and cognitive behaviour could be achieved. Other stress paradigms such as restraint stress are broader in their subsequent molecular changes, which makes interrogation of results more complex. In addition, restraint stress protocols can produced quite variable effects in terms of mouse behaviour. Previous research has shown that in mice CORT can be administered between a dose of between 25 and 100 mg/L (Notaras et al., 2017d; Schroeder et al., 2015). For cohort 1 the CORT concentration of 50mg/L was chosen with the assumption that the mice would increase water intake as they matured (Bachmanov et al., 2002), and this CORT concentration would maintain CORT intake relative to body weight. Increased water intake was not tested for, which is a limitation. A high CORT dose has been found in other models to create persistent stress phenotypes, which is important in a chronic model (Gourley and Taylor, 2009; Johnson et al., 2006). Corticosterone-hemisuccinate (Q1662-000 Steraloids Inc, United States) was dissolved in water to a final concentration of 50 mg/L. For cohort 3, the CORT concentration of 25mg/L was used to be consistent with previous research from the laboratory group (Klug et al., 2012). This lower dose was chosen to avoid possible side effects of weight gain and decreased locomotion, which would present confounding effects (Karatsoreos et al., 2010). It is important to note that during my PhD the laboratory were trialling a range of CORT concentrations as our previously used dose of 25mg did not seem to produce the same effect when the laboratory moved locations from MHRI to the new Florey building. This highlights the differences you can experience in animal behavioural testing from one location to another, and the need to report on the specifics of animal housing including caging, lighting etc. To achieve a CORT concentration of 25mg/L, 32.22mg of corticosterone-hemisuccinate (Q1662-000 Steraloids Inc, United States) was dissolved per litre of drinking water. The pH of the solution was raised to 12.4-12.7 using 10M of sodium hydroxide to cleave the hemisuccinate tail and then stirred at three degrees Celsius for five to seven hours. The pH of the solution was then lowered to 7.0-7.4 using 32.2% hydrochloric

acid. For both cohorts, control groups received water without CORT. Once treatment stopped at the end of week 8, cohorts 1 and 3 were left undisturbed for another 2 weeks. Behavioural assessment followed this two-week washout period to allow endogenous stress systems to recover from chronic CORT treatment. This would ensure that any behavioural changes observed were a result of long-term changes in response to chronic CORT treatment and to avoid measuring a semi-acute change in response to glucocorticoids.

	Week 6	Week 7	Week 8	Weeks 9-10	Week 11-14	Week 15
Animals: Male/ Female hBDNF ^{Val66Met} mouse model	CORT treatment during late adolescence - 25 mg/L in drinking water			Washout Period	Cognitive phenotyping	Animals euthanized and brains perfused

Figure 2.2 - Timeline of experiments for cohort 3

Male and female hBDNF^{Val66Met} mice underwent chronic CORT treatment during adolescence and were cognitively phenotyped in young adulthood. Genotypes included Val/Val and Met/Met animals. A two-week washout period was used to investigate the chronic glucocorticoid effects of CORT treatment. A week after testing finished, at approximately 15 weeks of age, mice were euthanized and perfused to collect their brains for confocal imaging.

2.2.5 Environmental Enrichment protocol

Mice in cohort 1 received EE from 7 to 9 weeks of age (see Figure 2.1), during which they were kept in larger-than-standard open top cages (44 × 30 × 15 cm, model RB2, Wiretrainers) with various toys, tunnels, housing, platforms and abundance of nesting material such as tissue paper and shredded paper (Figure 2.4A), to provide novel cognitive challenges. The number of items and variety of textures between cages were kept constant. These were changed once per week to maintain novelty and engagement with the enhanced environment. Control mice were housed in open-top standard mouse cages (34 × 16 × 16 cm, model MB1, Wiretrainers) with basic nesting materials and were designated “standard-housed” (SH) (Figure 2.4B). All mice were given 1 week to acclimatize to their environment when moved from open top to IVC cages at the end of week 9 (Figure 2.1).

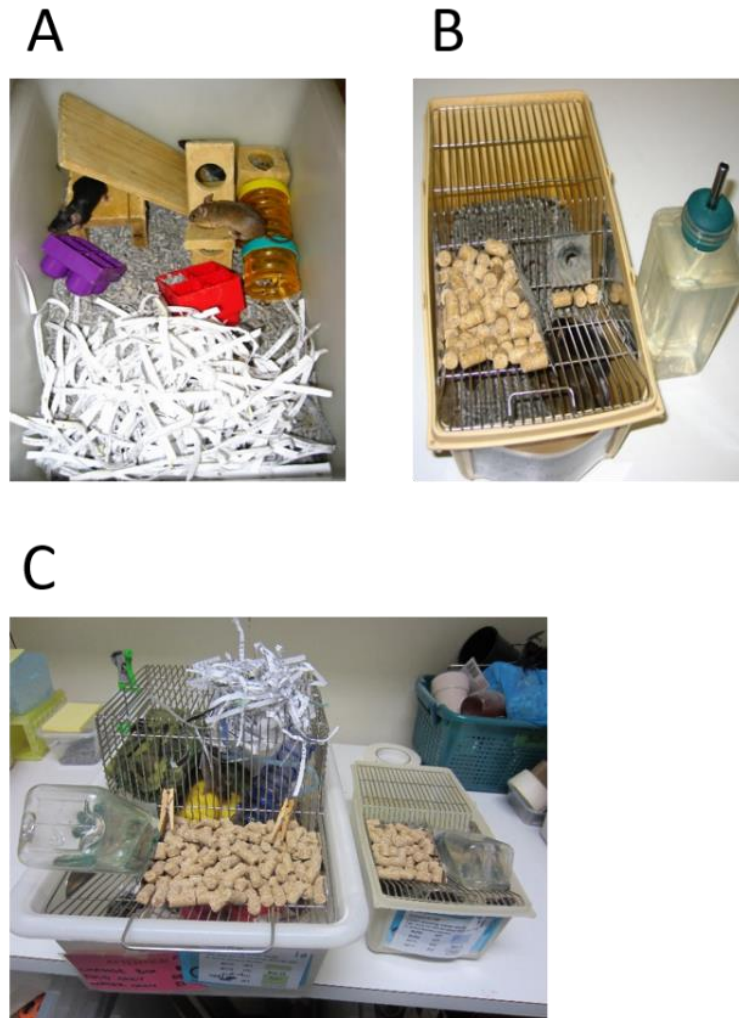


Figure 2.3 - Example images of environmental enrichment vs. standard housing

Panel A is an example of environmental enrichment housing, including toys and extra nesting materials. Panel B shows standard housing materials, which was only basic nesting materials. Panel C compares the size of the changes for the two conditions, with environmental enrichment including a larger cage size. Images courtesy of Behavioural Neuroscience Laboratory.

2.3 Behavioural assessment

Animals were transported to the behavioural suite and allowed to acclimatise in the holding room for 10 minutes prior to all behavioural experiments. All behavioural testing was conducted during the light phase and with dim light settings at approximately 100 lux. All experiments were recorded by a digital camera connected to a computer and a backup DVD recorder. Behaviour videos were then analysed using video tracking software (TopScan version 2.00, CleverSys Inc., Reston, VA, USA).

Table 2.1 - Order of Tests and Respective Mouse Age

Cohort	Test in order of occurrence	Age of mice at testing
1	Y-Maze	11 weeks
2	Y-Maze	10 weeks
2	NORT	10 weeks
2	EPM	11 weeks
2	Locomotor	11 weeks
2	Cheeseboard Maze	12 weeks

2.3.1 Y-Maze – short-term spatial memory

Short-term spatial memory was assessed using the hippocampal-dependent task the Y-Maze, which relies on the natural inclination of mice to explore novel environments (Dellu et al., 2000). The Y-maze consisted of three arms ($30 \times 8 \times 16$ cm) at 120° angles to each other and contain distinct geometric cues on the far end of each wall of the maze (see Figure 2.5). The Y-Maze was performed as previously described (Hill et al., 2014). Briefly, during the initial phase, the mouse was placed into the end of one arm (home arm) and was allowed to explore two arms for 10 minutes with one arm being closed (novel arm). After a 1 hour retention time in the home cage, the mouse was placed into the same Y-maze with all arms open for 5 minutes. Behaviour was analyzed with video tracking software (TopScan, CleverSys Inc., Reston, VA, USA). For cohort

1 the whole second phase of 5 minutes was analysed while for cohort 2 the first 3 minutes were examined. This was due to observations that after 3 minutes cohort 2 mice became disengaged and moved less, thereby masking any true effects.

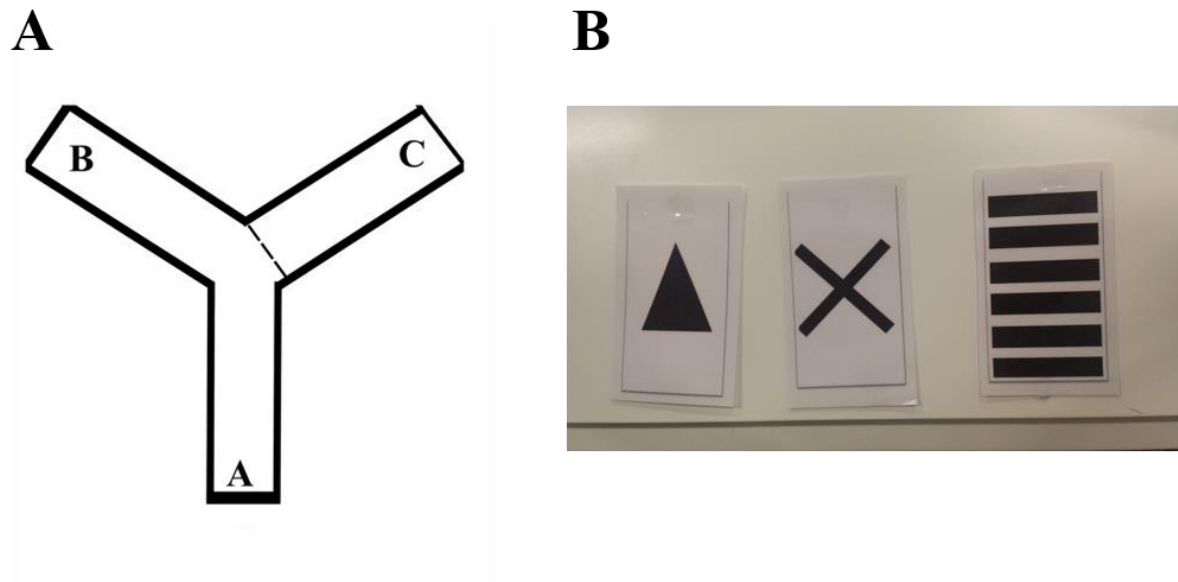


Figure 2.4 - The Y-Maze protocol for cohort 2

Panel A shows that the Y-Maze has three arms. During the first phase of the test only arms A and B are open, C is closed. A is the start location and the B is the familiar arm. Panel B shows the geometric cue on the end wall of each arm. Panel A adapted from (Grech et al., 2018a).

2.3.2 Elevated Plus Maze – a test of anxiety

The elevated plus-maze (EPM) is a test of anxiety in mice. It is designed on the basis that mice will tend to prefer darker and more enclosed areas than in open and brighter spaces. The EPM was made of Perspex consisting of two open arms (5×30 cm) and two enclosed arms ($5 \times 30 \times 14$ cm) extending at 90° angles from a central platform (5×5 cm). The maze was mounted on a base raised 60 cm above the floor (see Figure 2.6). The EPM was performed as previously described (Du et al., 2018). To begin the experiment the mouse was placed in the middle of the maze, facing

an open arm and tracking was started by a computer keyboard command. Mouse behaviour was recorded for 10 minutes. Behaviour analysed by TopScan version 2.00 includes entries into different arms and time spent in open or closed arms.

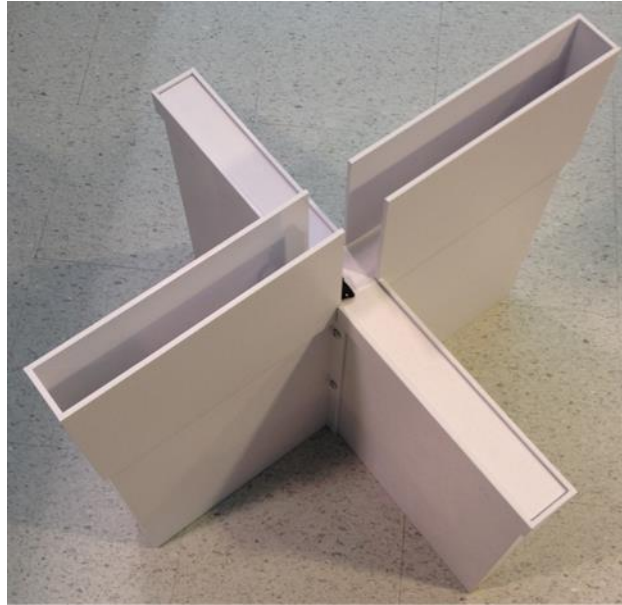


Figure 2.5 - The Elevated Plus Maze

The Elevated Plus Maze measures anxiety based on the time spent by a mouse in the open vs closed arms. Mice tend to prefer darker and more enclosed areas than open and brighter spaces.

Figure adapted from New Jersey Institute of Technology (2019).

2.3.3 Locomotor activity – measurement of basal activity

Baseline activity level is assessed using locomotor cages (28.5 cm × 28.5 cm × 20 cm) (see Figure 2.7). This was to ensure that behaviour observed in other behavioural tests is not due to group differences in activity level. Locomotor cages were cleaned with 80% ethanol before mice were removed from their home cages and placed into the locomotor cages. Mouse movement was recognised by infrared sensors and recorded by the automated recording system (Klug and Van

Den Buuse, 2013). Behaviours including distance, speed and rearing were recorded over the 1 hour test period.

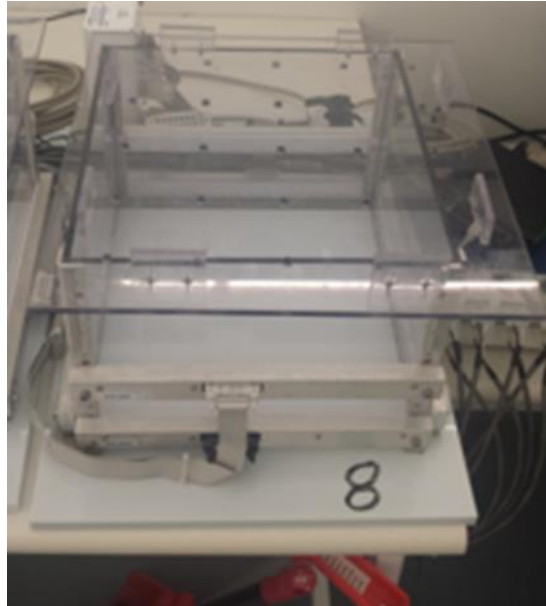


Figure 2.6 - Measuring locomotor activity

Basal activity levels of mice were analysed using locomotor cages. The Locomotor cage was a clear Perspex box with a lid. The mouse was placed in the cage for an hour and tracking sensors measured the mouse's movement around the cage. Image author's own.

2.3.4 Novel Object Recognition Task – measurement of non-spatial memory

This task relies on the natural inclination of mice to explore novelty as a measurement of non-spatial recognition memory. NORT was performed in a plastic box (37 cm × 37 cm × 28.5 cm) (see Figure 2.8A). The NORT was performed as previously described (Schroeder et al., 2015). Two days of habituation preceded the NORT session whereby mice were transported to the testing room at least 10 minutes before habituation. They were placed individually in the testing arena for 10 minutes in the testing room under test conditions. Once habituation was over, they were

returned to their home cage and stayed in the testing room until all animals had been habituated. On the third day habituation was performed however boxes were not cleaned after the habituation period. This provided familiar smells for the mice by their own excretions and was assumed to provide more accurate behaviour as the animals would be less stressed during the experiment proper. After habituation, the mouse was returned to their home cage. Once objects had been placed into the box the experiment proper was initiated. Two identical objects (see Figure 2.8B) were cleaned with 80% ethanol then placed and firmly secured in the left and right hand far corner of the box. To begin the experiment the mouse was placed in the box with its nose pointing away from the objects and at the midpoint of the wall opposite the glass bottles. Recording began by TopScan version 2.00 recognising the mouse. The mouse was allowed to explore the objects for 10 minutes. The mouse was returned to the home cage for a 1 hour retention period. Objects were cleaned with 80% ethanol before use. One object from phase A was placed and firmly secured in one corner of the box, and the novel object, (see Figure 2.8A), in the opposite corner. The position of the novel object was randomised. The mouse was allowed to explore the objects for 10 minutes. The first 5 minutes of the second trial were analysed for behaviours such as nose bouts to the objects and time spent exploring an object. Memory deficit were assumed by an animal showing no preference in exploring the novel object.

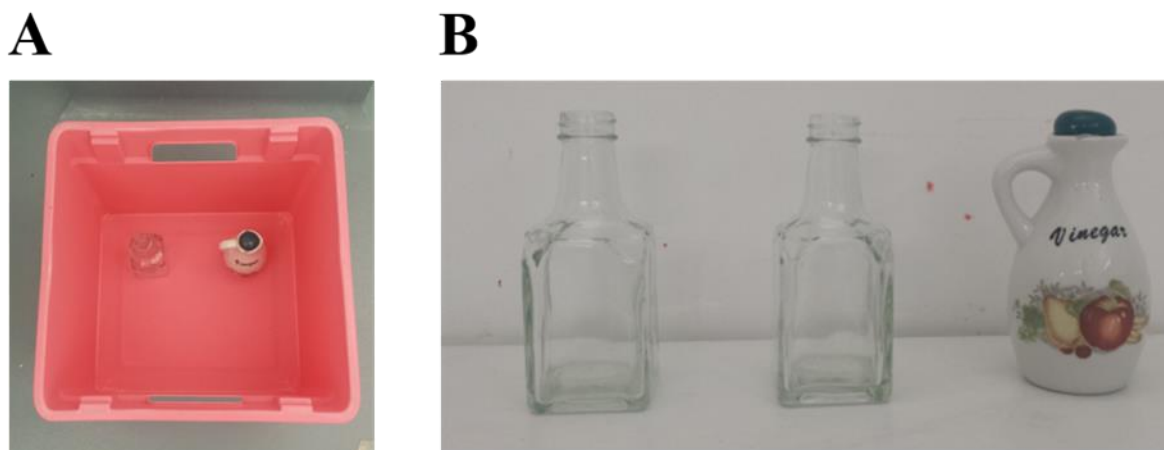


Figure 2.7 - Novel Object Recognition Task

The Novel Object Recognition Task is a novelty test of non-spatial recognition memory. Panel A shows the box used for testing, and orientation of objects during the test. Panel B shows the familiar and novel object. Image author's own.

2.3.5 Cheeseboard Maze – long term spatial memory and reversal learning

The cheeseboard maze is a long-term spatial memory test as well as a measure of cognitive flexibility. The cheeseboard maze is a gray painted circular wooden board (94 cm diameter), covered by 32 wells (3.25 cm diameter, 1 cm depth) and is elevated 30 cm off the floor (see Figure 2.9A). The exact organisation of the 32 wells is as follows: 8 lines of 4 wells each radiate evenly from the centre of the board, the inner well of each line is 14 cm from the centre and there is a 5 cm gap between each well. The outer most well is 5 cm from the edge of the board. At each point of the compass a spatial cue was placed, approximately 30cm from the maze (see Figure 2.9B). Experiment lighting is kept at ~80 lux during all experiment days. The Cheeseboard protocol was based upon Cheng et al (Cheng et al., 2014). Before each trial the maze and wells are cleaned with 10% ethanol and painted with diluted condensed milk (1:4 ratio) to prevent the mice from being distracted or guided by odour cues. Figure 2.10 outlines the experimental protocol. Mice were

food deprived from habituation day 1 and maintained at 85% body weight for the duration of the task. The experiment begins with two days of habituation, with two 2-minute trials with a 20 minute inter-trial interval conducted on each day. No wells are baited for these two days.

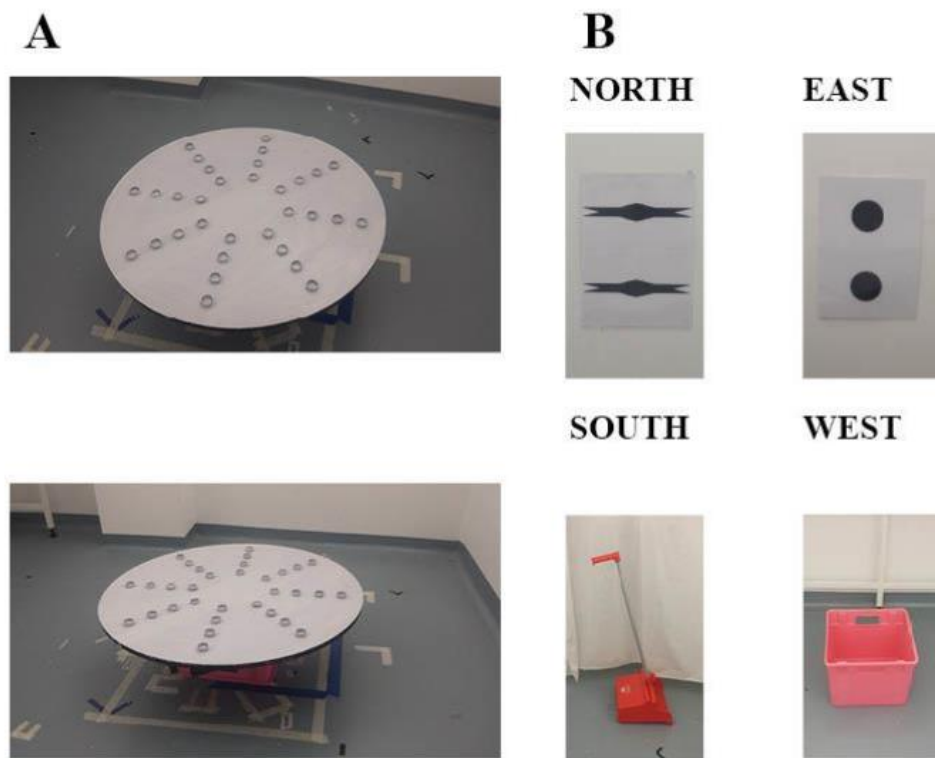


Figure 2.8 -The Cheeseboard Maze

The Cheeseboard Maze is a test of spatial learning and cognitive flexibility. Panel A shows the Cheeseboard Maze design, which consists of 32 evenly spaced and identical wells. It is raised off the floor. Each mouse has an assigned baited well across testing. Panel B shows the spatial cues that were placed at each cardinal point, which the mice were meant to use to learn the position of their assigned baited well.

For habituation, the mouse is placed on the maze and allowed to explore for the duration of the trial. Following the habituation trial, the mouse is fed 100µl of diluted condensed milk (1:4 ratio). For the following 6 days, the mice are trained to find the food reward over 2 trials with a 20 minute inter-trial interval. The location of the baited well is different for each mouse and is kept constant across trials and days for each individual mouse. Locations are counterbalanced across genotypes. If the target well is not located during the trial, mice are placed next to the target well and allowed to consume the food reward to eliminate chance differences in the amount of positive reinforcement received during learning. Mice should learn to use the spatial cues placed around the maze to find the baited well to receive the reward, relying on spatial memory. Spatial memory should be strengthened after multiple trials, and this should be reflected in reduced time taken to reach the baited well. Impaired spatial memory is indicated by a longer time to find the baited well. Latency was assessed post-experiment using TopScan version 2.00. Memory retrieval is tested on day 7, where no wells are baited, and mice are allowed to explore the maze freely. Intact spatial memory of baited well location should be demonstrated by mice spending longer in the zone where the baited well was previously. Following the probe trial on days 8 and 9 the location of the food reward is changed to a well 180° away from the original target well. Day 8 works as a training day and day 9 is a measure of reversal working memory. This can also be a measure of cognitive flexibility and is testing the ability of the mouse to ignore the initial position of the reward to learn the new location of the second reward. For the probe trial on day 7 and 9, the board is divided up into 8 zones corresponding to each of the 8 lines of wells and the time spent in each zone (% time) was measured by TopScan version 2.00. Centre body tracking data was analysed. These 8 zones were then grouped into 4 different quadrants (Target, Clockwise, Anti-clockwise, Opposite) for each mouse based on the location of the Target well.

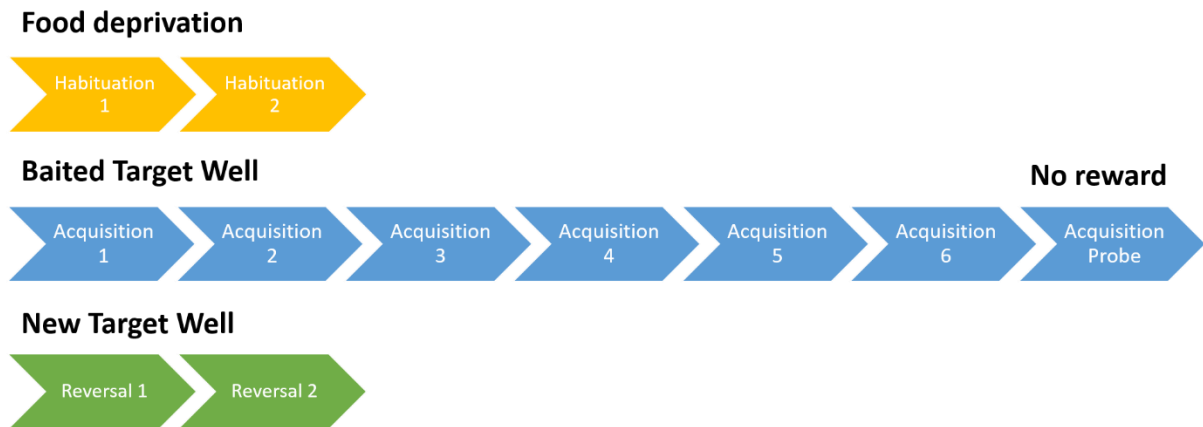


Figure 2.9 - The Cheeseboard Maze timeline

Mice are food deprived from habituation day 1, before training begins at Acquisition day 1. Mice are assigned a target well which is baited with condensed milk. On day 7 the reward is absent and memory retrieval is probed over a 2 minute trial. On reversal day 1 the target well is reversed by 180° on the Cheeseboard, and reversal working memory is tested on reversal day 2.

Search strategy for probe trials was investigated in several ways using the trace for each mouse. The trace is a record of mouse movement throughout a trial (Figure 2.11). The first basic approach was to count the number of quadrants crossed by each mouse using the trace and defined these as Indirect (crossing 4 quadrants), Mid (crossing 2-3) quadrants and Direct (crossing 1 quadrant). The second approach involved qualitatively assessing the traces and categorising by classic Morris Water Maze (MWM) strategy. Focal and Directed searches were grouped as “Focal”; Scanning, Chaining and Thigmotaxis as “Non-focal”; and Random was a solo group. Thirdly, whether the first entry into a quadrant was the Target Quadrant was investigated.

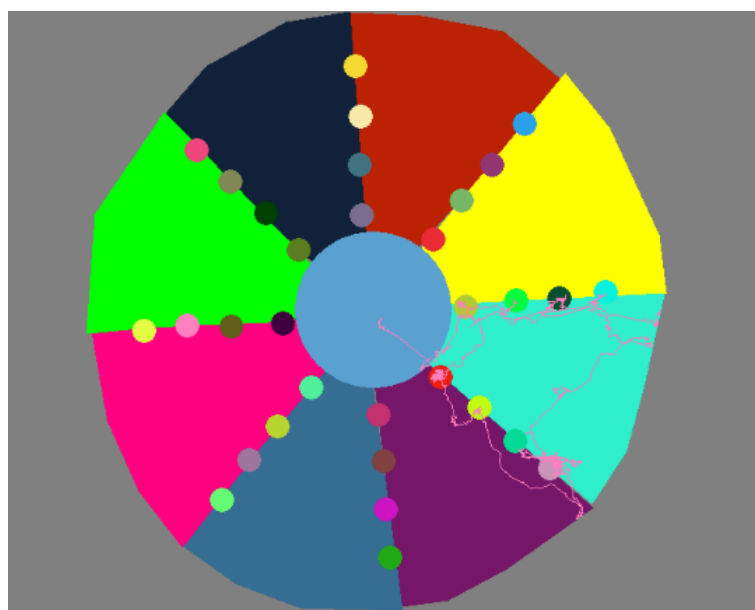


Figure 2.10- Example trace image of mouse movement across the Acquisition Probe trial

Each coloured circle is a well. Each coloured segment represents an eighth of the maze, and dependent on where the Target well was, two eights were combined to create the Target quadrant. The purple line shows where the mouse explored.

2.4 Tissue collection and preparation for molecular analysis

2.4.1 Brain Dissection and Protein Extraction

2.4.1.1 Brain dissection

Cohort 1 mice were randomly killed by cervical dislocation at 16 weeks of age and their brains were collected, snap-frozen on dry ice and stored at -80°C until required. Mice were randomly culled between 9am-5pm to attempt to balance any effect of circadian rhythm on protein expression profiles. Dissections were performed on a cold dissection plate ($\sim 0^{\circ}\text{C}$) mounted within a container of wet ice mixed with pieces of dry ice. Brains were positioned with the ventral side up in a stainless-steel adult mouse brain matrix (Zivic Instruments, Pennsylvania, USA; model number #BSMA001-1). The olfactory bulb was removed using a sharp razor blade. Following

this, two coronal slices of 2 mm thickness were extracted. Relevant brain regions were subsequently dissected according to coordinates from the Paxinos & Franklin mouse brain atlas (Paxinos and Franklin, 2004). The first 2 mm slice contained the mPFC and the second 2 mm slice contained the posterior cingulate cortex, nucleus accumbens and caudate putamen. The remaining brain was not sliced and was dissected. The hypothalamus was removed using small forceps. The cortex was then separated from the brainstem using curved forceps. Small forceps were used to roll the hippocampus out of the posterior end of the cortex. The hippocampus was bilaterally dissected and separated into dorsal and ventral hippocampus (~50/50). The dissected tissues were placed into labelled Eppendorf tubes and stored at -80°C until required.

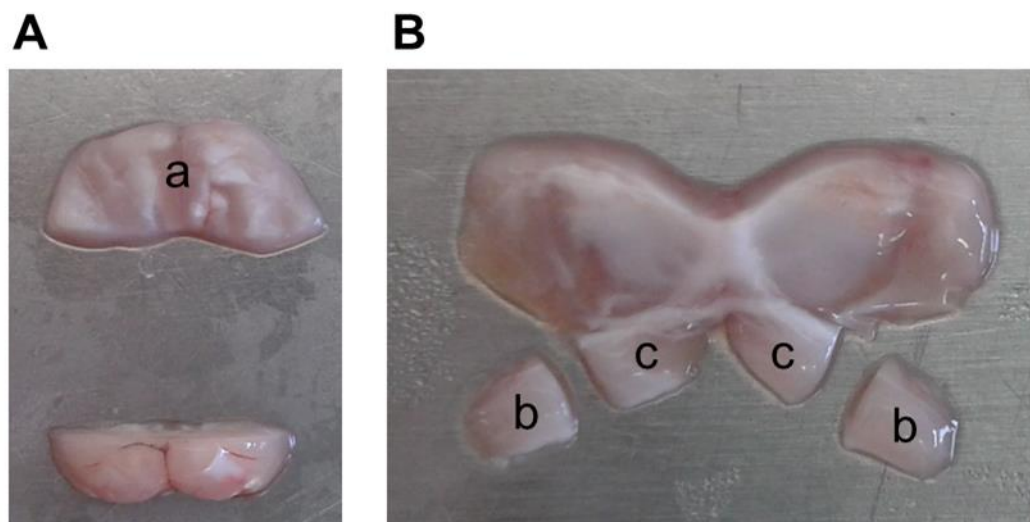


Figure 2.11 – Brain dissection of the mPFC and hippocampus

The bottom of panel A shows dissection of the mPFC by removal of the posterior part of the frontal brain for the separation of the PFC (label a). Panel B shows the rolled out hippocampus from the posterior cortex end and dissection of the hippocampus into ventral (b) and dorsal (c) hippocampi.

2.4.1.2 *Protein extraction*

Dissected tissue samples were weighed and kept on dry ice. 100 µl Radioimmunoprecipitation (RIPA) lysis buffer (50Mm Tris pH 8.0, 0.1% Sodium dodecyl sulfate (SDS), 1% Triton X-100, 150 mM NaCl, in 1L of Millipore water) containing protease inhibitor (1:200, 524628 Merck, Kilsyth, VIC, Australia) and phosphatase inhibitor (1:50, 539134 Merck, Kilsyth, VIC, Australia) was added for each 0.01 g of tissue. Tissues were homogenised with a hand-held electric pestle that used replaceable polypropylene tips (Sigma Aldrich, Missouri, USA). Samples were left on ice for 10 minutes after homogenisation. Samples were solubilised on a rotator at 4°C for 60 minutes and subsequently lysates were centrifuged for 15 minutes at $14,000 \times g$ at 4°C. Resulting supernatant was transferred to a new labelled Eppendorf tube and stored at -80 °C.

2.4.1.3 *Bicinchoninic acid assay*

Protein concentrations were screened using the Bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, USA). Sample protein was diluted to a 1:10 concentration by diluting 3 µL of protein stock in 27 µL of Millipore water for a 30 µL solution. 10 µL duplicates of samples and protein standards were pipetted into a 96-well plate. Each well was then mixed with 190 µl of the supplied BCA mixture (50:1, BCA reagent A and B). The plate was sealed with plastic wrap and incubated at 37°C for 30 minutes. The absorbance was measured at 562 nm on a plate reader (BioTek Instruments, Winooski, VT, USA). The protein standard for the BCA is from the Pierce BCA Protein Assay Kit (23227) from ThermoScientific. The standard is Bovine serum albumin (2.0mg/ml in 0.9% aqueous NaCl solution with sodium azide (product #23209). A standard curve was generated from averaged protein standards (concentrations included: 0, 0.025, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2 thousand ug/ml), and a regression equation was derived to analyse protein concentrations present in experimental samples. Aliquots of 50 µg of protein were prepared for

each experimental sample based on the regression equation. This is diluted x2 after the addition of loading buffer, so the concentration run in the gels is 2.5 µg/µl but importantly a total of 50ug is added to each well. Depending on protein concentration, samples were topped up with Millipore water to achieve a total volume of 10 µL. Protein aliquots were stored at -20°C until required and protein stocks returned to -80°C. The protein was diluted to a constant concentration across samples so that the small volume of sample would be loaded per well.

2.4.2 Western blot: Gel preparations, SDS-Page, Transfer and Imaging

2.4.2.1 Gel preparations

Polyacrylamide gels were typically prepared the day before the experiments and stored at 4°C overnight, or on the day of the experiment. A total of 4 gels were typically used to accommodate up to 56 samples in addition to a Rainbow Ladder for each gel. No internal control was used to standardize between gels. However, all gels were standardised to the average of the first gel. Importantly, each gel had an even mix of samples from each group. Firstly, resolving gels were pipetted in-between two glass plates held together by a gel casting stand until 4/5 of the way to the top, overlayed with 70% ethanol and left to set for 1 hour. When set the ethanol was poured off. Stacking gels were poured and a 15-comb, 1.5 mm, 40 µL mould (Bio-rad, California, USA) inserted immediately after. Gels were allowed to set for a further 30 minutes. Resolving and stacking gel reagents and preparations are presented in Tables 1 and 2. The percentage of acrylamide in resolving gel was based on the molecular weight of the protein of interest. The larger the size of the protein of interest, the lower the percentage of acrylamide gel required and vice versa.

Table 2.2 - Resolving gel reagents and preparations for Western Blot

Reagents	1 × Gel Preparation		
	8%	10%	15%
dH₂O	3.4 ml	2.7 ml	1.1 ml
1M Tris Ph 8.8	3.75 ml	3.75 ml	3.75 ml
10% SDS	100 µl	100 µl	100 µl
30% Acrylamide	2.64 ml	3.3 ml	4.95 ml
10% APS	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl

Table 2.3 - Stacking gel reagents and preparations for Western Blot

1× Stacking Gel Preparation	
dH ₂ O	3.4 ml
1M Tris Ph 6.8	0.62 ml
10% SDS	50 µl
30% Acrylamide	0.85 ml
10% APS	50 µl
TEMED	5 µl

2.4.2.2 *Gel electrophoresis and Transfer*

Pre-prepared gels were taken out of the fridge and left to sit on the bench for 15 minutes. Transfer buffer (14.4 g Glycine, 3.02 g Tris base, 800 ml dH₂O, 200 ml of methanol) was prepared fresh on the day and cooled in an esky of wet ice in a 4°C cold room. The well combs were removed from the gels and gels were then fitted to a Bio-Rad SDS-Page apparatus and submerged in running buffer (3.023 g Tris base, 14.41 g Glycine, 1 g SDS made up to 1L with dH₂O). Protein aliquots were taken out of the -20°C freezer and defrosted on ice before being mixed with an equal volume of loading buffer (0.4M Tris Ph 6.8, 37.5% Glycerol, 10% SDS, 1% 2-mercaptoethanol, 0.5% bromphenol blue). Following this, the samples were denatured at 95°C on a heat block for 10 minutes and finally centrifuged for 10 seconds to collect all contents within the Eppendorf tubes. 4 µl of Rainbow Ladder (GE Healthcare, Little Chalfont, United Kingdom) was loaded into the first well on the right side of each gel and experimental samples were subsequently loaded into the remaining wells from right to left. Gel electrophoresis was run using a Bio-Rad Power Pack at 120V until the dye front reached the bottom of the gel (approximately 1.5 hours).

Following gel electrophoresis, the SDS-PAGE tank was disassembled to expose the gel. The stacking gel and bottom 2 mm were cut off, using the Rainbow Ladder as a guide. The remaining gel was placed into a container to soak in transfer buffer. Two rectangles of Whatman 3 MM filter paper (BioRad, Hercules, CA, USA) and two foam pads (BioRad) were soaked in containers of transfer buffer. A nitrocellulose membrane (pore size= 0.22 µm) (Santa Cruz, CA, USA) was cut to the size of the gel and soaked in a separate container of transfer buffer. Bio-Rad transfer cassettes were assembled as follows: the cassette was laid on its black side, one piece of soaked foam pad, one piece of soaked filter paper, the gel with the rainbow marker on the right-hand side followed by 2 ml of transfer buffer over the top of the gel, the membrane, a second piece of soaked filter

paper and a second soaked foam pad. After the addition of each layer, a roller was applied to roll out air bubbles. Completed cassettes were then placed into the Bio-Rad transfer tank with the black side of the cassette facing the black side of the electrode module. They were submerged in transfer buffer and insulated by an ice block in the tank, as well as wet ice in an insulated box and transfer was performed in a 4°C cold room. Transfer conditions were dictated by the protein of interest and the percentage gel used. For proteins larger than 100 kDa, overnight transfer at 30V (approximately 18 hours) followed by 70V for 1 hour the next morning was performed and generally 8-10% gels were used. For proteins smaller than 100 kDa, generally 15% gels were used and transferred at 120V for 1.5 hours.

2.4.2.3 *Antibody staining*

To confirm successful transfer, the nitrocellulose membrane was rinsed briefly in Ponceau staining mixture (Sigma-Aldrich) and washed with Millipore water. Membranes were examined to confirm the presence and integrity of protein bands. Following transfer of the proteins and rainbow ladder onto the membrane all nitrocellulose membranes were cut at specific points guided by the rainbow ladder so that multiple markers of interest could be investigated without stripping blot. This was performed according to the expected molecular weight of the protein of interest using the Rainbow ladder as a guide. All membranes were destained from the Ponceau solution by washing in tris-buffered saline Tween 20 (TBST) (20 ml 1M Tris base pH 7.5, 30 ml 5M NaCl and 5 ml 20% TWEEN in 1L dH₂O), then blocked in 5% skim milk in TBST for at least 1 hour on a shaker at room temperature. After blocking, the membrane was washed in TBST and primary antibody was diluted based on the recommended dilution supplied by the manufacturer in 5% Bovine Serum Albumin (Sigma Aldrich) in TBS. Table 3 summarizes all primary antibodies and dilutions used. Antibody dilution was guided by manufacturer instructions and determined by internal laboratory

validation testing. Specificity was confirmed by using internal laboratory controls and noting the number and molecular weight of bands. All membranes were incubated in primary antibody overnight with shaking at 4°C. The next morning, membranes were washed twice for 15 minutes in TBST on a shaker at room temperature. Depending on the primary antibody used, either mouse, rabbit or rat secondary horseradish peroxidase (HRP) conjugate antibody (1:2000, Cell Signaling Technology, Danvers, MA, USA) was diluted in 5% non-fat milk in TBST and applied to the membrane sections. Secondary antibodies were incubated for 1.5 hours on a shaker at room temperature and then washed three times for 15 minutes with TBST on a shaker at room temperature. LumiGlo (GeneSearch, QLD, Australia) or Ultra Chemiluminescence Solution (Perkin Elmer, Massachusetts, USA) was prepared and 1 ml added to each membrane for 1 minute incubation prior to imaging. Blots were imaged using the Luminescence Image Analyzer (Fuji film LAS-4000, FujiFilm Life Science, Stamford, CT, USA). Captured pictures of the protein bands were analysed using TotalLab Quant Analysis Software (TotalLab Ltd, Newcastle upon Tyne, United Kingdom). The density ratio of each protein band was normalized against the β -actin or α -tubulin loading control. All experimental groups were normalised to their respective control group. Western blots were repeated at least twice to confirm results.

2.4.2.4 Stripping and re-probing

In order to examine more than one protein on the same blot due to multiple proteins of interest having close molecular weights, membranes were stripped and re-probed. Following imaging, blots were placed into a container with stripping buffer (25 ml Tris pH 6.8, 8g SDS and 2.8 ml 2-mercaptoethanol in 400 ml dH₂O) in a shaker at 50°Celsius for 30 minutes. Membranes were washed three times for 15 minutes in TBST and blocked for 1 h in 5% non-fat milk in TBST. The

primary antibody of interest was applied and the remaining procedures were followed as previously described.

Table 2.4 - Primary antibodies used to quantify target protein levels by Western Blot

Protein	Molecular Weight (kDA)	Antibody	Dilution Used
Alpha-tubulin	50	Abcam ab 7291	1:5000
Beta-actin	42	Sigma a5316	1:10,000
mBDNF	13	Santa Cruz sc-20981	1:200
Calretinin	28	SWANT 7699/4	1:2000
pERK	42/44	Cell Signaling Technology 9102	1:1000
phosphoERK	42/44	Cell Signaling Technology 9106	1:2000
GAD67	67	Sigma G5419	1:500
NMDAR1	120	Cell Signaling Technology 4204	1:1000
NMDAR2A	180	Cell Signaling Technology 4205	1:1000
NMDAR2B	190	Cell Signaling Technology 4212	1:1000
NT-4	14	Santa Cruz sc-545	1:200
Parvalbumin	12	Millipore MAB1572	1:1000
Somatostatin	14	Millipore MAB354	1:1000
TrkB (H181)	Full length band140 and truncated band 95	Santa Cruz sc-8316	1:1000
pTrkB (705)	140	Signalway 11328	1:1000
pTrkB (515)	85	Abcam ab109684	1:1000
pTrkB (816)	140	Millipore ABN1381	1:500

2.5 Immunohistochemistry protocol

2.5.1 Transcardial Perfusion

For cohort 2, 6 mice/group underwent transcardial perfusion 3-7 days after last behavioural test was performed at approximately 16 weeks of age. For cohort 3, mice were culled 1 week following behavioural testing and were approximately 15 weeks of age. 5-6 animals per group were culled. Mice were randomly culled between 9am-5pm to attempt to balance any effect of circadian rhythm on protein expression profiles.

A perfusion pump (Masterflex Easy-Load, Cole-Parmer, Chatswood, NSW, Australia) set at speed 1 and attached to a blunted needle (18G) was used for perfusions. Phosphate-buffered saline (PBS) (27.5 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.9 g NaH_2PO_4 , 9.0 g sodium chloride (NaCl), make up to 1 litre, pH 7.4) and 4% paraformaldehyde (PFA) (105 ml 38% formalin solution, 895 ml PBS) were made on the day and kept on ice during the procedure. Pre-perfusion checks included firstly clearing the perfusion line with dH_2O followed by ~30ml of PBS to confirm that solution flowed freely without blockages or leaks.

All mice were anaesthetised with a single intraperitoneal injection of 100 μl pentobarbitone diluted 1 in 10 in syringe (Virbac, NSW, Australia), at a dosage of 3.25 mg. This is approximately a third of the dose (~130mg/kg) for euthanasia (about 350mg/kg). Following injection, anaesthesia was confirmed by loss of the flexor withdrawal reflex. Once anaesthetised but before the heart has stopped the mouse was pinned to the surgery board in a supine position with one pin through the skin of each limb. Abdomen fur was sprayed with 80% ethanol and skin layer removed with large scissors. The sternum was grasped with forceps and large scissors were used to make an incision in the abdominal wall just below the diaphragm. Using small scissors dissection was made through

the diaphragm and down through the rib-cage either side of the lungs. The ribcage was clamped up and away with a haemostat to fully expose the heart. Fat deposits and connective tissue were cut away from the heart. Perfusion needle was inserted into the ventricle approximately 5 mm deep. The right atrium was punctured with sharp scissors and the pump started. Approximately 50 ml of PBS was pumped through each mouse followed by 50 ml of the fixative, 4% PFA. Once the mouse was perfused, indicated by liver colour changes (from red to light brown) and stiffness of extremities, the pump was turned off. The head was separated from the trunk using scissors. Brains were collected from the head and moved into a labelled 50 ml tube containing 4% PFA. Post-fix whole brains were kept in 4% PFA for 24 hours at 4°C. The next day, brains were placed into labelled 50 ml pots of 15% sucrose solution for 24 hours at 4°C. Following this, the 15% sucrose solution was replaced with the 30% sucrose solution and cryoprotected for another 48-72 hours at 4°C or until the brains sunk. Brains were washed with PBS and flash-frozen in isopentane chilled with dry ice and stored at -80°.

2.5.2 Cohort 2 – Immunofluorescence Co-Expression Protocol

Coronal sections were cut on a cryostat by Angela Vais, Histology Senior Officer, Monash Histology Platform, at 20 µm thickness and collected at 1:6 intervals. mPFC was sectioned from 2.8 to 1.72mm relative to Bregma, and hippocampus was sectioned from -1.94 to -2.92mm relative to Bregma. Sections were mounted onto gelatine coated slides. Firstly, sections were washed twice in PBS, incubated in ice-cold 0.3% H₂O₂ methanol for 30 minutes and then treated with the Vector Mouse on Mouse (M.O.M.) immunodetection kit according to manufacturer's instructions, which included a blocking step. Blocking involved incubation for 1 hour in in a humidified chamber at room temperature with IgG blocking solution provided by the M.O.M. kit (Vector Laboratories, Burlingame, CA, USA). Primary antibodies (anti-mouse Parvalbumin, MAB1572, 1:2000,

Millipore and anti-rabbit TrkB H181, sc-8316, Santa Cruz, 1:500) were prepared in M.O.M diluent, applied to the slides and left to incubate overnight at 4 °C in a humidified chamber. On the second day, sections were washed three times in PBS before applying fluorescent secondary antibodies (donkey anti-mouse IgG 488 (1:400) (715-546-150) and donkey anti-rabbit IgG 594 (1:400) (711-586-152)) prepared in M.O.M diluent for an incubation period of 1.5 hours in the dark. After the incubation period, slides were washed three times in PBS and cover slips were secured with VectaShield Mounting Medium for fluorescence with 4',6-Diamidino-2-Phenylindole (DAPI) (H-1200, Vector Laboratories Inc., Burlingame, CA, 94010) at a concentration of 1.5 µg/ml and left to dry overnight in the dark.

2.5.3 Stereological Cell Quantification, Image Acquisition and Analysis

Co-expression of PV and TrkB was determined on images of immunostained brain sections from male PV-Cre and male PV-Cre: Fl^{+/+} mice (Figure 2.13). 5-6 mice per group were analysed. Stacks of images spanning 20 µm in the z-plane were taken in the dorsal hippocampal areas to be analysed. A Nikon C1 Confocal microscope with an Andor Zyla 4.2 sCMOS Camera was used to capture images at 40× magnification and 3 µm step z-stacks. The mPFC sections examined related to Bregma co-ordinates 2.08, 2.32 and 2.68 mm and the dorsal hippocampus sections examined related to Bregma co-ordinates -1.42, - 1.82 and -2.18mm. A maximum projection image was created from each stack using the Nikon C1 Confocal software. Co-expression of markers and area measurements were determined using the FIJI ImageJ 1.52g software. Cells labelled with DAPI, PV and TrkB were quantified in serial (1:6) coronal sections in the mPFC and DHP. The experimenter was blinded to mice numbers when counting and determined brain region boundaries based upon DAPI stain. To determine co-expression, a macro was used to create a

mask for the 488-PV channel and used to confirm co-expression with 405-DAPI and 594-TrkB.

These cells were then counted using the Cell counter plugin.

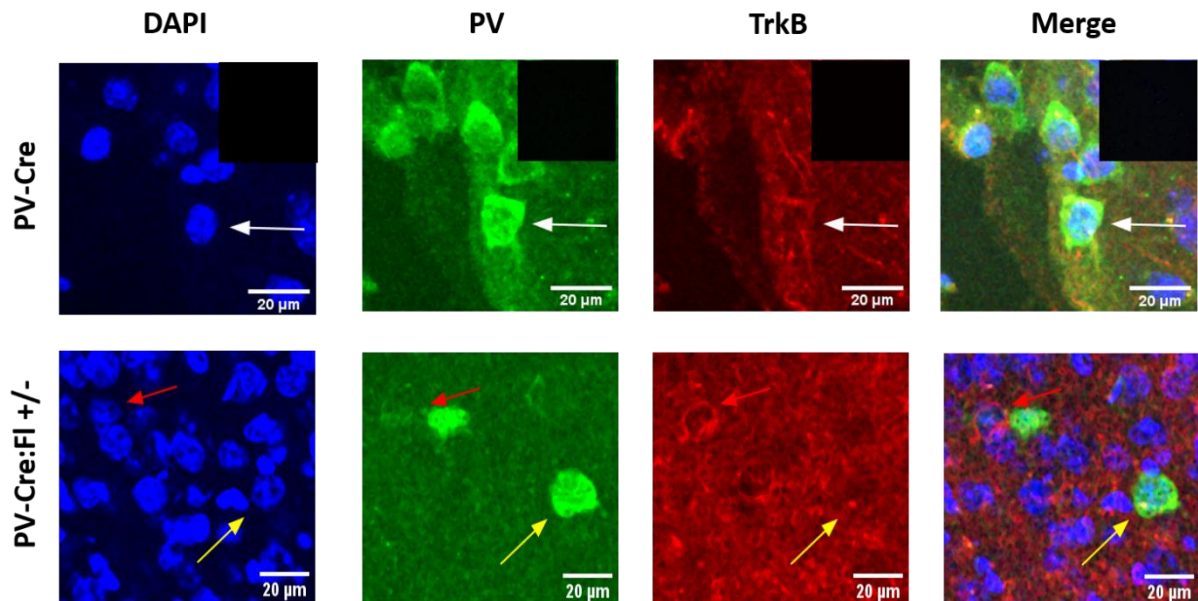


Figure 2.12 - Example parvalbumin and TrkB staining for cohort 2

Example staining in the mPFC for co-expression of parvalbumin (PV) (green) and TrkB (red) with DAPI (blue) for nuclei staining, for PV-Cre control (Top panel) and PV-Cre: Fl^{+/−} mice (bottom panel). Negative controls are shown in the top right corner of PV-Cre panels to ensure specificity of staining. Negative control was secondary antibodies added to sections but no primary to ensure that there was no non-specific binding occurring. White arrows point to co-expressed cells. Yellow arrows point to PV cells without TrkB expression. Red arrow points to TrkB only staining.

2.5.4 Cohort 3 – Free-floating immunohistochemistry protocol for confocal microscopy

Free floating 20 μm tissue sections were cut by Mauricio Sepulveda, Behavioural Neuroscience Laboratory, La Trobe University and stored in cryoprotectant (300ml ethylene glycol, 150 g sucrose, 275 ml dH₂O, 275 ml 0.1 PB pH 7.4) in 24-well cell culture plates. Experiments were performed in 24-well cell culture plates (Costar 3524, Corning Incorporated, Corning, NY, USA) to cause minimal disruption to tissue. Solutions were extracted with a 1000 μl pipette. 300 μl of

solutions were added throughout the protocol. Sections were sorted to find representative sections of Bregma -1.46, -1.82, -2.18, -2.54, -2.80 and -3.16 to image throughout the dorsal and ventral hippocampus. On experimental day 1 sections were washed in phosphate buffer (PB) (diluted 10 times from 0.1M PB: 27.5 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 3.8 g $\text{Na}_2\text{H}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ dissolved in 1 litre dH_2O , pH 7.4) for 10 minutes on a shaker. PB wash buffer was removed and blocking solution (10% Normal Donkey Serum (NDS) + 1% triton-x-100 in PB) added. Samples were incubated in blocking solution for 1 hour at room temperature. Blocking solution was removed and primary antibody solution (primary antibodies + 2% NDS + 0.3% triton-x-100 in PB) added overnight on a rocker at room temperature. Antibodies used are summarised in Table 4. On experimental day 2 samples were washed in PB 3 x 5 minutes. Samples were then incubated in secondary antibody solution (secondary antibodies + 2% NDS in PB) for 3 hours. Following this, samples were washed in PB 3 x 5 minutes. Samples were mounted to a glass slide in a container deep enough that the glass slide could be partly submerged in PB buffer. Sections were transferred to the container using a pasture pipette that had a narrow end cut off or a brush into the container with buffer. Tissue sections were gently brought to the top of the PB buffer surface using a brush. Sections were allowed to even out and stretch on top of PB buffer surface, and the glass slide was positioned underneath the section and lifted out. Tissues were allowed to become opaque but not dry before adding a small line of Dako Fluorescence Mounting Medium (Dako, North America Inc., CA, USA). A cover slip was lowered to cover the glass slide with forceps and edges sealed with nail polish. Sections were stored in -20°C freezer.

Table 2.5 - Primary and secondary antibodies used for confocal microscopy

Primary Antibody and Dilution	Secondary Antibody and Dilution
rabbit anti-Calretinin (1:1500, Swant, 7691)	donkey anti-rabbit AlexaFluor 594 (1:400, Jackson Immunoresearch)
guinea pig anti-NeuN (1:1000, Merck, ABN90)	donkey anti-guinea pig Dylight 405 (1:200, Jackson Immunoresearch)
mouse anti-Parvalbumin (1:1000, Sigma-Aldrich, Sig P30881)	donkey anti-mouse AlexaFluor 488 (1:400, Jackson Immunoresearch)
rat anti-Somatostatin (1:100, Merck, MAB354)	donkey anti-rat AlexaFluor 647 (1:200, Jackson Immunoresearch).

2.5.5 Stereological Cell Quantification, Image Acquisition and Analysis

Stacks of images spanning 20 μm in the z-plane were taken in the hippocampal areas to be analysed. The DHP samples examined related to Bregma co-ordinates -1.42, - 1.82 and -2.18mm. The VHP samples examined related to Bregma co-ordinates -2.54mm, -2.80mm and -3.16mm. A Nikon C1 Confocal microscope with an Andor Zyla 4.2 sCMOS Camera was used to capture images at 20 \times magnification and 3 μm step z-stacks. A maximum projection image was created from each stack using the Nikon C1 Confocal software (see Figure 2.14). Expression of markers and area measurements were determined using the FIJI ImageJ 1.52g software. The experimenter was blinded to mice numbers when counting and determined brain region boundaries based upon neuronal nuclear protein (NeuN) stain. To determine marker expression, a macro was written with help from Dr. Shane Cheung, Monash Micro Imaging – MHTP, The Hudson Institute of Medical

Research. This macro created an individual mask for the 488-PV channel, 594-CAL and 647-SST channel. Each of these masks were applied to the 405-NeuN channel to confirm co-expression of NeuN with each marker, confirming that the observed immunofluorescence is indeed a neuronal cell. The macro then automatically counted particles between the 50-300 microns.

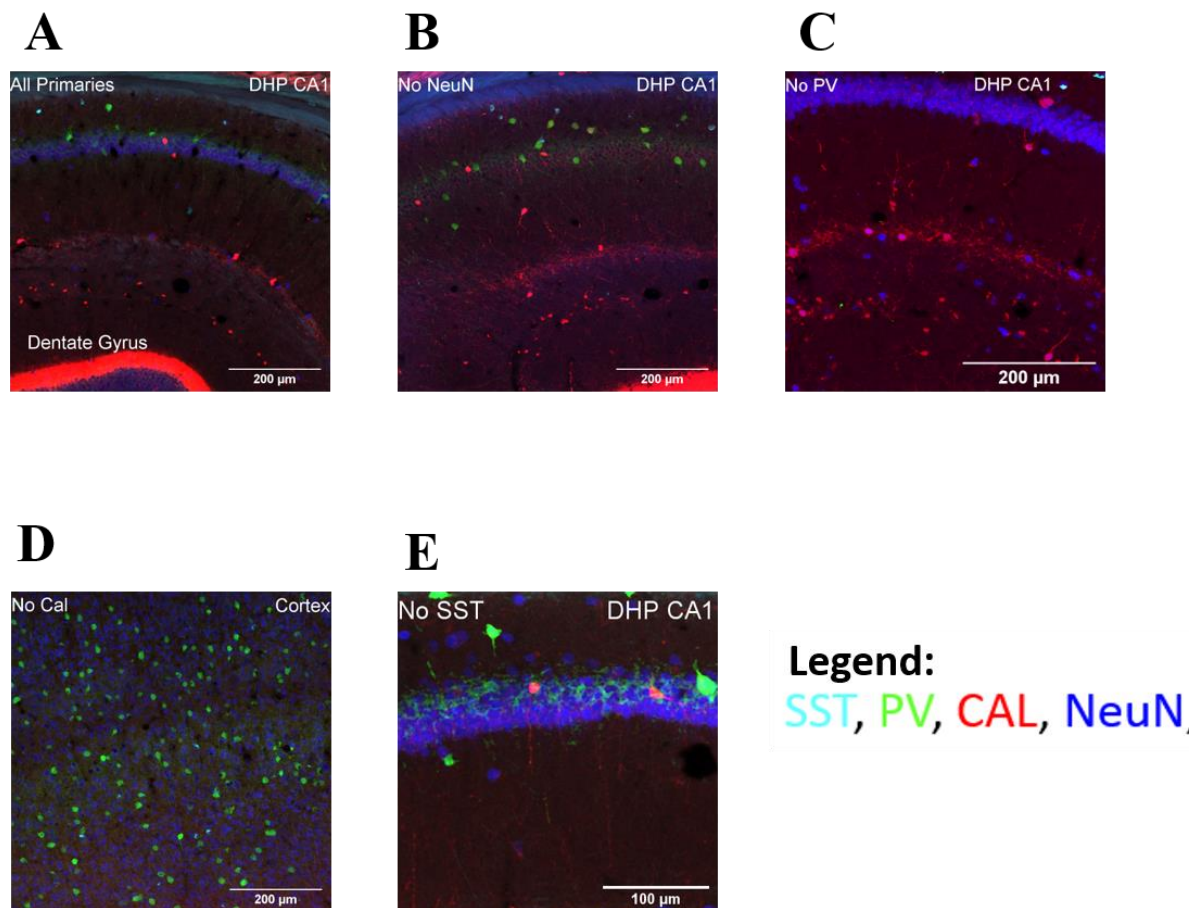


Figure 2.13 - Control staining for cohort 3

Example images of control staining showing specificity of secondary antibodies. Panel A shows section that has been stained with all primaries and secondaries. Panel B shows staining with NeuN primary omitted and all secondaries included. Panel C shows staining with PV primary omitted and all secondaries included. Panel D shows staining with CAL primary omitted and all secondaries included. Panel E shows staining with SST primary omitted and all secondaries included.

2.6 Statistical data analysis

Behavioural and molecular data were analysed using SYSTAT 13 (Systat Software Inc., San Jose, Ca, USA), IBM SPSS Statistics 24 software (International Business Machines, New York, USA) or Prism 7.01 and 8.0 (GraphPad, California, USA). All graphs were created using Prism 7.01 or 8. All data are expressed as the mean \pm the standard error of the mean (SEM). Based on the type of data different analysis programs and parameters were used, which are specifically outlined below. For cohort 1, groups were compared by Analysis of Variance (ANOVA) with the independent factors being sex (male or female), genotype (WT or HET), treatment (water or CORT) and environment (SH or EE), using the SYSTAT 13 program (Systat Software Inc., San Jose, Ca, USA). Thus, there were 16 experimental groups. Post-hoc comparisons were done with Tukey's test. Group differences were considered significant when $p < 0.05$. For cohort 2, immunohistochemistry, Elevated Plus Maze and Locomotor activity were checked for normal distribution and two-way ANOVA was performed in Prism, with independent factors of sex (male or female) and genotype (PV-Cre or PV-Cre:TrkB Fl $^{+/-}$). For the Y-Maze a three-way ANOVA of sex (male or female), genotype (PV-Cre or PV-Cre:TrkB Fl $^{+/-}$) and arm (Home, Familiar or Novel) was performed, followed by unpaired t-tests for each individual group. For the Cheeseboard Maze, Mauchley's test of sphericity was tested for Repeated Measure ANOVA. Greenhouse-Geiser correction was applied when necessary and post hoc comparisons were done with Bonferroni test. Group differences were considered significant when $p < 0.05$. For cohort 2 supplementary data, Chi square was used for search strategy analysis. For cohort 3, groups were firstly compared by Univariate ANOVA with the independent factors being sex (male or female), genotype (hBDNF $^{Val/Val}$ or hBDNF $^{Met/Met}$) and treatment (CORT or water) using the IBM SPSS statistics 24 software. To assess the effects of different hippocampal subregions (CA1, CA2, CA3, DG) a Repeated Measures ANOVA was performed. If region interacted with genotype, sex or

treatment, data were split accordingly and analysed by two-way ANOVAs using GraphPad PRISM software with Sidak's multiple comparison where justified. Group differences were considered significant when $p < 0.05$.

Chapter 3. Sex-Dependent Effects of Environmental Enrichment on Spatial Memory and Brain-Derived Neurotrophic Factor (BDNF) Signaling in a Developmental “Two-Hit” Mouse Model Combining BDNF Haploinsufficiency and Chronic Glucocorticoid Stimulation

3.1 Chapter 3 Introduction

Our laboratory has previously published using the ‘two-hit’ model, that combines genetic BDNF heterozygosity (“first hit”) with a chronic stress physiological paradigm in adolescence (“second hit”). Post-mortem brains of people with schizophrenia have shown around ~50% decrease in BDNF (Durany et al., 2001; Hashimoto et al., 2005a; Weickert et al., 2003) ., and to mimic this BDNF heterozygous mice, which express 50% less BDNF expression in the frontal cortex and hippocampus (Hill and van den Buuse, 2011), were chosen. Chronic corticosterone administered via drinking water was chosen to model stress to directly asses the interaction of glucocorticoid receptors and BDNF-TrkB signalling on excitatory/inhibitory markers. We have found that this model leads to spatial memory deficits that may have relevance to the cognitive symptoms in individuals with schizophrenia (Klug et al., 2012). Schizophrenia has no specific treatment options for cognitive symptoms, and there is an argument for focusing on preventative treatments for schizophrenia (Arango et al., 2018). Schizophrenia is most likely a group of heterogenous syndromes, which will need individual treatment approaches. As a result, potentially the most effective strategy moving forward is to mitigate symptom severity using preventative approaches. In humans, this could be through having positive physical and mental health habits, i.e. a healthy lifestyle (Arango et al., 2018). In rodents, it is possible to model this through a protocol known as environmental enrichment. This can include access to novel objects, tunnels and running wheels (Nithianantharajah and Hannan, 2006). Following on from our previous studies using the two-hit model, we wanted to investigate whether an environmental enrichment (EE) protocol would prevent the spatial memory deficits that emerge in this model. Chronic corticosterone was administered during weeks 6-8 as this is the period of sexual maturation, ergo adolescence (Hill et al., 2012b).. To determine if EE could restore spatial memory impairments induced by this stress paradigm, it began a week later to allow CORT effects to occur to determine if these effects could be

reversed. Previously, one week of corticosterone treatment has been found to significantly alter hippocampal MR levels in rats. This was an intermediate result between vehicle treated and chronic treatment of 3 weeks (Karten et al., 1999).

As explored above and in the thesis introduction, there is a need to mitigate the trajectory of schizophrenia due to a lack of appropriate treatments for cognitive symptoms. While there is evidence that some preventative treatments can help, including ‘healthy lifestyle approaches’ (Arango et al., 2018) and identification of ultra-high risk for pharmaceutical and therapy conjunctive treatments (McGorry et al., 2002), it is not clear whether the positive benefits occur through the BDNF-TrkB signalling pathway and what effect this may be having on excitatory/inhibitory markers. These systems are clearly involved in cognition, but how to therapeutically target them effectively is not yet known. A key aim of this project was whether EE would restore spatial memory through BDNF-TrkB signalling, and therefore, could this be potential therapeutic target for cognitive deficits in schizophrenia. Supplementary data includes investigation of inhibitory markers and the downstream ERK1/2 signaling pathway. While only the dorsal hippocampus was investigated in the published manuscript due to its established pivotal role in spatial memory, for the supplementary data both dorsal and ventral regions of the hippocampus were investigated. This was to investigate whether the experimental conditions affected other inhibitory and BDNF-TrkB signalling markers and whether these reached more broadly across the hippocampus. For the supplementary data, dorsal and ventral regions of the hippocampus were investigated, with sex and region-specific changes emerging from adolescent exposure to positive and negative environments. The rationale for undertaking these experiments was to comprehensively profile the excitatory/inhibitory molecular changes occurring in response to the two-hit model, and to investigate whether these modulated differentially by EE according to BDNF genotype, sex, and CORT treatment.

The following published hypotheses were investigated:

- 1) It was hypothesised that an environmental enrichment protocol would reverse the spatial memory deficit in our “two-hit” model of BDNF haploinsufficiency and CORT treatment and how this would compare to EE effects in WT controls.
- 2) It was hypothesised we could replicate molecular changes to the BDNF-TrkB signaling pathway and NMDAR system in the dorsal hippocampus in the two-hit model, and that these are modulated differentially by EE according to BDNF genotype, sex, and CORT treatment

The following hypotheses were investigated and presented as supplementary data:

- 3) It was hypothesised that inhibitory markers and the ERK1/2 signaling pathway protein expression would be decreased by BDNF heterozygosity, CORT and the combination of both treatments.
- 4) It was hypothesised that EE would restore the expression of inhibitory markers and the ERK1/2 signaling pathway protein expression decreased by BDNF heterozygosity, CORT and the combination of both treatments.

3.2 Published Manuscript



Sex-Dependent Effects of Environmental Enrichment on Spatial Memory and Brain-Derived Neurotrophic Factor (BDNF) Signaling in a Developmental “Two-Hit” Mouse Model Combining BDNF Haploinsufficiency and Chronic Glucocorticoid Stimulation

Adrienne M. Grech^{1,2}, Udani Ratnayake², Anthony J. Hannan²,
Maarten van den Buuse^{3,4,5*} and Rachel A. Hill^{1,2}

¹ Department of Psychiatry, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, VIC, Australia, ² The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC, Australia, ³ School of Psychology and Public Health, La Trobe University, Melbourne, VIC, Australia, ⁴ Department of Pharmacology, University of Melbourne, Melbourne, VIC, Australia, ⁵ The College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia

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Edited by:

Mathias V. Schmidt,
Max-Planck-Institut für Psychiatrie,
Germany

Reviewed by:

Simone Macri,
Istituto Superiore di Sanità (ISS), Italy
Alessandra Berry,
Istituto Superiore di Sanità (ISS), Italy

*Correspondence:

Maarten van den Buuse
m.vandenbuuse@latrobe.edu.au

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Neurodevelopmental disorders are thought to be caused by a combination of adverse genetic and environmental insults. The “two-hit” hypothesis suggests that an early first “hit” primes the developing brain to be vulnerable to a second “hit” during adolescence which triggers behavioral dysfunction. We have previously modeled this scenario in mice and found that the combined effect of a genetic haploinsufficiency in the brain-derived neurotrophic factor (BDNF) gene (1st hit) and chronic corticosterone (CORT) treatment during adolescence (2nd hit), caused spatial memory impairments in adulthood. Environmental enrichment (EE) protocols are designed to stimulate experience-dependent plasticity and have shown therapeutic actions. This study investigated whether EE can reverse these spatial memory impairments. Wild-type (WT) and BDNF heterozygous (HET) mice were treated with corticosterone (CORT) in their drinking water (50 mg/L) from weeks 6 to 8 and exposed to EE from 7 to 9 weeks. Enriched housing included open top cages with additional toys, tunnels, housing, and platforms. Y-maze novel preference testing, to assess short-term spatial memory, was performed at 10 weeks of age. At week 16 dorsal hippocampus tissue was obtained for Western blot analysis of expression levels of BDNF, the BDNF receptor TrkB, and NMDA receptor subunits, GluNR1, 2A and 2B. As in our previous studies, spatial memory was impaired in our two-hit (BDNF HET + CORT) mice. Simultaneous EE prevented these impairments. However, EE appeared to worsen spatial memory performance in WT mice, particularly those exposed to CORT. While BDNF levels were lower in BDNF HET mice as expected, there were no further effects of CORT or EE in males but a close to significant

female CORT \times EE \times genotype interaction which qualitatively corresponded with Y-maze performance. However, EE caused both sex- and genotype-specific effects on phosphorylated TrkB residues and GluNR expression within the dorsal hippocampus, with GluNR2B levels in males changing in parallel with spatial memory performance. In conclusion, beneficial effects of EE on spatial memory emerge only following two developmental disruptions. The mechanisms by which EE exerts its effects are likely via regulation of multiple activity-dependent pathways, including TrkB and NMDA receptor signaling.

Keywords: brain-derived neurotrophic factor, spatial memory, environmental enrichment, hippocampus, corticosterone, stress, neuroplasticity

INTRODUCTION

Cognitive impairment is a common symptom in a range of neurodevelopmental disorders, including schizophrenia, major depressive disorder (MDD) and anxiety. In schizophrenia, cognitive impairment occurs in $\sim 80\%$ of cases and includes deficits in learning and memory, which have carry-on effects to social and intellectual functioning (Heinrichs and Zakzanis, 1998; Lewis, 2012). It has been reported that individuals with MDD and anxiety can have cognitive impairments in multiple domains including memory and attention (Gualtieri and Morgan, 2008; McIntyre et al., 2013).

One theory for the pathophysiology of neurodevelopmental disorders is the two-hit hypothesis. The “two-hit hypothesis” postulates that the combination of genetic predisposition and environmental insults during critical periods of development can culminate in significant behavioral disruption in adulthood (Klug et al., 2012). The “first hit” (genetic factor) during development creates a vulnerable brain, and when coupled with the “second hit” (environmental factor) triggers the onset of the disorder (Bayer et al., 1999).

Brain-derived neurotrophic factor (BDNF) is an essential neurotrophin responsible for a broad range of neuronal functions (Adachi et al., 2014) and is associated with neurodevelopmental disorders. Post-mortem studies have reported reduced levels of BDNF and its cognate receptor, Tropomyosin-related kinase B (TrkB) in the prefrontal cortex (PFC) and hippocampus of individuals with schizophrenia (Thompson Ray et al., 2011; Reinhart et al., 2015), suggesting a role of BDNF-TrkB signaling in the illness. There is support in the literature for altered BDNF in humans with depression (Lee and Kim, 2010; Zaletel et al., 2017) and anxiety (Soliman et al., 2010; Castrén, 2014). Stress has been recognized as a major environmental risk factor in the pathophysiology of schizophrenia models (van Os et al., 2010; Brown, 2011; Magariños et al., 2018), and depression and anxiety (Binder and Nemeroff, 2010; Zaletel et al., 2017). We therefore modeled the “two-hit” hypothesis by combining genetic haploinsufficiency in the BDNF gene (1st hit) with adolescent chronic corticosterone treatment (2nd hit). We previously found that these animals show short-term spatial memory deficits (Klug et al., 2012; Hill et al., 2014).

Prolonged corticosterone (CORT) administration in rodents is a well-established model to mimic the physiological parameters of chronic stress and disrupt the HPA axis (Buret and van den Buuse, 2014; Shahanoor et al., 2017). The hypothalamic pituitary adrenal (HPA) axis is the well-conserved control center for the body’s stress response. While its role is to moderate the stress response, it can cause damage through prolonged release of glucocorticoids (GC) (Du and Pang, 2015). In humans this is cortisol and the rodent equivalent is corticosterone (Papadimitriou and Priftis, 2009), and these can act in a negative feedback loop to regulate the HPA axis in their respective mammalian systems (Du and Pang, 2015). Dysregulation of this loop can have a range of negative effects upon behavior and cognition. Indeed, in the hippocampus there is a dense expression of glucocorticoid receptors (GR), and it is thought that the excess activity of GC here could be contributing to the cognitive deficits associated with chronic stress (Mirescu and Gould, 2006; Jayatissa et al., 2008; Rainer et al., 2012; Du and Pang, 2015).

For humans, leading a healthy lifestyle or having a “positive environment,” in both the physical and emotional sense, helps prevent and create resilience to neurodegenerative and mental health issues (Maass et al., 2014; Brown et al., 2017; Lee et al., 2018). A recent, comprehensive review by Arango et al. (2018) outlines that environmental risk factors such as poverty, stressful urban environments and negative social interactions such as bullying and abuse during childhood and adolescence can act synergistically to increase susceptibility to developing a neurodevelopmental disorder (Arango et al., 2018). It goes on to demonstrate that a range of interventions, including age-appropriate stimulation, proper nutrition and exercise can be important buffers against neurodevelopmental disorders. Another recent review by Devoe et al. suggested that cognitive behavioral therapy and family therapy are useful in the long-term reduction of attenuated psychotic symptoms (Devoe et al., 2018). This resilience is thought to be linked to a holistic health approach, which includes a “stimulating environment.” A stimulating environment encapsulates many domains, including social, physical, and cognitive. Research in adulthood has found that focus on social groups and music therapy can prevent and alleviate depressive symptoms (Cruwys et al., 2013) and schizophrenia patient outcomes (Fachner et al., 2013; Geretsegger et al., 2017; Erkkilä et al., 2018). This is consistent with the

Arango et al. review that argued appropriate stimulation is necessary for a healthy mind (Arango et al., 2018). Positive environments in preclinical animal model research generally refer to environmental enrichment (EE), an experimental protocol that aims to provide the laboratory animals with a habitat with an enhanced sensory environment, in order to stimulate experience-dependent plasticity (Nithianantharajah and Hannan, 2006; Novkovic et al., 2015). Rodent EE studies vary in their protocols to create an enriched environment, and include larger living areas, giving the animals access to toys or other stimulating materials, living in larger social groups, and exercise (Clemenson et al., 2015). This has been found to have positive effects including improved cognitive functioning (Yuan et al., 2012), delay of disease progression (Garofalo et al., 2015) and recovering of disease symptoms, with learning and memory also modulated by EE (Burrows et al., 2015). However, some studies have shown that EE can also have a stressful and negative impact upon laboratory animals, including increased aggression (McQuaid et al., 2012).

It is well established in the literature that the hippocampus has a central role in cognition, is affected in human neurodevelopmental disorders (Lavenex et al., 2006; Barnea-Goraly et al., 2014; Ledoux et al., 2014; Blair et al., 2017), and in rodent studies has been particularly responsive to EE-induced effects (Teather et al., 2002). This is hypothesized to occur through the BDNF-TrkB signaling pathway (Novkovic et al., 2015). BDNF binding to TrkB induces receptor dimerization and subsequent phosphorylation of tyrosine residues (Minichiello, 2009), the most important being 705, 515, and 816. Y705 has been called the initiator of receptor autophosphorylation (Benmansour et al., 2016) and has an overall role in TrkB activation, with the extent of phosphorylation of this residue correlating with TrkB activity levels (Huang and McNamara, 2010). The tyrosine residue 515 (Y515) is the Shc adapter protein docking site (Ambjorn et al., 2013; Benmansour et al., 2016), which catalyzes multiple signaling cascades including pathways involved in learning and memory (Yang et al., 2011). Y816 is linked to the phospholipase (PLC) γ 1 pathway, has a role in synaptic plasticity, cell survival and axon elongation (Ming et al., 1999; Atwal et al., 2000; Minichiello, 2009), and contributes to ERK activation (Ambjorn et al., 2013).

Several studies have demonstrated that EE increases BDNF levels in the hippocampus (Cao et al., 2014; Ramírez-Rodríguez et al., 2014; Novkovic et al., 2015) and, consequently exerts its positive effects upon cognition (Novkovic et al., 2015). Conversely, stress has been shown to negatively impact BDNF-TrkB signaling (Buckley et al., 2007). Chronic treatment with corticosterone (CORT) has been shown to decrease levels of BDNF mRNA and protein, as well as intracellular BDNF content (Nitta et al., 1999). Thus, we hypothesized that EE may recover the spatial memory deficit previously found in our two-hit model via regulation of the BDNF-TrkB signaling pathway. This hypothesis was tested by measuring protein expression of mature BDNF, TrkB, and multiple TrkB phosphorylation sites in the dorsal hippocampus.

Dysfunction of the inhibitory circuits and consequently the tilting of the excitatory/inhibitory balance toward over-excitation, is a major contributor to cognitive deficits present in neurodevelopmental disorders (Daskalakis et al., 2002; Heckers and Konradi, 2014; Fee et al., 2017; Selten et al., 2018). Excitotoxicity is characterized by increased extracellular concentrations of glutamate, which overactivate N-methyl-D-aspartate receptors (NMDAR) and allow an excess of Ca^{2+} influx. This activates a range of enzymatic effects that may cause cell damage or even cell death, resulting in a variety of detrimental neuronal and cognitive consequences. NMDAR are heteromeric tetramers consisting of different combinations of NMDAR subunits; usually including one NMDAR-1 (GluN1) subunit and at least one or more GluN2(A-D) or GluN3(A,B) subunits (Paoletti et al., 2013). NMDAR are located both at the pre- and post-synaptic sites, positioning them to play vital roles in long-term potentiation (LTP) and plasticity (Paoletti et al., 2013). Both of these processes are highly implicated in cognitive processes such as learning and memory (Nithianantharajah and Hannan, 2006; Vierk et al., 2014).

The first aim of this study was to investigate whether EE could reverse the spatial memory deficit in our “two-hit” model of BDNF haploinsufficiency and CORT treatment and how this would compare to EE effects in wildtype (WT) controls. The second aim of this study was to investigate any molecular changes to the BDNF-TrkB signaling pathway and NMDAR system in the dorsal hippocampus, and if these were modulated differentially by EE according to BDNF genotype, sex, and CORT treatment.

MATERIALS AND METHODS

Animals

Male and female BDNF heterozygous (HET) mice (Ernfors et al., 1994) and WT littermate controls were obtained from a breeding colony at the Florey Institute, Melbourne, Australia. All mice were on a C57Bl/6 background and breeders were originally obtained from The Jackson Laboratory (USA). 10 pairs of breeders were set up of WT female \times HET male. Tail tissue samples were sent to Transnetyx (Cordova, TN, USA) for genotyping. Mice were weaned at 3 weeks and WT and HET mice were housed together. Males and females were housed separately, with an average of 3 mice per cage. Offspring were randomized into 8 experimental groups: (1) Group-housed males, water and standard housed (SH) (WT $n = 7$, HET $n = 7$), (2) Group-housed males, CORT, and SH (WT $n = 11$, HET $n = 10$), (3) Group-housed males, water, and EE (WT $n = 9$, HET $n = 9$), (4) Group-housed males, CORT, and EE (WT $n = 11$, HET $n = 12$), (5) Group-housed females, water, and SH (WT $n = 15$, HET $n = 11$), (6) Group-housed females, CORT, and SH (WT $n = 8$, HET $n = 10$), (7) Group-housed females, water, and EE (WT $n = 10$, HET $n = 10$), and (8) Group-housed females, CORT, and EE (WT $n = 10$, HET $n = 8$). No obvious competition or dominance ranking within the groups was observed. No overt aggressive behavior was observed for the EE groups. Six animals per group were used for molecular analysis. Mice had *ad libitum* access to food and water in a temperature controlled room maintained at $\sim 22^\circ\text{C}$ and on a 12/12 h light/dark cycle. All procedures were

performed during the light phase. All procedures performed were done according to guidelines set by the National Health and Medical Research Council of Australia and approved by the Florey Institute for Neuroscience and Mental Health Animal Ethics Committee.

CORT Treatment

Adolescent/young adult mice were treated with corticosterone in the drinking water from 6 to 8 weeks of age (see **Figure 1**). These time points were based upon previous studies by our laboratory that show sexual maturation occurs during this period (Hill et al., 2012). Previous research has shown that in mice CORT can be administered between a dose of between 25 and 100 mg/L (Schroeder et al., 2015; Notaras et al., 2017). The CORT concentration of 50 mg/L was chosen with the assumption that the mice would increase water intake as they matured, and this CORT concentration would maintain CORT intake relative to body weight. A high CORT dose has been found in other models to create persistent stress phenotypes, which is important in a chronic model (Johnson et al., 2006; Gourley and Taylor, 2009). Corticosterone hemisuccinate (Q1662-000 Steraloids Inc, United States) was dissolved in water to a final concentration of 50 mg/L. CORT bottles were covered with aluminum foil to be protected from light degradation and were changed every 3–4 days. Bottles were weighed before they were replaced to measure CORT intake by the mice. CORT-treated mice tended to drink between 10 and 20 mg/kg/day. Once treatment stopped at the end of week 8, mice were left undisturbed for another 2 weeks. Control groups received water without CORT.

Environmental Enrichment

Mice received EE from 7 to 9 weeks of age (see **Figure 1**), during which they were kept in larger open top cages ($44 \times 30 \times 15$ cm) with various toys, tunnels, housing and platforms to provide novel cognitive challenges. These were changed once per week. Control mice were housed in open-top standard mouse cages ($34 \times 16 \times 16$ cm) with basic nesting materials and were designated “standard-housed” (SH). All mice were given 1 week to acclimatize to their environment when moved from open top to Individually-Ventilated Cages (IVC, $39.1 \times 19.9 \times 16$ cm, Tecniplast, Italy) at the end of week 9.

Y-Maze Short-Term Spatial Memory Test

The Y-maze paradigm was performed as previously described (Hill et al., 2014) at week 11. The maze consisted of three arms ($30 \times 8 \times 16$ cm) at 120° angles to each other including geometric cues on the far end walls. Briefly, during the initial phase, the mouse was placed into the end of one arm (home arm) and was allowed to explore two arms for 10 min with one arm being closed (novel arm). After a 1 h retention time in the home cage, the mouse was placed into the same Y-maze with all arms open for 5 min. Behavior, including the time spent in each arm, was analyzed with video tracking software (TopScan, CleverSys Inc., Reston, VA, USA). A Discrimination Index (DI) was calculated, which was the amount of time spent in the novel arm divided by the average amount of time spent in the home arm and other familiar arm. Mice with

intact spatial memory typically spend more time in the novel arm, reflective of intact memory of the original two familiar arms, and the DI tends to be around 1.5. A DI of around 1.0 represents equal times in all three arms (i.e., chance level) and is interpreted as no recollection of the two arms being familiar.

Western Blot Analysis

Mice were killed by cervical dislocation at 16 weeks of age and their brains were collected and stored at -80°C . The hippocampus was bilaterally dissected and separated into dorsal and ventral hippocampus ($\sim 50/50$). Protein extraction and Western blot analysis were performed as previously described (Hill et al., 2014). Primary antibodies were rabbit anti-BDNF (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA or Almone Labs, Israel), rabbit anti-NT-4 (1:200, Santa Cruz), rabbit anti-pTrkB Y705 (1:1,000, Signalway Antibody LLC, Maryland, USA), rabbit anti-pTrkB Y515 (1:1,000, Abcam, Cambridge, MA, USA), rabbit anti-pTrkB Y816 (1:500, Millipore, CA, USA), rabbit anti-TrkB (1:1,000, Santa Cruz), rabbit anti-NMDAR subunit 1 (GluNR1, 1:1,000, Cell Signaling Technology Inc, Danvers, MA, USA), rabbit NMDAR subunit 2A (GluN2A, 1:1,000, Cell Signaling Technology), rabbit NMDAR subunit 2B (GluN2B, 1:1,000, Cell Signaling Technology), or mouse anti- β -actin (1:10,000, Sigma-Aldrich). Secondary antibodies included anti-mouse or anti-rabbit IgG HRP-linked secondary antibodies (1:2,000; Cell Signaling Technology; Danvers, MA, USA).

Statistical Analysis

All data are expressed as the mean \pm the standard error of the mean (SEM). Groups were compared by ANOVA with the independent factors being sex (male or female), genotype (WT or HET), treatment (water or CORT), and environment (SH or EE), using the SYSTAT 13 (Systat Software Inc., San Jose, CA, USA). Thus, there were 16 experimental groups. *Post-hoc* comparisons were done with Tukey's test. Group differences were considered significant when $P < 0.05$.

RESULTS

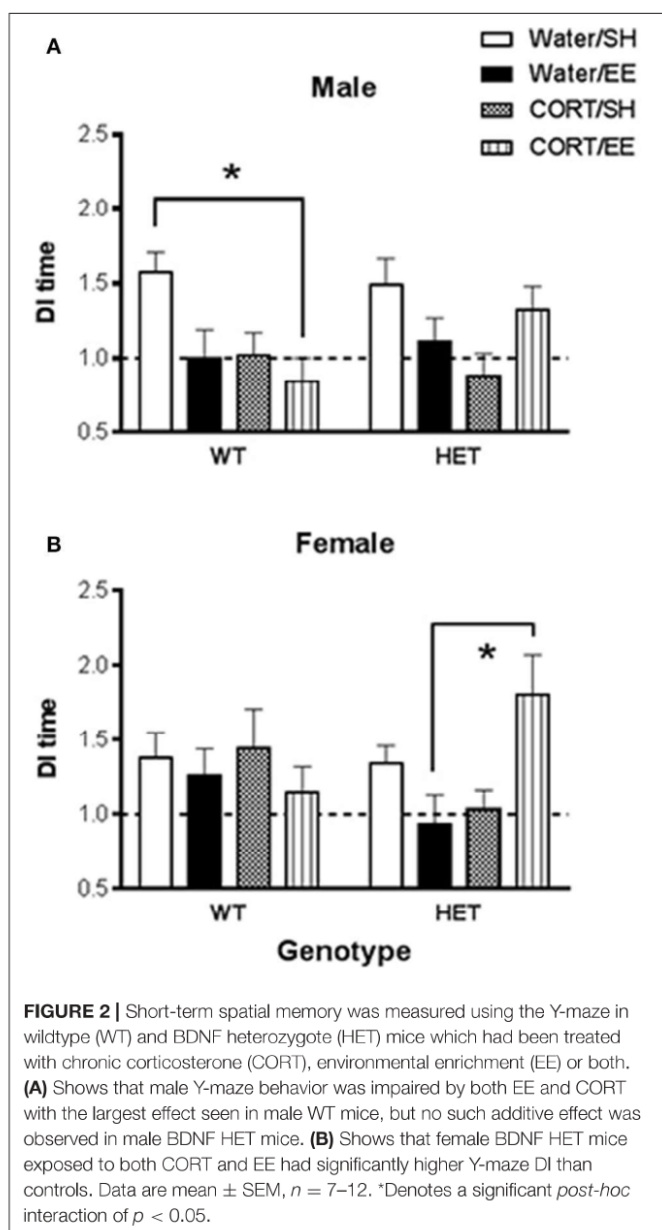
Y-Maze Behavior

Univariate ANOVA of the DI of time in the Y-Maze arms revealed that, while there were no main effects of either CORT or EE, there was a significant CORT \times EE interaction [$F_{(1, 142)} = 10.62$, $P = 0.001$], suggesting that any effect of CORT depended on whether the animals also underwent EE. Furthermore, this interaction appeared to depend on the genotype of the animals [CORT \times EE \times Genotype interaction: $F_{(1, 142)} = 6.81$, $P = 0.010$; EE \times Genotype interaction, $F_{(1, 42)} = 5.30$, $P = 0.023$]. Because there was also a CORT \times Sex interaction [$F_{(1, 142)} = 5.69$, $P = 0.018$], further interrogation of the data was done in males and females separately (**Figure 2**).

In males, there was again a CORT \times EE interaction [$F_{(1, 68)} = 7.37$, $P = 0.008$] although the CORT \times EE \times genotype interaction did not reach significance ($P = 0.079$). There was also a main effect of CORT treatment [$F_{(1, 68)} = 6.31$, $P = 0.014$]. Subsequent

	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 16
Mice: Male/Female WT/HET	Water or CORT treatment (50 mg/L in drinking water)				Adapt to IVC cages	Y-Maze	Animals euthanized and brain tissue collected
		Standard Housed or Environmental Enrichment					

FIGURE 1 | Time line of the experiments. Male and female WT and BDNF heterozygote (HET) mice were used to investigate whether environmental enrichment could ameliorate an established spatial memory deficit in a neurodevelopmental “two hit” model. BDNF heterozygosity was used as the first hit with the second hit being chronic corticosterone (CORT) administered in the drinking water. Environmental enrichment (EE) was administered during and after chronic CORT treatment.



pair-wise comparison with Tukey's test (**Figure 2A**) showed that DI values were significantly reduced in WT CORT/EE mice ($p = 0.045$) compared to WT water/SH.

In females, the CORT \times EE \times Genotype interaction was again significant [$F_{(1, 74)} = 7.16$, $P = 0.009$] suggesting differential effects of EE and CORT depending on the genotype. In HET mice, but not WT mice, pair-wise comparison with Tukey's test revealed that the combination of EE and CORT treatment resulted in significantly higher DI values compared to EE alone ($P = 0.009$) (**Figure 2B**).

Molecular Results

For the majority of investigated markers there was a main effect of sex when male and female data were combined, so it was decided to analyse the sexes separately. These main effects of sex were: mBDNF: $F_{(1, 67)} = 30.31$, $P < 0.001$; FL-TrkB: $F_{(1, 77)} = 227.13$, $P < 0.001$; Y705 ratio: $F_{(1, 72)} = 3105.0$, $P < 0.001$; Y816 ratio: $F_{(1, 68)} = 1061.98$, $P < 0.001$; GluN2A: $F_{(1, 51)} = 924.53$, $P < 0.001$; and GluN2B: $F_{(1, 67)} = 9.34$, $P = 0.003$.

mBDNF and NT4

Analysis of mBDNF levels revealed no effects of genotype, CORT, or EE on mBDNF expression in male dorsal hippocampus (**Figure 3A**). In females, there was similarly no main effect of genotype, CORT or EE, however there was a close to significant genotype \times CORT \times EE interaction [$F_{(1, 27)} = 4.13$, $P = 0.052$]. mBDNF expression levels qualitatively correspond with Y-maze performance with the female BDNF HET + CORT + EE group showing the highest expression (**Figure 3B**).

Analysis of NT-4 levels showed no effects of genotype, CORT, or EE either in males or females (**Figures 3C,D**).

TrkB and pTrkB

There were no effects of genotype, CORT or EE or interactions on FL-TrkB in the male and female DHP (**Figures 4A,B**).

Phosphorylation of TrkB was assessed at positions 705, 816, and 515. There was no main effect of genotype, CORT, or EE or interactions on pTrkB-Y705 in either males or females (**Figures 4C,D**). With respect to pTrkB-816, in the males there was a significant main effect of CORT [$F_{(1, 36)} = 16.45$, $P < 0.001$] and of EE [$F_{(1, 36)} = 8.22$, $P = 0.007$, **Figure 4E**], but no main effect of genotype or interactions. Inspection of the data (**Figure 4E**) shows that both CORT and EE increased the Y816 ratio and appear to have a cumulative effect when co-administered. This cumulative effect was also evident from a close to significant EE \times CORT interaction [$F_{(1, 36)} = 3.82$, $P = 0.058$] for Y816.

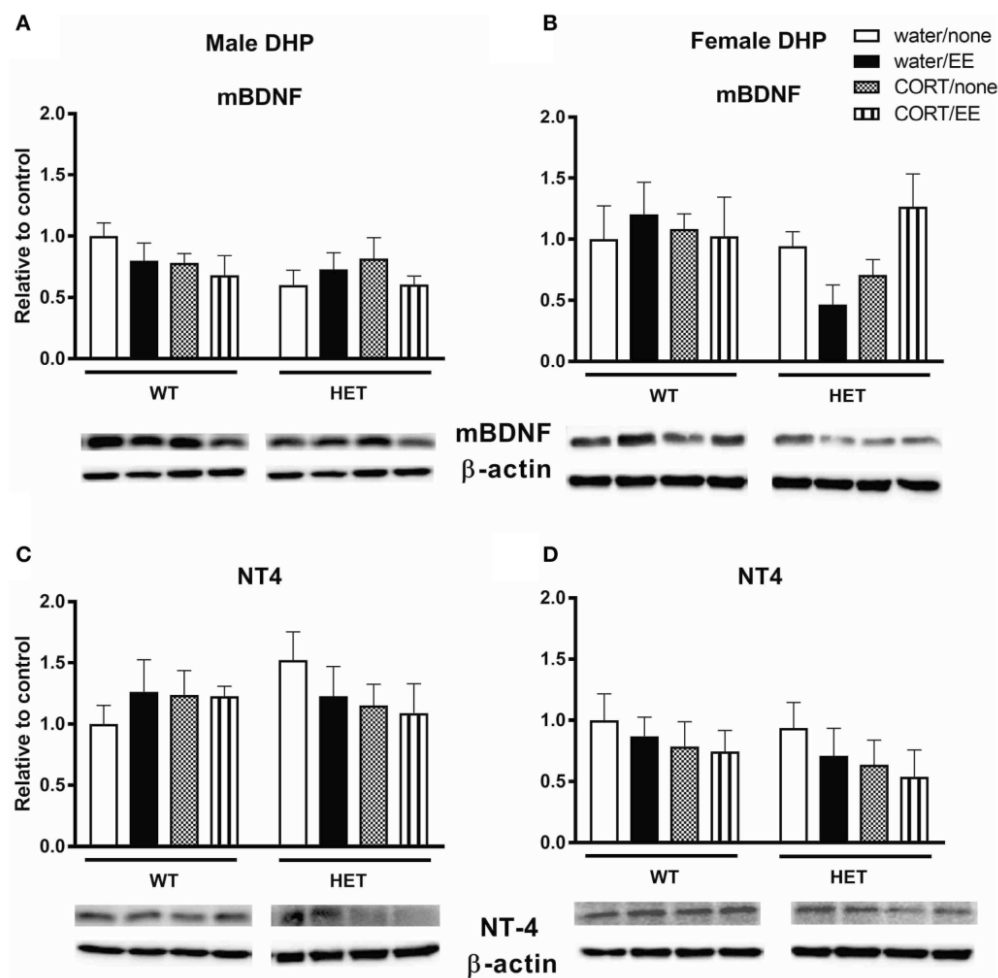


FIGURE 3 | Western blotting was used to measure protein expression of mature BDNF (mBDNF) and neurotrophin 4 (NT4) in the dorsal hippocampus (DHP) of wildtype (WT) and BDNF heterozygous (HET) mice after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both. Results are presented as standardized protein expression relative to control. There was a close to significant genotype \times CORT \times EE interaction on mBDNF in female DHP (**B**). There were no significant changes to protein expression of BDNF in males (**A**) or to NT-4 in males and females (**C, D**). Data are mean \pm SEM, $n = 3-6$, no significant main effects.

In female mice, there was a significant main effect of genotype [$F_{(1,36)} = 16.87$, $P < 0.001$] and CORT [$F_{(1,36)} = 18.99$, $P < 0.001$], but not EE, on Y816 levels (**Figure 4F**). There was also a significant EE \times CORT interaction [$F_{(1,36)} = 5.21$, $P = 0.028$], reflecting that CORT tended to increase expression while EE tended to reduce expression of Y816, particularly in WT mice. This differential effect of EE in WT compared to BDNF HET mice was supported by a genotype \times EE interaction [$F_{(1,36)} = 4.94$, $P = 0.033$]. However, there was no genotype \times CORT \times EE interaction.

Analysis of pTrkB515 expression in male mice showed a significant genotype \times CORT interaction [$F_{(1,39)} = 4.24$, $P = 0.046$, **Figure 4G**] whereby CORT treatment increased expression in the WT mice, but reduced expression in BDNF HET mice. There was no main effect of genotype, CORT, EE on Y515 in male mice. No significant effects of genotype, CORT, EE or interactions were found in female mice (**Figure 4H**).

NMDAR Subunits

Analysis of GluN1 protein expression in the male dorsal hippocampus revealed a main effect of genotype [$F_{(1,39)} = 5.73$, $P = 0.022$] but no effects of CORT or EE or interactions (**Figure 5A**). Male BDNF HET mice had higher protein expression of GluNR1 compared to male WT mice. Similarly, in females, there was a main effect of genotype [$F_{(1,28)} = 4.54$, $P = 0.042$], but no effects of CORT or EE, with BDNF HET mice having a higher GluN1 protein expression levels compared to WT mice (**Figure 5B**).

In male mice, we found a significant genotype \times CORT interaction for GluN2A protein expression [$F_{(1,39)} = 5.40$, $P = 0.001$, **Figure 5C**] but no effect of genotype, CORT or EE. This significant interaction resulted from reduced GluN2A protein expression in the male WT CORT/SH group but increased protein expression in male BDNF HET that underwent CORT/SH. *Post-hoc* Tukey's comparisons found while BDNF HET water/SH was significantly decreased ($p = 0.034$), BDNF

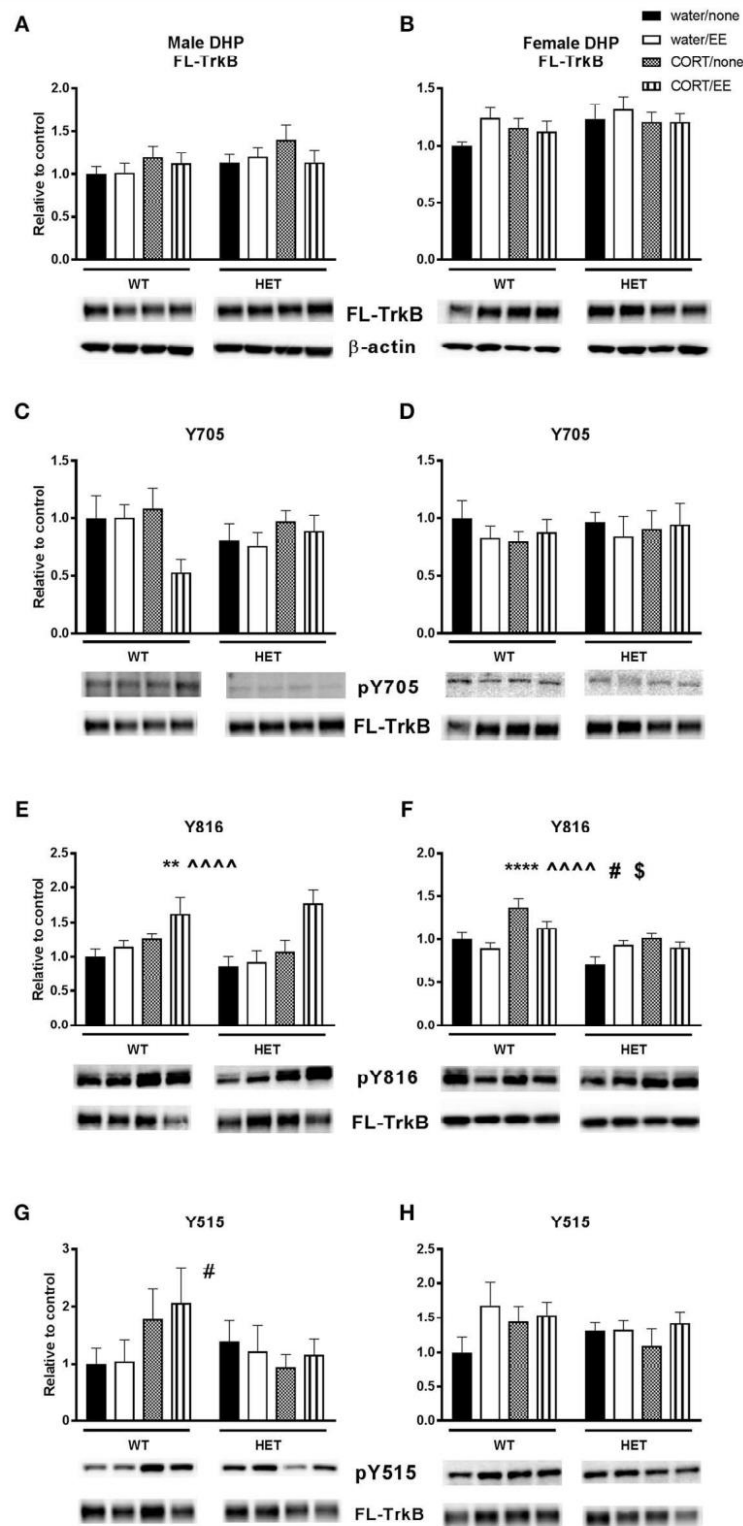


FIGURE 4 | Western blotting was used to measure protein expression of TrkB receptor and phosphorylated TrkB receptor residues after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus of wildtype (WT) and BDNF heterozygous (HET) mice. Results are presented as standardized protein expression relative to control. Phosphorylated TrkB residues are always divided by full length TrkB. For male results refer to (E), which shows a significant effect of CORT (^^^), and of EE (**) and (G) which shows a significant genotype \times CORT interaction (#). For female results refer to (F), which shows a significant main effect of genotype (**), significant main effect of CORT (i, significant genotype \times EE interaction (#) and significant EE \times CORT interaction (§). There were no significant changes to protein expression of FL-TrkB in males and females (A,B), Y705 in males and female (C,D) and Y515 in females (H). Data are mean \pm SEM, $n = 4-6$, main effect $**P < 0.005$, $^^^P < 0.001$, $****P < 0.001$, significant interaction $\#P < 0.05$ and $\$P < 0.005$ for protein expression based on ANOVA. For full details of ANOVA results, see main text.

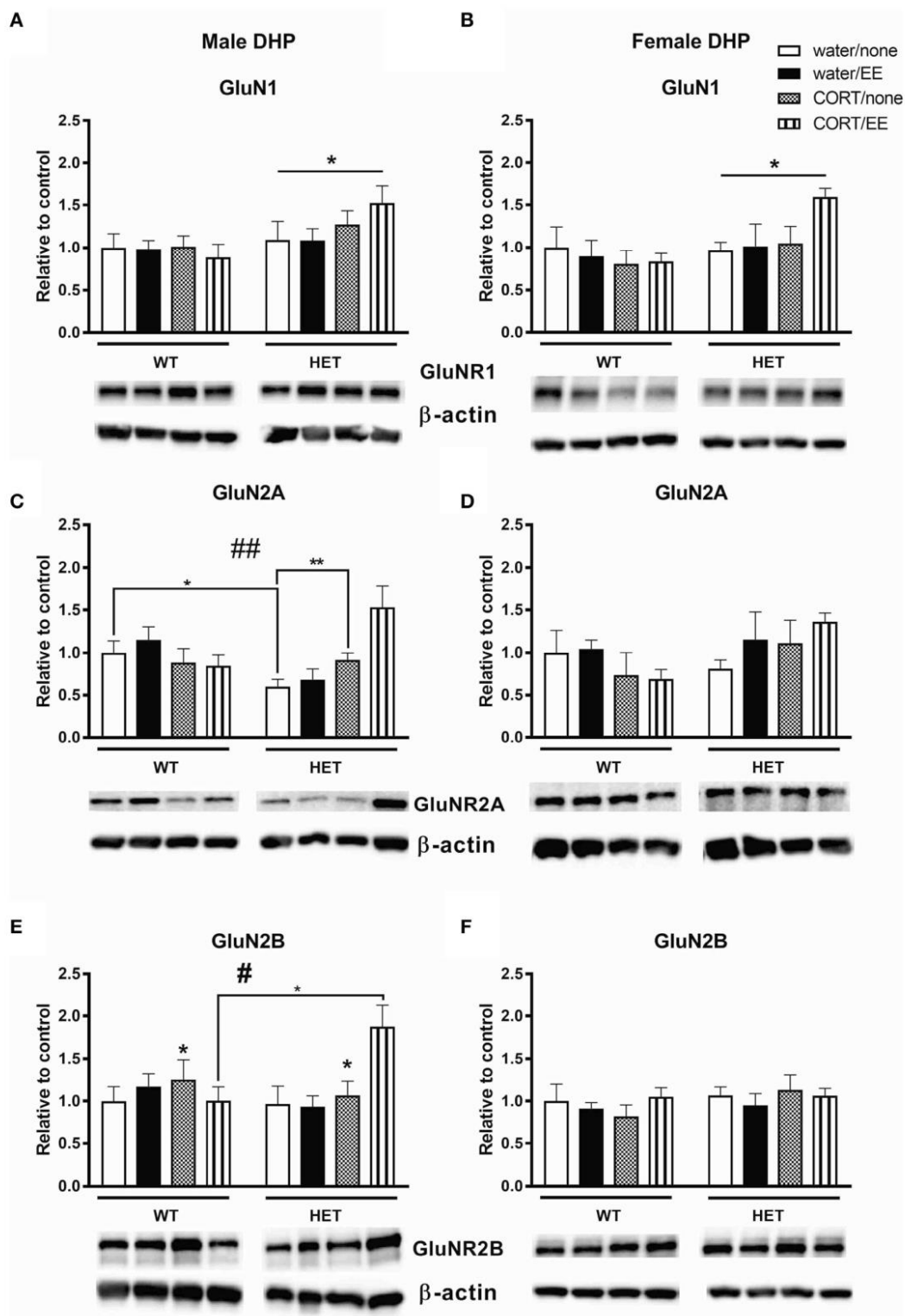


FIGURE 5 | Western blotting was used to measure protein of NMDAR subunits after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus of wildtype (WT) and BDNF heterozygous (HET) mice. Results are presented as standardized protein expression relative to control. **(A,B)** Show protein expression of NMDAR subunit GluN1, which had a significant main effect of genotype in males and females. **(C)** Shows male GluN2A protein expression had a genotype \times CORT interaction and **(E)** shows male GluN2B protein expression had significant main effect of CORT and a significant genotype \times EE \times CORT interaction. There were no significant changes to GluN2A and GluN2B protein expression in females **(D,F)**. Data are mean \pm SEM, $n = 3-6$, main effect or *post hoc* comparison $*p < 0.05$, significant interaction $\#p < 0.05$ and $\#\#p < 0.005$ for protein expression based on ANOVA and Tukey's *post-hoc*. For full details of ANOVA results, see main text. ******Indicates a significant *post-hoc* interaction of $p < 0.005$.

HET CORT/SH was significantly increased ($p = 0.003$) compared to controls. In female mice, there were no main effects of genotype, CORT, EE or interactions for GluN2A (Figure 5D).

Analysis of GluN2B levels in male dorsal hippocampus revealed a main effect of CORT [$F_{(1, 39)} = 4.45$, $P = 0.041$] whereby CORT increased GluN2B protein expression (Figure 5E). We also found a significant genotype \times CORT \times EE interaction [$F_{(1, 39)} = 5.50$, $P = 0.024$], and *post-hoc* comparisons found that the BDNF HET CORT/EE group had higher GluN2B protein expression compared to WT CORT/EE ($p = 0.04$). There were no main effects of genotype or EE for GluN2B and no main effects of genotype, CORT, EE or interactions for GluN2B in the females (Figure 5F).

DISCUSSION

This study investigated the possible preventative benefits of environmental enrichment on memory impairments in a two-hit neurodevelopmental model. The current study is an extension of previous research by our group (Klug et al., 2012), showing in the two-hit model that chronic adolescent stress, modeled here by chronic CORT treatment, combined with BDNF haploinsufficiency leads to a spatial memory deficit in the Y-maze. The primary aim of this study was to investigate if this deficit can be prevented through environmental enrichment. In humans, negative environmental factors can act synergistically with other risk factors to trigger the onset of neurodevelopmental disorders. However, a range of environmental interventions including age-appropriate stimulation, proper nutrition, and exercise have been found to be important preventatives against the onset of neurodevelopmental disorders and mental health issues (Arango et al., 2018).

In the current study, while we anticipated positive modulation on cognition by EE alone, it appeared to have a detrimental effect on spatial memory performance, particularly in male WT mice. In this group, Y-maze DI was reduced in both the EE and CORT groups, with the WT CORT-EE reaching significance compared to the control WT. In contrast, albeit not at the level of a statistical genotype \times CORT \times EE interaction, in male BDNF HET mice, this additive effect was not observed, and EE/CORT-treated BDNF HET mice had Y-maze DI values not different from control BDNF HET mice. Analysis of data from both sexes combined showed a significant genotype \times CORT \times EE effect and this interaction remained statistically significant in female mice, where a significantly higher DI was found in the EE/CORT-treated BDNF HET group compared to EE only.

Previous studies examining the effects of EE on hippocampal-dependent memory tasks have shown beneficial effects of EE on long-term memory in the Morris Water maze (Leggio et al., 2005; Garthe et al., 2015) and spatial working memory in the radial arm maze (Leggio et al., 2005). In addition, EE has been shown to improve performance in the novel object recognition task, and this study showed that NR2B transgenic mice with enhanced NR2B function show much longer recognition memory when exposed to EE, and furthermore, they showed increased expression of NR1, NR2B, and NR2A subunits following EE

exposure (Tang et al., 2001). This aligns with our study where we found significant effects of EE on NR2B subunits, however, we showed that this effect was specific to males, and these differential effects of EE on NMDA receptor subunits were contingent upon prior exposure to stress and BDNF genotype.

EE exposure for 4 weeks prior to behavioral testing has been shown to enhance spontaneous alternation in the Y-maze paradigm in mice (He et al., 2017) and rats (Jin et al., 2017). In addition, 6 weeks of EE treatment recovered Y-maze spatial memory preference for the novel arm in transient receptor potential channel (TRPC1)-/- mice (Xing et al., 2016). Mice exposed to chronic restraint stress for 4 weeks show impairments in spatial recognition memory in the Y-maze and here, simultaneously living in an enriched environment was able to ameliorate this deficit (Chen et al., 2010).

The above studies consistently show beneficial effects of EE on Y-maze performance, however, we found that EE alone appeared to negatively impact Y-maze novelty preference. This may be due to a number of important methodological considerations. Firstly, it has been shown that including a running wheel in the EE set up is critical particularly to the spatial memory-enhancing effects of EE (Lambert et al., 2005; Rogers et al., 2016). Our protocol did not include a running wheel. In addition, Zeleznikow-Johnston et al. (2017) also used an EE protocol that did not include a running wheel and while they found that EE enhanced visual discrimination and reversal learning, it had no effect on pattern separation or working memory in healthy mice. Thus, it appears that the beneficial effects of EE on hippocampal-dependent spatial memory tasks is heavily reliant upon physical activity as a component of EE. Secondly, the age at which EE is initiated is critical with most previous reports on the beneficial effects of EE initiating EE immediately post-weaning and continuing to maintain EE up to the time of behavioral testing (Simpson and Kelly, 2011). Our study began EE at 7 weeks of age and did not maintain EE until time of testing, thus the mice having experienced an enriched environment would have been in a state of deprivation prior to behavioral testing. Overall, in WT mice EE in our study appears to have functioned as another form of “stress,” with the combined effect of EE and CORT being negatively additive. In contrast, a restorative effect of EE emerged only after CORT treatment in BDNF HET mice. This may represent a variation of the “inoculation hypothesis,” which suggests that EE is a chronic mild stress, and as such creates a resilience to subsequent stressors (Crofton et al., 2015). It should be noted that in our study EE commenced after the introduction of CORT treatment, and mice were returned to standard housing prior to behavioral testing, thus EE followed by standard housing appeared to be another form of stress when compared to standard housing throughout the experimental timeline. Our results would suggest that this mechanism is particularly clear against a background of reduced BDNF levels. Two-hit animals (i.e., BDNF HET mice that received CORT treatment) may have been “inoculated” against EE, and as such EE was restorative to this group.

In the current study we found that Y-maze performance was impaired by chronic CORT treatment in both WT and BDNF HET male but not female mice, suggesting males are more

vulnerable to the effects of chronic corticosterone treatment. Female rats and mice are known to have higher levels of circulating corticosterone than males and a previous report in rats found that EE prevented a chronic stress-induced rise in corticosterone in females but not males, and showed a desensitization of the HPA axis to further exposure to an acute stress in female rats (Welberg et al., 2006). This may be due to interactions between female sex hormones such as estradiol and the HPA axis.

In our study, the close to significant genotype \times CORT \times EE interaction for mBDNF protein expression in the female dorsal hippocampus qualitatively follows the female pattern of Y-maze results, which had a significant genotype \times CORT \times EE interaction. This was sex-specific, as qualitatively the male Y-maze behavior did not align as closely to the measured mBDNF protein levels. Within the literature there is a broad range of results regarding EE and mBDNF levels. Most likely caused by the wide variety of protocols, studies have shown EE to both increase mBDNF (Cao et al., 2017) and to have no impact (Rogers et al., 2016). The diversity in approach is exemplified with two studies; Cao et al. exposed their mice to EE for 8 weeks, changing the cage every 4 days (Cao et al., 2017), while Rogers et al. (2016) had a similar set up to our study, with 4 weeks of EE and changing the cage weekly. It may be that the frequency of novelty and duration of exposure to EE are major considerations for effects on mBDNF protein expression. The current study found that the water/EE condition did not increase BDNF protein expression, which contrasts with other studies that show EE to increase BDNF (Chourbaji et al., 2012). BDNF is secreted in an activity-dependent manner (Hashimoto et al., 2000). However, in our study at the time-point the brains were collected, the mice were no longer exposed to EE. It could be that EE altered BDNF levels while it was on-going but after EE was stopped BDNF levels returned to pre-EE levels in response to the return to standard housing. In addition, sex is an obvious modifier of BDNF levels and, similar to our findings, a previous report in rats showed that female rats have higher levels of hippocampal BDNF and the effect of EE on increasing hippocampal BDNF levels was greater in females compared to males (Bakos et al., 2009).

In both sexes, both CORT and EE altered the expression of the phosphorylated Y816 TrkB residue. While CORT increased the Y816 ratio in both sexes, EE had a sexually divergent effect whereby it increased the Y816 ratio in males and decreased in females. A recent paper by Bengoetxea et al. (2018) found that only 1 week of EE in male rats increased the expression of TrkB, and improved performance in the Morris Water Maze. Comparatively in our study, when comparing both male genotypes' Y816 ratio to the respective Y-maze data, it would appear that the Y816 ratio shows the inverse trend compared to the Y-maze. This is indicating that while this residue has increased activation, this is not reflective of improved Y-maze performance. However, it should be noted that hippocampal lysates were analyzed 5 weeks following behavioral testing and thus do not reflect TrkB activation at the time of behavioral testing. A similar phenomenon is observed in the females, however it was more genotype

specific to the female two-hit mice. Again, while the Y816 ratio is increased, the behavior did not reflect this, perhaps indicating that despite long-term increased activation of the residue this did not result in improved spatial memory performance.

An interesting divergence emerges in the male Y515 ratio results. The recorded genotype \times CORT interaction demonstrated that CORT treatment increased Y515 ratio in WT but decreased it in the two hit animals. However, despite this divergence at the molecular level, possibly because of statistical power issues, the analysis did not show a CORT \times genotype interaction for spatial memory performance, although this group clearly showed the lowest DI. Because both Y515 and Y816 contribute to the ERK1/2 signaling pathway, it is possible that both are needed to be upregulated to improve behavior.

It has been reported that chronic glucocorticoid treatment disrupts the interaction between glucocorticoid receptors and TrkB with subsequent dampening of the PLC γ signaling pathway (Numakawa et al., 2013), with implications for cognition. Yan et al. (2016) recently demonstrated that chronic CORT treatment decreased pTrkB, and Barfield and Gourley (2017) showed truncated TrkB, the inactive form, to be increased in relation to FL-TrkB after chronic adolescent CORT treatment. Our study adds to this literature by investigating the activation of the distinct residues, and the results discussed above contribute to creating a comprehensive molecular map of how glucocorticoids and the BDNF-TrkB signaling pathway dynamically interact.

Of the three NMDA receptor subunits investigated, the most interesting changes occurred on GluN2B in the males. CORT increased GluN2B protein expression but despite the role of NMDAR in cognition, Y-maze performance was impaired. It could be that this increase of GluN2B is excitotoxic and this is a possible mechanism through which CORT impairs spatial cognition in male mice. This is consistent with previous research by our lab (Klug et al., 2012), which found that CORT treatment increased NR2B protein levels in male BDNF HET mice and paralleled impaired spatial memory as measured by the Y-maze (Klug et al., 2012).

This study was specifically interested in EE as a preventative treatment during adolescence, however a study design limitation is the lack of ability to compare this treatment window with longer-term EE treatment that continues into behavioral testing and up until the point of brain collection. Another limitation of this study is that we present here findings from only one short-term spatial memory task. Future studies should include other spatial memory tasks as well as address other cognitive domains, such as working memory and recognition memory. Overall our study shows that the effects of EE on spatial memory are heavily dependent on BDNF genotype and prior exposure to stress, with our results showing benefit only in two-hit (BDNF HET \times CORT) mice. In addition, we show an increased vulnerability of males to chronic CORT and EE and this coincides with male-specific alterations to phosphorylated TrkB residues and NMDA receptor subtypes following both CORT and EE exposure. Female spatial memory performance, however, aligned with mBDNF expression levels in the dorsal hippocampus. These results show that the timing and nature of EE, prior exposure to a model

of stress, BDNF genotype, and sex are all critical modifiers of EE-induced spatial memory and molecular outcomes.

AUTHOR CONTRIBUTIONS

AG performed all Western blot analysis and data analysis and wrote the first draft of the paper. UR performed the behavioral testing and its analysis. AH advised on the original project and on data analysis and interpretation. MvdB designed the project,

oversaw behavioral testing, performed data analysis, and edited the manuscript. RH co-designed the project, oversaw Western blot analysis, performed data analysis, and edited the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.3 Supplementary Material Results

There were significant sex effects for most markers in the DHP (see Table 3.1), with only PV having a non-significant effect of sex. The sexes were subsequently separated for further analyses and ventral hippocampus was separated by sex to be consistent.

3.3.1 Inhibitory Markers

3.3.1.1 *GAD67*

For males and females there were no significant effects of genotype, CORT or environmental enrichment on the protein expression of GAD67 in the DHP and VHP (Figure 3.1).

3.3.1.2 *Parvalbumin*

Male mice showed no significant effects of genotype, CORT or environmental enrichment on the protein expression of PV in the DHP and VHP (Figure 3.2A and B). There were no significant effects of genotype, CORT or environmental enrichment on PV protein expression in the female DHP (Figure 3.2C). In the female VHP (Figure 3.2D) there was a significant genotype \times CORT interaction ($F(1,36) = 4.80$, $p = 0.035$), with no significant post hoc comparisons observed. Here, HET animals had decreased PV protein expression after CORT treatment, but WT animals were unchanged.

Table 3.1 - Statistical results for main effect of sex for majority of markers

	Dorsal Hippocampus
GAD67	$F(1, 78) = 497.12, p < 0.0001^*$
Parvalbumin	$F(1, 67) = 0.2, p = 0.65$
Calretinin	$F(1, 68) = 254.72, p < 0.0001^*$
Somatostatin	$F(1, 80) = 47.18, p < 0.0001^*$
pERK1	$F(1, 78) = 23.65, p < 0.0001^*$
ERK1	$F(1, 77) = 19.55, p < 0.0001^*$
pERK1/ERK1	$F(1, 72) = 0.71, p = 0.4$
pERK2	$F(1, 78) = 133.49, p < 0.0001^*$
ERK2	$F(1, 79) = 615.74, p < 0.0001^*$
pERK2/ERK2	$F(1, 69) = 3.69, p = 0.059^\#$

* $p < 0.005$, # approaching significant

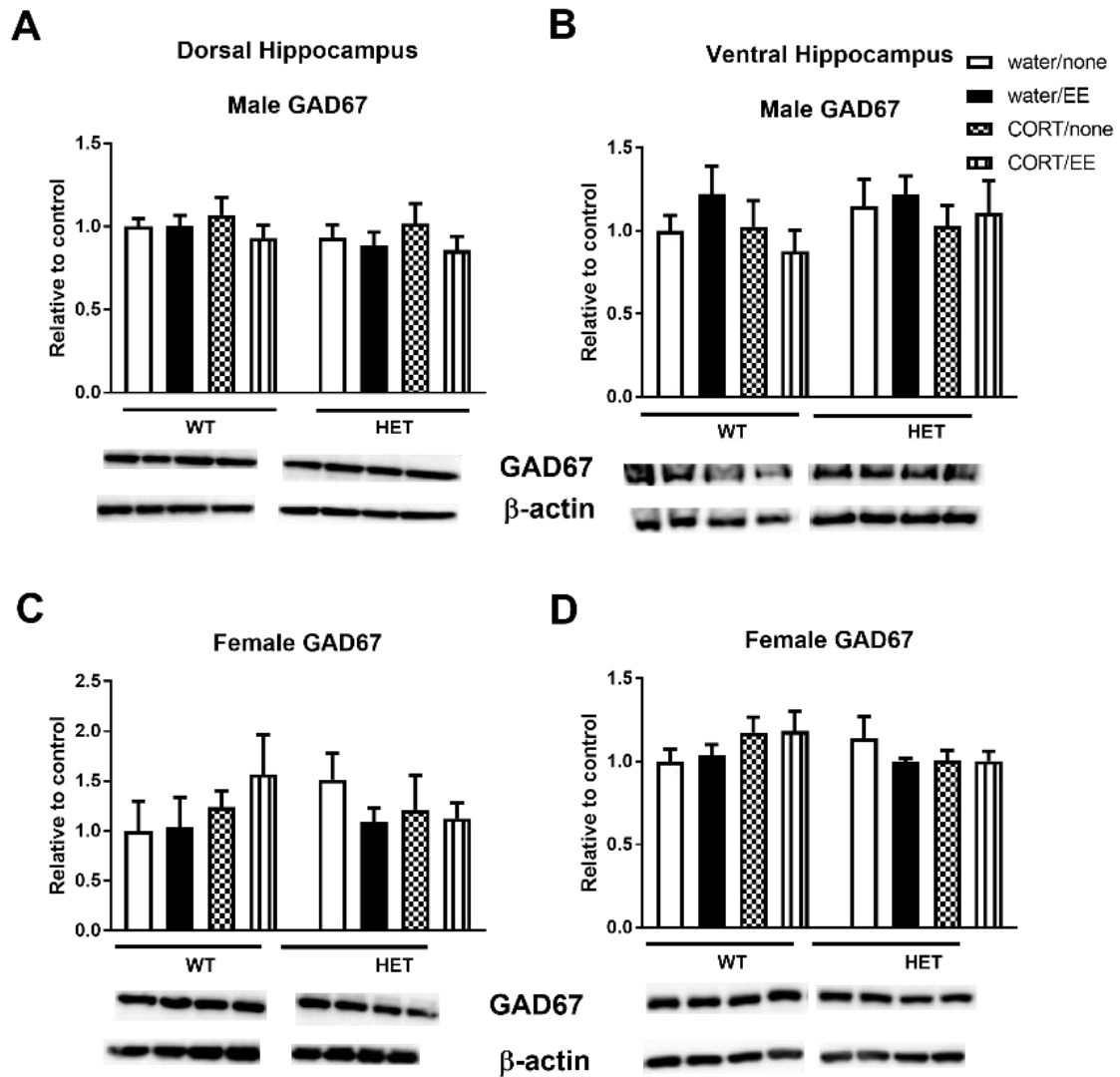


Figure 3.1 - GAD67 protein expression in the hippocampus

Western blotting was used to measure protein expression of GAD67 after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus (DHP) and ventral hippocampus (VHP) of male and female 2-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Data are mean \pm SEM, $n = 4-6$, no significant main effects.

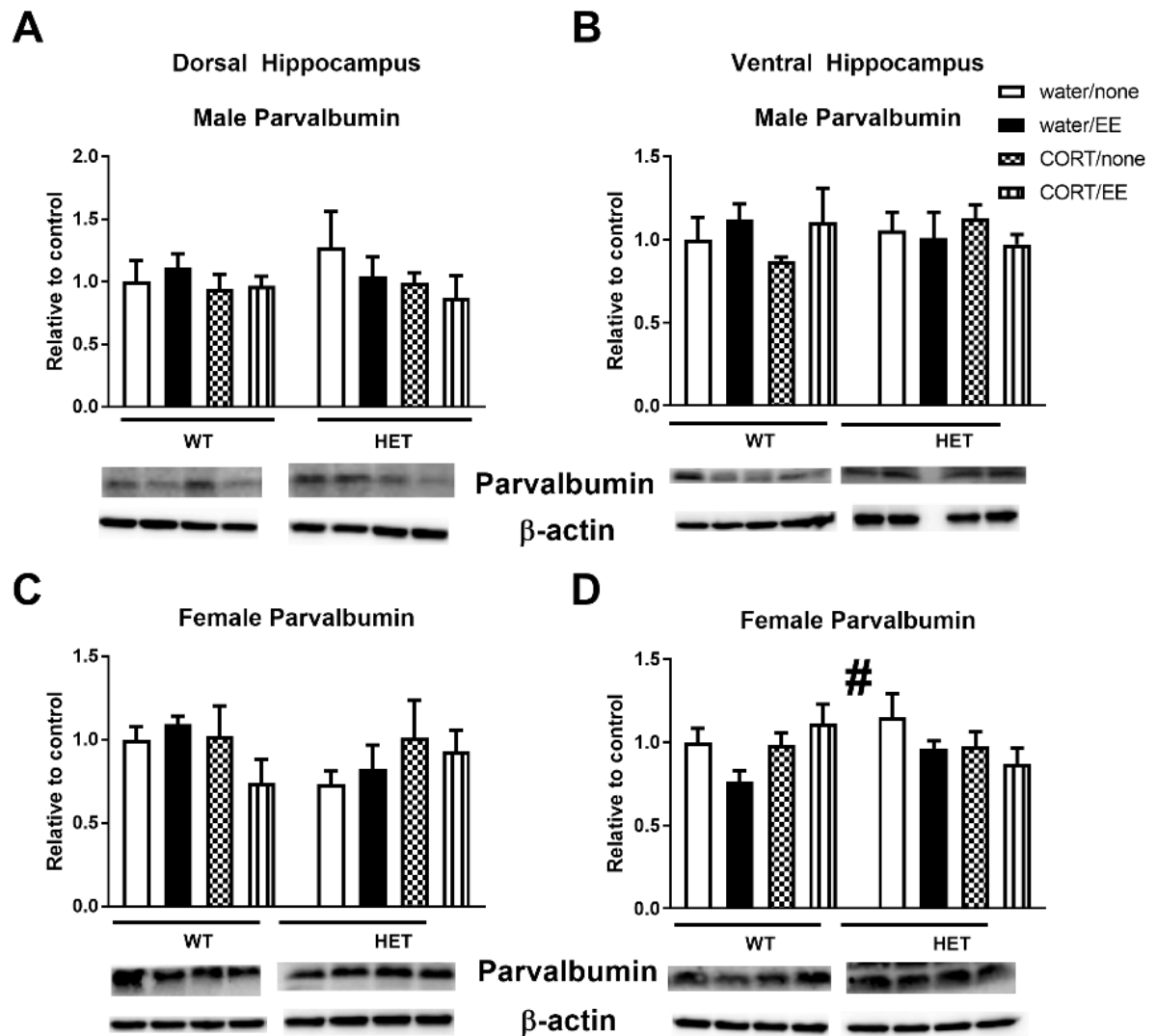


Figure 3.2 - Parvalbumin protein expression in the hippocampus

Western blotting was used to measure protein expression of parvalbumin (PV) after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus (DHP) and ventral hippocampus (VHP) of male and female wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Male DHP (panel A), male VHP (panel B) and female DHP (panel C) show no significant effects of genotype, EE or CORT on PV protein expression. Female VHP (panel D)

shows a significant genotype \times CORT interaction (#) for PV protein expression. Data are mean \pm SEM, n = 3-6, # p < 0.05.

3.3.1.3 Calretinin

A significant genotype \times CORT interaction was found ($F(1,37) = 5.21$, $p = 0.028$) for male DHP (Figure 3.3A), with no significant Tukey's post hoc comparisons observed. Here, HET animals show decreased CAL protein expression after CORT treatment while WT animals show elevated levels. This was hippocampal region specific, with no significant effects of genotype, CORT or environmental enrichment on male VHP CAL protein expression (Figure 3.3B). Female mice showed no significant effects of genotype, CORT or environmental enrichment on the protein expression of CAL in the dorsal and ventral hippocampus (Figure 3.3C, D).

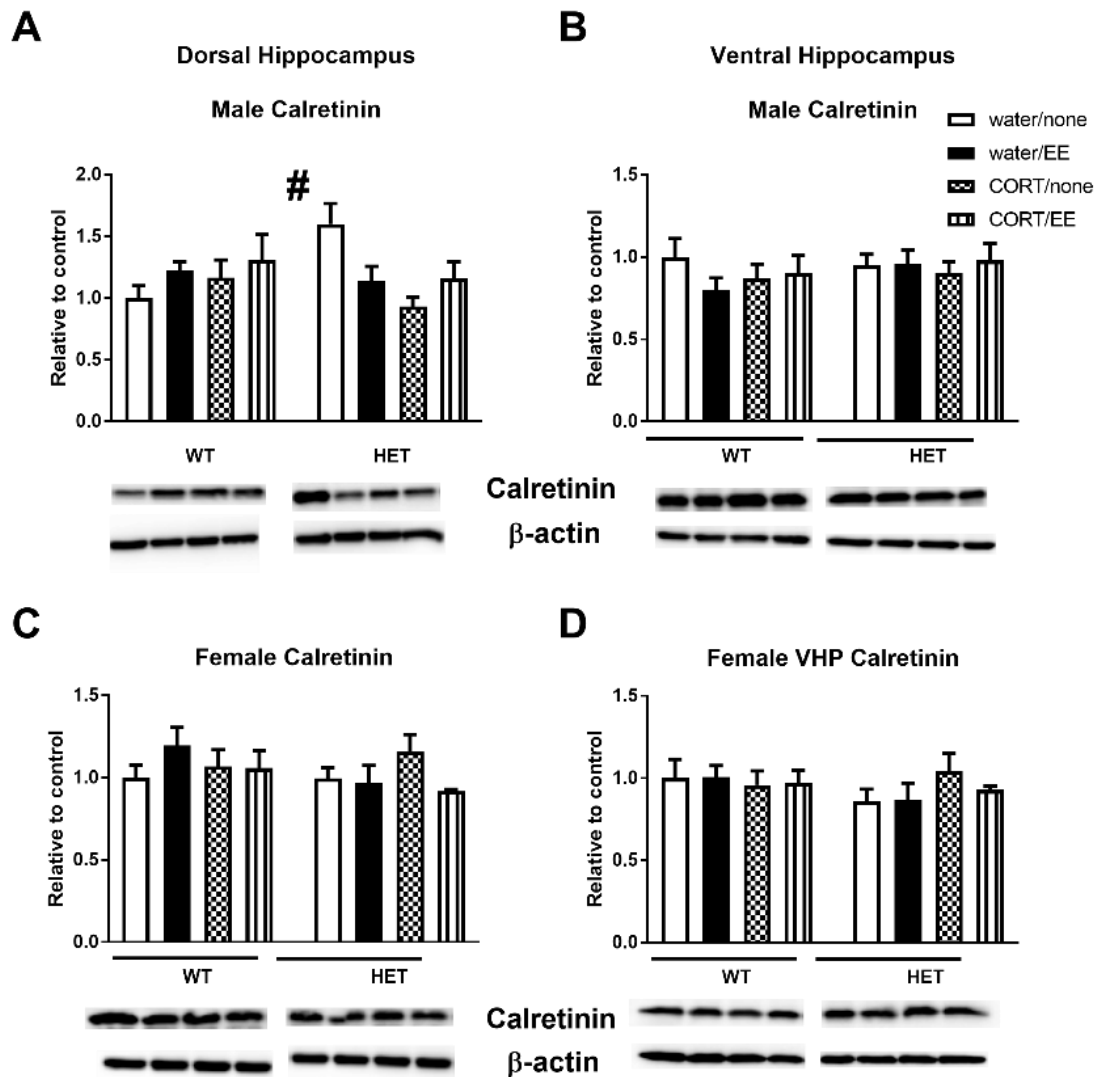


Figure 3.3 - Calretinin protein expression in the hippocampus

Western blotting was used to measure protein expression of calretinin (CAL) after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus (DHP) and ventral hippocampus (VHP) of male and female wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Male DHP (panel A) shows a genotype x CORT interaction (#) for CAL protein expression. Male VHP (panel B), female DHP (panel C) and female VHP (panel D) showed no significant effects of genotype, EE or CORT. Data are mean \pm SEM, $n = 3-6$, # $p < 0.05$

3.3.1.4 Somatostatin

Male mice had no significant main effects of genotype, CORT or environmental enrichment on the protein expression of SST in the dorsal and ventral hippocampus (Figure 3.4A, B). A significant genotype \times CORT interaction ($F(1,40) = 9.7$, $p = 0.003$) was observed for SST protein expression in the female DHP (Figure 3.4C). Tukey's post hoc comparisons revealed that the HET CORT group had significantly lower SST protein expression compared to the WT SH group ($p = 0.036$) and the WT CORT group ($p < 0.0001$). There were no significant effects of genotype, CORT or environmental enrichment on female VHP SST protein expression (Figure 3.4D).

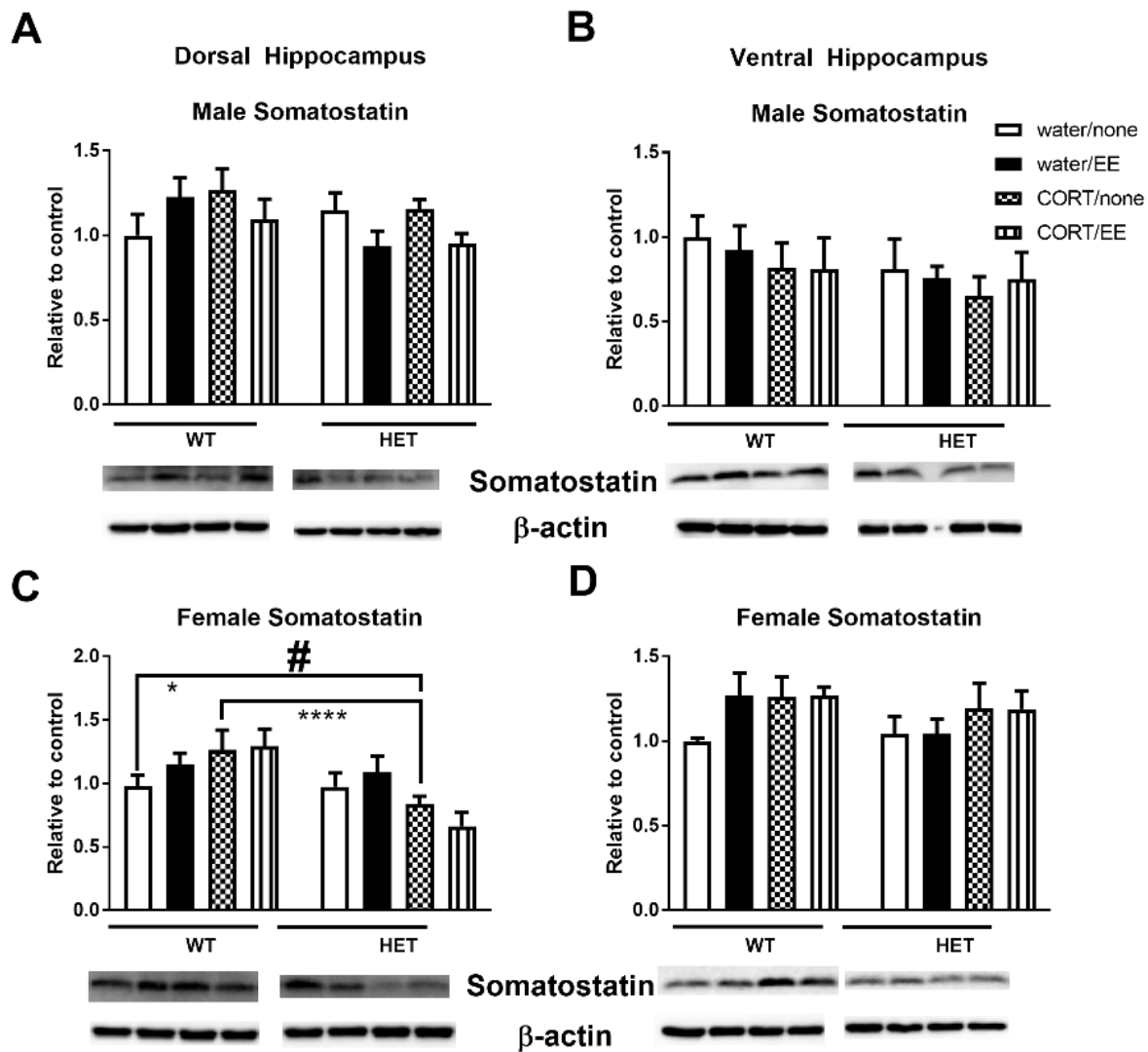


Figure 3.4 - Somatostatin protein expression in the hippocampus

Western blotting was used to measure protein expression of somatostatin (SST) after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus (DHP) and ventral hippocampus (VHP) of male and female wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Panel A and B show no significant effects of genotype, EE or CORT in the male DHP and male VHP respectively. Panel C shows a genotype \times CORT interaction (#) for

SST protein expression in the female DHP. Panel **D** shows no significant effects of genotype, EE or CORT in the female VHP. Data are mean \pm SEM, $n = 3-6$, # $p < 0.05$, post hoc comparisons * $p < 0.05$, **** $p < 0.0001$.

3.3.1.5 Erk1/2 Phosphorylation: Male Dorsal Hippocampus

3.3.1.5.1 ERK1

In male DHP total protein expression of phosphorylated ERK1/2 (pERK1/2) was measured (Figure 3.5). For pERK1 there was a significant main effect of genotype ($F(1,38) = 4.712$, $p = 0.036$), whereby HET groups had higher pERK1 protein expression compared to WT groups (Figure 3.5A). There were significant main effects of CORT ($F(1,38) = 15.704$, $p < 0.0001$) and EE ($F(1,38) = 10.248$, $p = 0.003$). Similar patterns were observed for these effects (Figure 3.5A). CORT and EE groups of both genotypes expressed higher pERK1 protein expression than the relevant SH group. This was excluding the WT EE group, which had unchanged pERK1 protein expression compared to WT SH. No significant interactions between the three independent factors were found. There were no significant main effects of genotype, CORT, EE or interactions for total ERK1 protein expression (Figure 3.5B). For the pERK1/ERK1 ratio a significant main effect of EE was observed ($F(1, 38) = 7.77$, $p = 0.008$), here EE increased the ERK1 ratio (Figure 3.5C). There was no significant effect of genotype, CORT or interaction for the ERK1 ratio in the male DHP.

3.3.1.5.2 ERK2

In male DHP a significant effect of EE ($F(1,37) = 10.01$, $p = 0.003$) was found, whereby EE increased protein expression of pERK2 (Figure 3.5D). A significant effect of CORT ($F(1,37) = 7.74$, $p = 0.008$) was observed, and here CORT also increased pERK2 protein expression in WT.

It is interesting to note that a cumulative effect can be observed in the CORT-EE group (Figure 3.5D). There were no significant effects of genotype, EE or CORT for total ERK2 protein expression in male DHP (Figure 3.5E). A significant effect of EE ($F(1,35) = 5.57$, $p = 0.024$) was observed again, for the pERK2/ERK2 ratio (Figure 3.5F). Here, as for pERK2 expression, EE increased the pERK2/ERK2 ratio.

3.3.1.6 Erk1/2 Phosphorylation: Male Ventral Hippocampus

3.3.1.6.1 ERK1

For pERK1 in the male VHP there was a main effect of genotype ($F(1,35) = 5.36$, $p = 0.027$), whereby WT groups had higher expression of pERK1 than HET groups (Figure 3.6A). There was a significant effect of EE ($F(1,35) = 5.86$, $p = 0.021$), and here EE appears to decrease pERK1 protein expression. There were no significant effects of genotype, EE or CORT on total ERK1 expression (Figure 3.6B) and pERK1/ERK1 ratio in the male VHP (Figure 3.6C).

3.3.1.6.2 ERK2

There were no significant effects of genotype, EE or CORT on pERK2 and ERK2 expression or pERK2/ERK2 ratio in the male VHP (Figure 3.6).

Male Dorsal Hippocampus

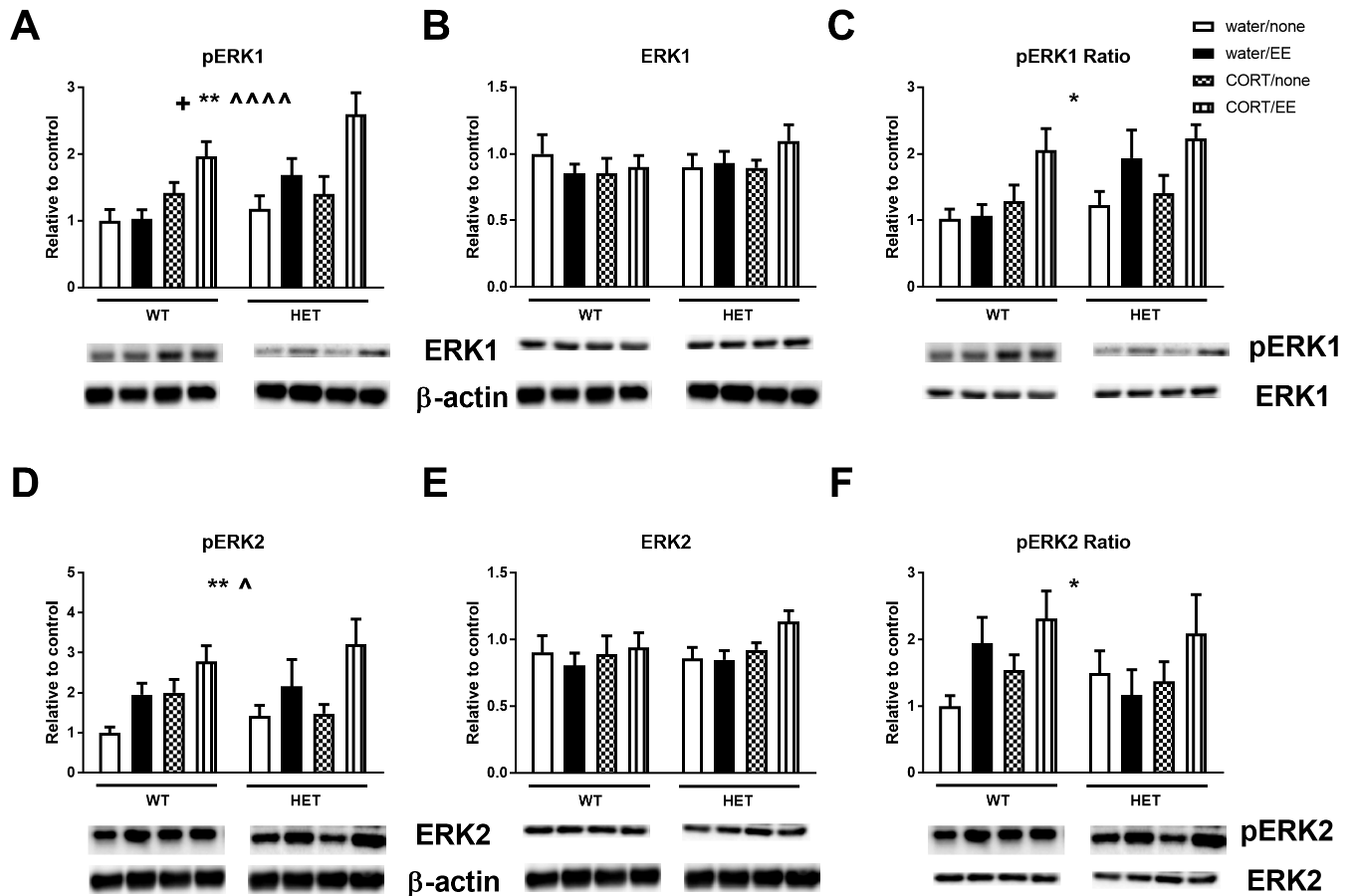


Figure 3.5 - ERK1/2 protein expression in male dorsal hippocampus

Western blotting was used to measure protein expression of pERK and ERK after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus (DHP) of male wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Panel A shows a main effect of genotype (+), EE (**) and CORT (^^^)^ for pERK1 protein expression in male DHP. Panel B shows no significant effects of genotype, EE or CORT in male DHP. Panel C shows a main effect of EE (*) for the pERK1/ERK1 ratio. Panel D shows a main effect of EE (**) and CORT (^) for pERK2 protein expression in male DHP. Panel F shows a main effect of EE (*) for the

*p*ERK2/ERK2 ratio in male DHP. Data are mean \pm SEM, *n* = 5-6, +, * or ^ *p* < 0.05, ** *p* < 0.005, ^^^ *p* < 0.0001.

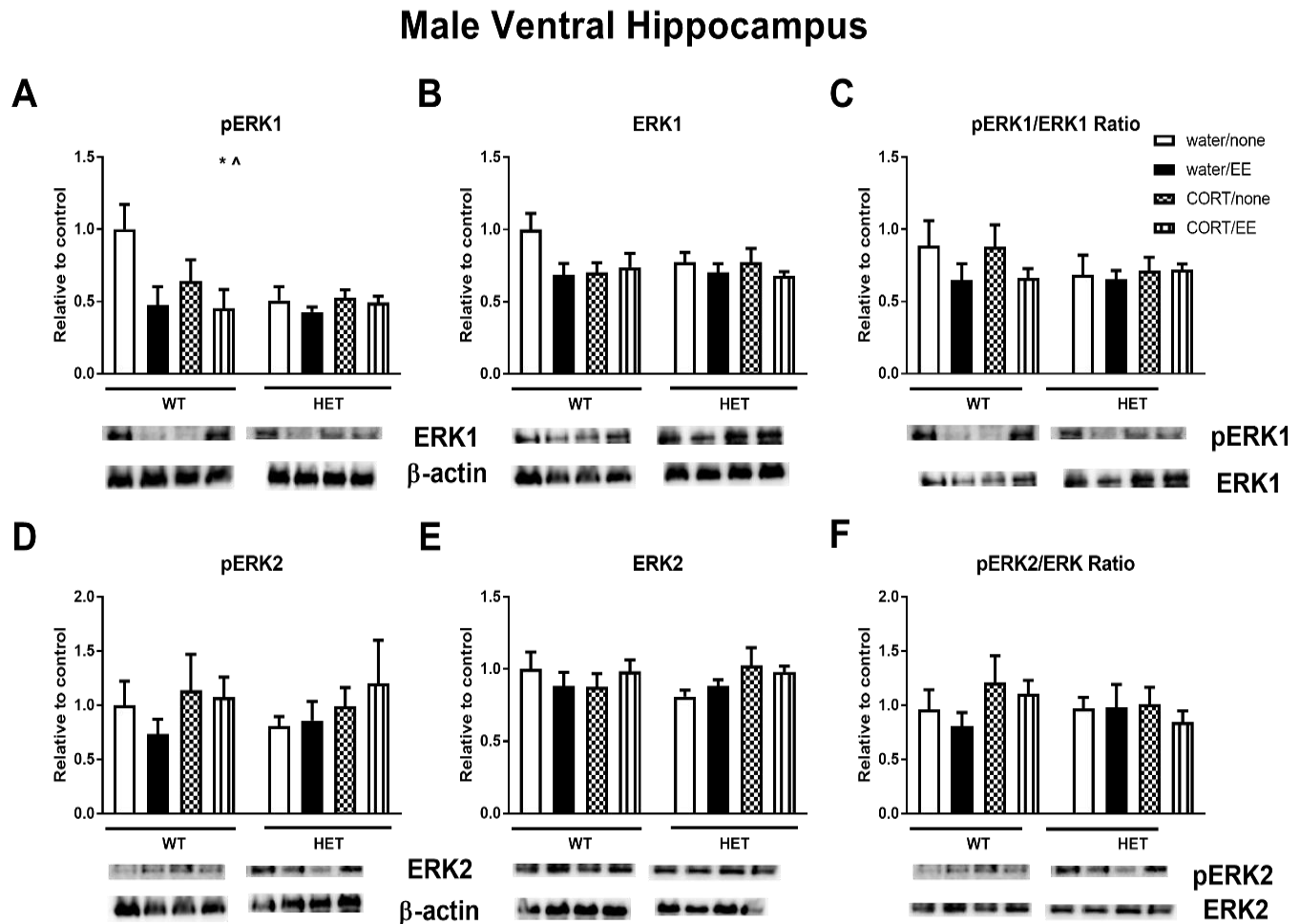


Figure 3.6 - ERK1/2 protein expression in the male ventral hippocampus

Western blotting was used to measure protein expression of pERK and ERK after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the ventral hippocampus (VHP) of male wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Panel A shows a main effect of genotype (*) and EE (^) for pERK1 protein expression in male VHP. Panels B, C, D, E and F

show no significant effects of genotype, EE or CORT in male VHP. Data are mean \pm SEM, $n = 4-6$, * or $^{\wedge} p < 0.05$.

3.3.1.7 Erk1/2 Phosphorylation: Female Dorsal Hippocampus

3.3.1.7.1 ERK1

For pERK1 expression in the female DHP there was a significant main effect of EE ($F(1,40) = 10.27$, $p = 0.003$). Here, EE decreased pERK1 expression (Figure 3.7A). There were no significant effects for ERK1 expression in the female DHP (Figure 3.7B). A significant main effect of EE ($F(1,37) = 11.21$, $p = 0.002$) was also found for pERK1/ERK1 ratio (Figure 3.7C). Similarly, to pERK1, EE decreased the pERK1/ERK1 ratio.

3.3.1.7.2 ERK2

In the female DHP a significant main effect of EE ($F(1,39) = 4.81$, $p = 0.034$) was found for pERK2 protein expression (Figure 3.7D). Here, like female DHP ERK1 results, EE decreased pERK2 expression. There were no significant effects of genotype, EE or CORT for ERK2 expression (Figure 3.7E) and pERK2/ERK2 ratio (Figure 3.7F).

3.3.1.8 Erk1/2 Phosphorylation: Female Ventral Hippocampus

3.3.1.8.1 ERK1

In the female VHP there was no significant effect of genotype, EE or CORT on pERK1 (Figure 3.8A). However, a significant main effect of genotype ($F(1,31) = 14.3$, $p = 0.001$) was found for total ERK1 protein expression, whereby HET groups appear to have lower expression compared to WT groups (Figure 3.8B). There were no significant effects of independent factors for the pERK1/ERK1 ratio in the female VHP (Figure 3.8C).

3.3.1.8.2 ERK2

There were no significant effects of genotype, EE or CORT for total ERK2 (Figure 3.8D) and pERK2 (Figure 3.8E) expression in the female VHP. However, a significant genotype \times EE interaction ($F(1,36) = 5.01$, $p = 0.031$) was observed for the pERK2/ERK2 ratio but no significant Tukey's post hoc comparisons were found (Figure 3.8F). Here EE appears to reduce pERK2/ERK2 ratio in WT mice but not in HET mice.

Female Dorsal Hippocampus

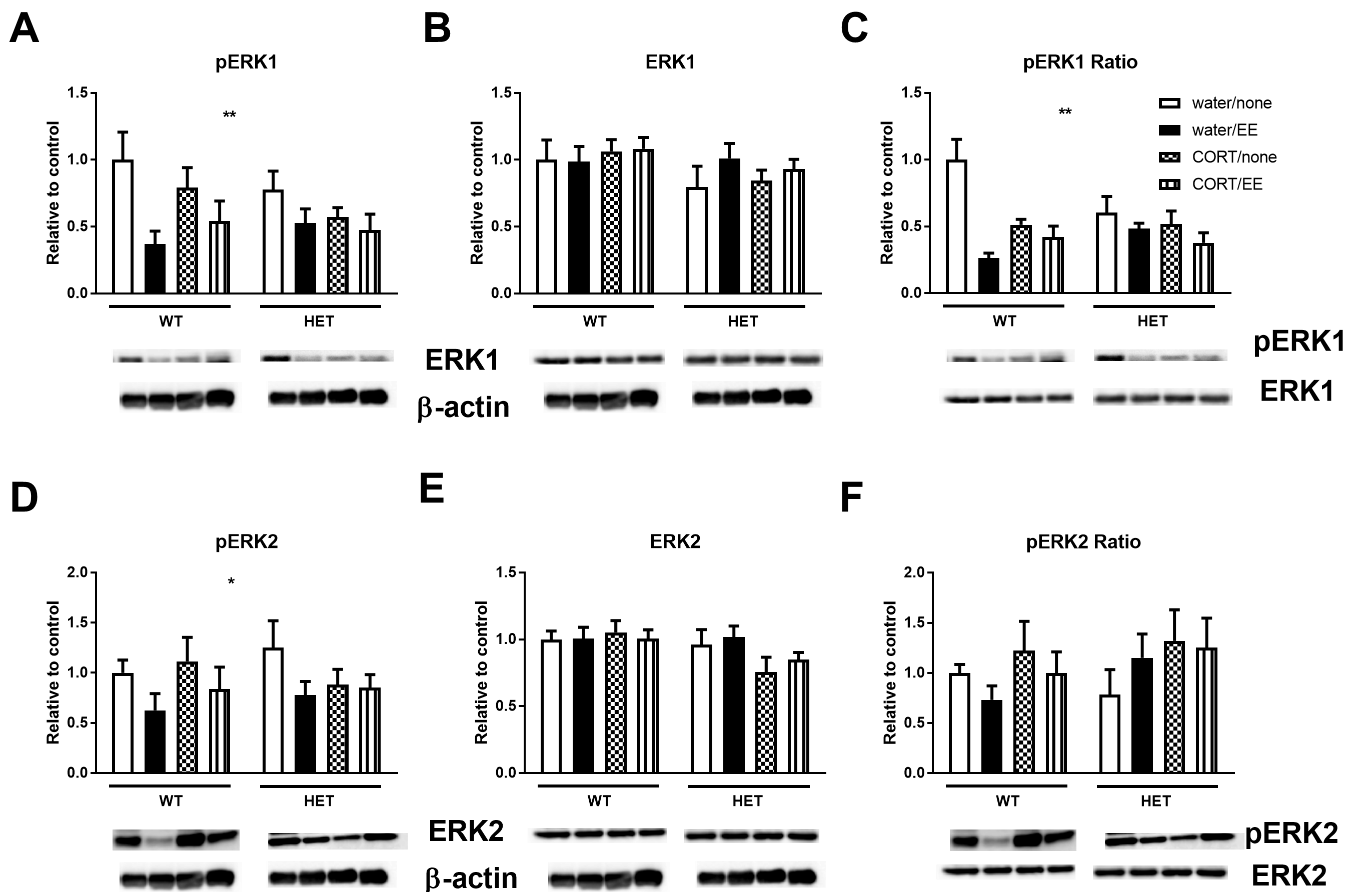


Figure 3.7 - ERK1/2 protein expression in female dorsal hippocampus

Western blotting was used to measure protein expression of pERK and ERK after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus (DHP) of female wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Panel A shows a main effect of EE for pERK1 protein expression in female DHP. Panel B shows no significant effects of genotype, EE or CORT in female DHP. Panel C shows a main effect of EE for the pERK1/ERK1 ratio in female DHP. Panel D shows a main effect of EE for pERK2 protein expression in female

DHP. Panel E and F show no significant effects for ERK2 protein expression and pERK2/ERK2 ratio respectively in the female DHP. Data are mean \pm SEM, $n = 4-6$, * $p < 0.05$, ** $p < 0.005$.

Female Ventral Hippocampus

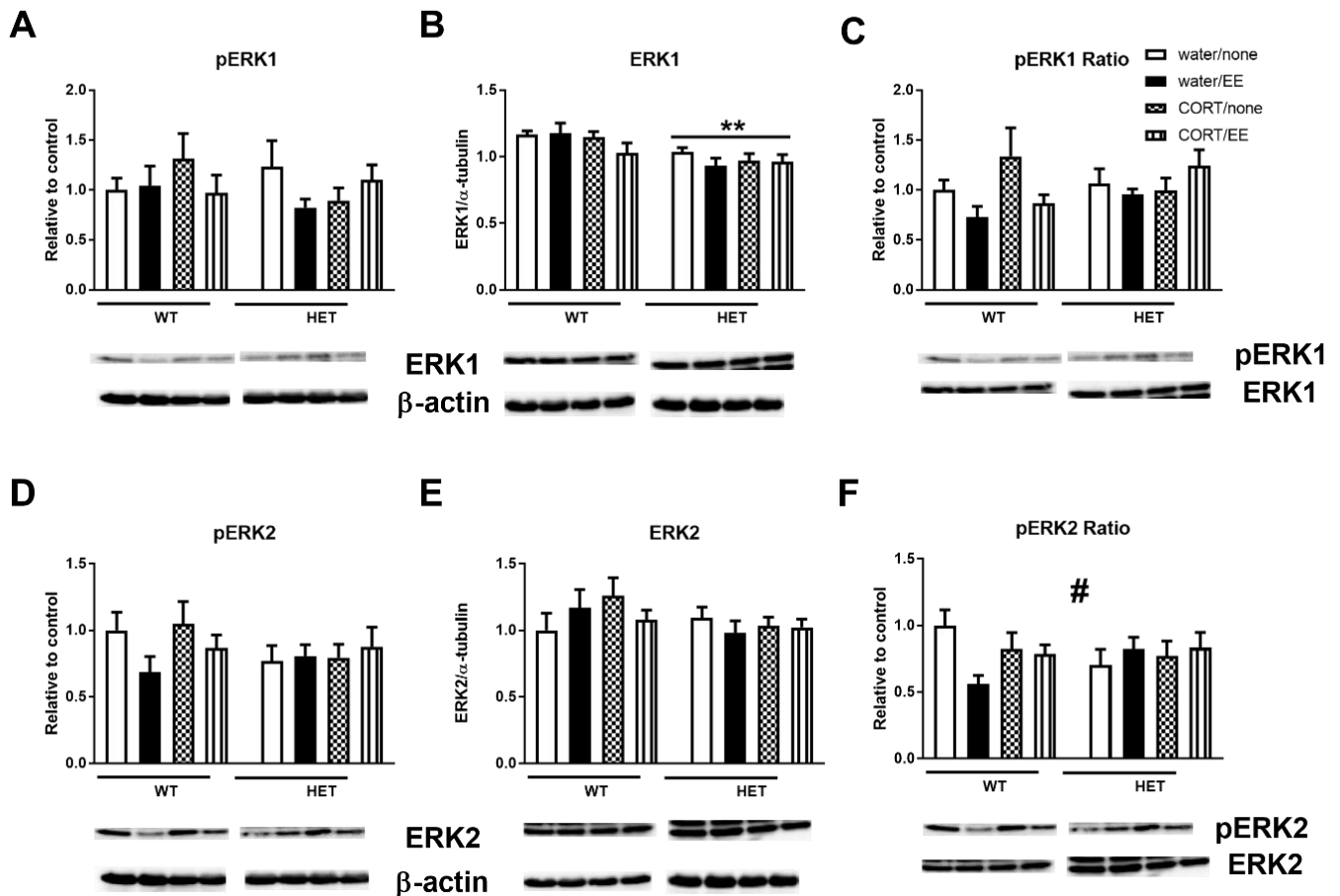


Figure 3.8 - ERK1/2 protein expression in female ventral hippocampus

Western blotting was used to measure protein expression of pERK and ERK after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the ventral hippocampus (VHP) of female wildtype-like (WT) and BDNF heterozygous (HET) mice. Results are presented as standardised protein expression relative to control. Panel A shows no significant effects of genotype, EE or CORT in female VHP. Panel B shows a significant genotype effect for ERK2 protein expression in female VHP. Panels C, D and E show no significant effects of genotype, EE or CORT for pERK1/ERK1 ratio, pERK2 and ERK2 protein expression respectively.

*Panel F shows a significant EE × genotype effect for pERK2/ERK ratio in female VHP. Data are mean ± SEM, n = 3-6, # p < 0.05, ** p < 0.005.*

3.4 Supplementary Material Discussion

I demonstrate here that protein expression of key excitatory and inhibitory markers differentially responded to key factors of genotype, CORT and environmental enrichment. In these mice, E/I systems expression are divergently driven by either a ‘positive’ or a ‘negative’ environment. These environments compounded upon each other on downstream signaling pathways.

3.4.1 Genotype × CORT interaction common to all inhibitory markers but sex and region dimorphic

There was a common genotype × CORT interaction for inhibitory markers, whereby HET animals had decreased inhibitory marker protein expression and WT animals were increased following CORT exposure. This was region and sex-dimorphic. The interaction between sex and inhibitory markers has previously been established in our laboratory (Du et al., 2018; Wu et al., 2014), as well as the interaction between BDNF heterozygosity and sex (Wu et al., 2015). Sex dimorphic effects on CAL expression after stress have been previously observed in rats (Xu et al., 2018b), and in our study we found male-specific changes to CAL expression. Here, only male HET exposed to CORT showed decreased CAL protein expression in the DHP. This is a key brain region for the Y-Maze spatial memory task (Blum et al., 1999). CAL-IN are moderators of other interneurons (Cauli et al., 2014), and in male HET mice decreased expression inferred decreased functionality. However, this did not lead to difference in DI (see published manuscript). CAL-IN have a disinhibitory role (Cauli et al., 2014). So, while they do not directly mediate learning and memory processes, they are an important regulator of the circuits that do. Interestingly, in CAL knockout mice, while there is an excess of GABA in the hippocampus which disrupts LTP, basal

synaptic function remains intact (Schurmans et al., 1997). This raises the question of whether the Y-Maze is the appropriate test here. Possibly a more demanding task such as the touchscreen battery would elucidate subtler functional differences. Comparatively, female HET animals exposed to CORT had decreased DHP SST expression, while WT CORT treated females had increased SST expression. SST-IN can target the pre-synapse of pyramidal cells and PV cells, as well as apical dendrites (Funk et al., 2017). Increased SST expression in female WT animals correlated with a lack of CORT induced spatial memory deficits. Potentially here, higher SST expression lead to increased presynaptic inhibition and was able to be an early block to incorrect signaling. Comparatively, the HET animals treated with CORT had decreased expression of SST and assumedly decreased SST functionality. Therefore, the decreased inhibition by SST-IN may contribute to the spatial memory deficit shown by HET +CORT treated animals.

While PV is the inhibitory marker strongly associated with spatial memory (Murray et al., 2011), it was only changed in female animals in the VHP. Here, there was a genotype \times CORT interaction whereby female HET CORT mice had decreased PV protein expression, but WT animals were unchanged. Functionally female BDNF HET CORT mice had impaired spatial memory, as determined by a DI value of 1 (see published manuscript). Comparatively WT CORT had intact spatial memory with a DI value of 1.5. While the literature indicates that the VHP has a stronger role in emotional memory, rather than spatial memory (Campbell and MacQueen, 2004; Cioocchi et al., 2015), communication between the dorsal and ventral regions of the hippocampus does occur (Fanselow and Dong, 2010). Therefore, it could still be contributing to a disturbance in the E/I balance. This could be a cumulative effect, with disruptions to SST in the DHP and PV in the VHP contributing discord to the hippocampal circuits, leading to spatial memory deficits.

Discrete dysfunction of different inhibitory markers has been shown to result in similar cognitive outcomes. This is possible due to the complex circuits that contribute to cognition. The E/I balance is pivotal, complicated and sensitive to change. The specific vulnerabilities and mechanisms that diverge between the sexes is important to unravel, as sex-specific approaches to psychiatric disorders becomes more supported and a higher research priority.

3.4.2 ERK1/2 Signaling Cascade impacted primarily by Environmental Enrichment

EE was seen to impact the protein expression of ERK1/2 signaling in both males and females, a system associated with healthy cognition (Samuels et al., 2008; Yang et al., 2011). Here, we found that EE alone increased the male DHP ERK1 and ERK2 ratios. Additionally, a cumulative increase was observed for CORT-EE groups of both genotypes, despite no significant effect of CORT. There are multiple systems involved in functional cognition that converge onto ERK1/2 signaling, including the glutamatergic system (Cahill et al., 2014). In male DHP, GluNR subunit protein expression (see published manuscript) correlated with ERK1/2 expression. However, we found no major effects of EE on GluNR subunit protein expression despite EE significantly affecting ERK1/2. There is evidence that synaptic vs. extrasynaptic NMDAR activation can activate and not activate ERK respectively (Ivanov et al., 2006). Additionally, other neurotransmitter systems converge onto this pathway, including dopaminergic signaling (Cahill et al., 2014) as well as neurotrophin and estrogen receptor pathways to name a few (Singh 1999). ERK1/2 phosphorylation results thus represent the sum of all activity onto this system, and it is difficult to determine which system is contributing the most through protein expression analysis. However, it is an important caveat that the ERK1/2 phosphorylation is dynamic, and in this study, mice were killed 4 weeks after the Y-Maze was performed. Therefore, it is difficult to interpret

this data in context of behaviour. Future studies should attempt to cull mice immediately following a spatial cognition test to attempt to align phosphorylated ERK with behavioural outputs.

CORT alone did not influence male DHP ERK1/2 expression, but it appeared to compound ERK1/2 expression in male mice that also received EE. In the published manuscript, the idea of an ‘inoculation hypothesis’ (Grech et al., 2018b) was put forward to explain the lack of cognitive benefit received from EE. We argued that the experience of EE and subsequent removal before cognitive testing was another form of stress. Here, it appears that exposure to CORT primes the system such that when it is subsequently exposed to EE, the ERK1/2 response to the exposure and removal of this condition is increased. This was male-specific and not cognitively beneficial, since male CORT-EE mice of both genotypes did not perform Y-Maze to the standard of control. Comparatively, for females only WT EE GluNR markers and ERK1 had similar expression patterns. As outlined above ERK is a highly convergent pathway. It is a common downstream signaling cascade for estrogen and BDNF-TrkB signaling (Singh et al., 1999), which may explain sex-dimorphic molecular responses.

3.5 Summary and Prelude to Chapter 4

Spanning both the published manuscript and the supplementary data, we have found sex- and region-specific changes to excitatory/inhibitory markers and the ERK1/2 signaling cascade in response to BDNF heterozygosity, CORT treatment and EE. Key results from the published manuscript included that EE was only a preventative treatment for female two-hit mice and this is possibly attributable to females being protected from excitotoxicity that was suggested in male two-hit mice. Primarily, inhibitory marker expression was dictated by genotype response to CORT treatment. Comparatively, ERK1/2 signaling was altered by EE. This study demonstrates how a positive vs negative environment can have specific molecular impacts, which eventually have

consequences for behaviour. Multiple systems contribute to cognition, and the E/I balance here was explored through protein expression of the excitatory/inhibitory system and the convergent downstream signaling ERK1/2 pathway.

Genotype and sex effects may be a result of impaired Bdnf exon IV activity. Bdnf exon IV has been shown to contribute significantly to activity-dependent secretion of BDNF (Lu, 2003). It is decreased by GC agonists (Chen et al., 2017a) and increased through EE (Dong et al., 2018). We have previously reported in another two-hit model (maternal separation + chronic CORT) that despite a male-specific increase in BDNF exon IV (amongst others) these mice experience a decrease in mBDNF and pTrKb protein expression in adulthood (Hill et al., 2014). Here, there was a male-specific spatial memory deficit as measured by the Y-Maze (Hill et al., 2014). Bdnf exon IV has been implicated in the beneficial effects of EE in a range of disease models including Huntington's Disease (Zajac et al., 2010) and memory deficits in neonatal anaesthesia in rats (Wu et al., 2016).

This study demonstrates that the E/I balance is influenced both by sex and genotype and that this is reflected in sex-dimorphic response to EE. When considering this data in the context of possible preventative approaches to severity of schizophrenia cognitive symptomology, it is imperative to consider sex and type of EE. Here, novel environment (toys etc.) were only restorative for female spatial memory and it may be that an EE treatment that included exercise (running wheel) would have been beneficial to male two-hit mice. As such, sex and type of EE should be considered when translating possible treatment approaches to humans.

However, BDNF heterozygosity is too gross a gross model of disrupted BDNF signaling. Therefore, a more specific model of disrupted BDNF signaling, specifically at IN was needed. PV is implicated in mental illness and cognition, so was the chosen marker. The following chapter is

a behavioural characterisation study exploring the cognitive consequences of dysfunctional BDNF-TrkB signaling at PV-positive interneurons.

Chapter 4. Sex-specific spatial memory deficits in mice with a conditional TrkB deletion on parvalbumin- interneurons

4.1 Chapter 4 Introduction

The experimental work presented in Chapter 3 illustrated that altered BDNF signaling in the context of a two-hit model disrupts the E/I balance. However, to understand more specifically the relationship between the BDNF-TrkB signaling pathway and IN, it was necessary to use a more precise model.

PV is a calcium-binding protein that is expressed in a subtype of IN (Eyles et al., 2002). PV-IN are responsible for generating gamma oscillations, which are rhythmic and repetitive neural firings of electrical impulses at a high frequency (30-80Hz) (Sohal et al., 2009). In individuals with schizophrenia, gamma oscillations are disorganised during cognitive tasks (Chen et al., 2014). Supporting this, post-mortem expression of PV has been found to be decreased in key learning and memory brain regions, including the mPFC and hippocampus (Sibille et al., 2011; Zhang and Reynolds, 2002). A functional relationship between the BDNF-TrkB signaling pathway and PV-IN has been suggested in the literature, with the BDNF receptor TrkB co-expressing with PV-IN (Gorba and Wahle, 1999) and found to be critical for PV-IN functioning (Zheng et al., 2011). Due to this literature, it was decided that PV-IN would be the chosen IN for this project. Both BDNF-TrkB signalling (Kemppainen et al., 2012; Shilpa et al., 2017) and PV-IN function (Kim et al., 2016b; Sohal et al., 2009) are integral to healthy cognition, but how they may interact to cumulatively contribute to cognitive deficits in schizophrenia is not yet elucidated. Known cognitive deficits occurring as part of schizophrenia symptomology include: learning and memory deficits (Chen et al., 2014; Gonzalez-Burgos et al., 2010), attention (Zhang et al., 2012), cognitive flexibility (Yu et al., 2015), visual working memory (Lynn et al., 2016) and navigation using allocentric strategies (Weniger and Irle, 2008).

The following chapter is a behavioural characterisation study exploring the cognitive consequences of dysfunctional BDNF-TrkB signaling at PV-IN. Published and supplementary

data includes immunohistochemistry to confirm knock down and affective and cognitive behavioural results and analysis. The rationale for investigating PV and TrkB co-staining in the mPFC and hippocampus using immunohistochemistry was that these are key brain region involved in the pathophysiology of cognitive deficits in schizophrenia. The rationale for performing the Y-Maze, Cheeseboard Maze and NORT was to investigate the cognitive domains of working memory, reasoning and problem solving and visual learning and memory, key cognitive domains that are impaired in schizophrenia (refer to section 1.9.2). Locomotor and Elevated Plus Maze were performed as affective controls, to ensure that changes in activity or anxiety were not contributing to the behavioural results observed.

The overall aim of this project was to investigate whether knock down of TrkB receptors on PV-IN in mice would result in cognitive deficits that mimic those observed in schizophrenia.

The following experiments were investigated and published in Behavioural Brain Research:

- 1) Knockdown of TrkB on PV using the cre-lox recombination system
- 2) Cognitive and affective characterisation of this model using an established behavioural battery

The hypotheses for this project were as follows:

- 1) It was hypothesised that TrkB staining would be decreased by up to 50% on PV-positive in PV-Cre:Fl^{+/-} mice compared to PV-Cre mice in both the mPFC and hippocampus
- 2) It was hypothesised that PV-Cre:Fl^{+/-} mice would perform worse on all cognitive tasks compared to PV-Cre mice. Additionally, it was hypothesised that males would perform worse than females.
- 3) It was hypothesised that allocentric search strategies would be impaired in PV-Cre:Fl^{+/-} mice as these are impaired in schizophrenia

4.2 Published Manuscript



Research report

Sex-specific spatial memory deficits in mice with a conditional TrkB deletion on parvalbumin interneurons

Adrienne Mary Grech^{a,b}, Xin Du^{a,b}, Simon S. Murray^c, Junhua Xiao^c, Rachel Anne Hill^{a,b,*}^a Department of Psychiatry, School of Clinical Sciences, Monash University, Monash Medical Centre, Clayton, Victoria, Australia^b The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria, Australia^c Department of Anatomy and Neuroscience, The University of Melbourne, Melbourne, Victoria, Australia

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ABSTRACT

Schizophrenia is a debilitating disorder characterised by three main symptom categories: positive, negative and cognitive. Cognitive symptoms emerge first, and currently do not have appropriate treatments, despite being a strong predictor of the severity and progress of the illness. Cognitive deficits are strongly associated with the dysfunction of GABAergic parvalbumin interneurons (PV-IN). PV-IN are supported by Brain-Derived Neurotrophic Factor (BDNF) via its receptor Tropomyosin-related Kinase B (TrkB). The main aim of this study was to investigate the cognitive and affective consequences of disrupted BDNF-TrkB signalling at PV-IN. We crossed PV-Cre mice with heterozygous TrkB floxed mice (PV-Cre:Fl^{+/-}) to knock-down TrkB receptors on PV-IN. Male and female mice underwent a battery of tests including: Y-Maze, Cheeseboard Maze, Elevated Plus Maze, and Locomotor activity. Co-expression of PV and TrkB in the hippocampus was assessed by fluorescent immunohistochemistry and detailed stereology.

Sex-specific spatial memory impairments were found in the Y-Maze. Only male PV-Cre:Fl^{+/-} mice showed no preference for the novel arm. Furthermore, there was a male specific genotype difference in memory retrieval in the Cheeseboard Maze. Male PV-Cre:Fl^{+/-} mice were more preservative in their learning than male PV-Cre control mice.

Overall, the evidence from this study suggests that sex had a developmental influence on this constitutive model. Male spatial memory was altered by the disruption to BDNF-TrkB signalling at PV-IN. This aligns with males showing more severe cognitive dysfunction in schizophrenia.

1. Introduction

Schizophrenia is readily recognised for its positive symptoms of hallucinations and delusions, but the major category of cognitive symptoms has been shown to precede positive symptoms [1] and to have the capacity to dictate the severity and illness course of schizophrenia [2]. Cognitive symptoms can include changes to working memory [3], attention [1] and cognitive flexibility [4]. Despite the impact on quality of life, there are currently no targeted pharmaceutical treatments which address cognitive impairment [2]. Brain-Derived Neurotrophic Factor (BDNF) is a major neurotrophin that has a plethora of roles within the central nervous system (CNS), many of which contribute to the development and maintenance of neuronal structures associated with cognitive processes [5]. Synaptic plasticity is one such process [6]. It has a well-established role in hippocampal long term

potentiation (LTP) [7], a more permanent form of synaptic plasticity. Upon binding of BDNF to its cognate receptor Tropomyosin-related Kinase B (TrkB), receptor dimerization occurs followed by auto-phosphorylation of discrete tyrosine residues. These residues are distinct in their activation time course, downstream signalling and contribution to cognitive processes [8,9]. Human studies have associated BDNF with healthy cognition [10,11] and modulation of psychiatric disorders [12,13]. Both BDNF and TrkB mRNA levels are decreased post-mortem in schizophrenia individuals, including in the hippocampus [14–16]. Significantly, decreased BDNF serum protein levels have been found across the schizophrenia illness trajectory including in first-episode [17], chronic [18] and relapsed [19] schizophrenia patients. This indicates that decreased BDNF is not a medication artefact. Instead, it is likely a pathophysiological feature of schizophrenia contributing to the manifestation of cognitive symptoms.

* Corresponding author at: Department of Psychiatry, School of Clinical Sciences at Monash Health, Monash University, Level 5, Block E, Monash Medical Centre, 246 Clayton Rd, Clayton VIC 3168, Australia.

E-mail address: Rachel.hill@monash.edu (R.A. Hill).

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Reduced BDNF has been shown to impair GABAergic function [20]. In particular, parvalbumin-positive interneurons (PV-IN) are a subtype of inhibitory GABAergic interneurons that significantly contribute to the maintenance of the excitatory/inhibitory balance [21,22] and are thought to contribute to healthy cognitive function [23,24]. The TrkB receptor directs developmental assembly of inhibitory synapses [25,26], co-expresses with PV-IN [27] and is critical for proper PV-IN functioning [28]. Importantly, reduced TrkB mRNA levels in schizophrenia have been correlated with GAD67 and PV mRNA levels [29]. However, an overall reduction in PV-INs was not tested for in this correlation. We have previously reported that male BDNF heterozygote mice had decreased PV-IN density across adolescent development [30], supporting a role for decreased BDNF-TrkB signalling influencing INs as a pathophysiological feature of schizophrenia. Subsequently, a critical question is whether BDNF loss in this specific cell type contributes to cognitive dysfunction. Our aim was to investigate the consequences of disrupted BDNF signalling on PV-IN upon spatial memory and cognitive flexibility. We crossed a PV-Cre parent with a TrkB heterozygous floxed parent to generate PV^{Cre} × TrkB^{flox} (PV-Cre:Fl^{+/-}) offspring and performed a battery of affective and behavioural tests. Currently available literature on this model indicates deficits in working and short-term spatial memory [31], fear memory [32] and motor function [31,32]. We hypothesised that spatial memory and cognitive flexibility would be impaired due to disrupted BDNF-TrkB signalling at PV-IN. There are sex differences in the onset and manifestation of schizophrenia in humans, with males developing schizophrenia earlier and with more severe negative and cognitive symptoms [33]. Therefore a second hypothesis was that there would be sex differences in spatial memory performance in response to disrupted BDNF-TrkB signalling at PV-IN.

2. Methods

2.1. Animal care and housing

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria, Australia. All animal experiments complied with the ARRIVE guidelines and in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). PV-Cre (B6;129P2-Pvalb^{tm1(Cre)Arbr}/J) mice were crossed with TrkB heterozygote floxed (Ntrk2^{tm1Lfp}) mice [34] (kindly donated by Dr. Simon Murray and Dr Junhua Xiao) on a C57BL/6 background to generate PV-Cre × TrkB heterozygote floxed mice (PV-Cre:Fl^{+/-}) in which TrkB receptors were knocked down on PV-IN. Lucas et al [32] reported that homozygote, but not heterozygote, knockout of TrkB lead to locomotor dysfunction. This was our rationale for using TrkB heterozygote floxed mice. Mice were group housed 2–7 per box in individually ventilated cages (Tecniplast Green Line IVC Sealsafe PLUS) for the duration of experimentation. No enriching structures beyond nesting materials were provided. Mice had ad libitum access to food and water, except for the duration of the Cheeseboard Maze where they were food restricted to 85% of their body weight to provide motivation. The room was on a 12/12 h light/dark cycle (lights on at 7am) and temperature and humidity controlled. All procedures were performed during the light phase.

Behavioural parameters

All mice were handled at least once per week from 6 weeks of age, with behavioural testing beginning at 10 weeks of age. Mice were given 2–3 days recovery between each behavioural test to minimise stress. All behavioural testing was performed during the light phase with dim experimental lighting set at approximately 100 lx. When possible, all behavioural testing was performed before 2 pm in order to avoid the

drowsiest period for mice. However, a number of mice from each group performed the Y-Maze after 2pm: (3) male PV-Cre, (4) male PV-Cre:Fl^{+/-}, (1) female PV-Cre and (1) female PV-Cre:Fl^{+/-}. Mice were transported from the animal house to the maze room before experimentation and given at least 10 min to acclimatise. Tests were performed in the following order: Y-Maze, Elevated Plus Maze, Locomotor activity, Cheeseboard Maze acquisition and Memory Retrieval. Animal numbers for each test are as follows:

2.2. Y-maze – short-term spatial memory test

The Y-Maze was performed as previously described [35]. The maze consisted of three arms (30 × 8 × 16 cm) placed at 120° to each other, with geometric spatial cues placed at the end of each arm. Phase one was run with only two arms open (home and familiar), with the third arm (novel arm) closed off. Mouse behaviour was recorded for 10 min before being returned to the home cage. Phase two was performed after a one hour retention period. The mouse was then returned to the maze with all arms available. Mouse behaviour was recorded for 5 min and the first 3 min were analysed. The maze was cleaned with 80% ethanol between each testing to remove odour cues. Behaviour analysed was duration percentage of time spent in other arms (average of home and familiar) compared to the novel arm. A Discrimination Index (DI) was calculated, which was the duration percentage of time spent in the novel arm divided by the average amount of time spent in the home arm and other familiar arm. Mice with intact memory typically spend more time in the novel arm, reflective of intact memory of their previous visit to the original two familiar arms. Intact spatial memory is represented by DI being around 1.5. DI of around 1.0 is representative of equal time spent in all three arms (i.e. chance level) and subsequently interpreted as disrupted spatial memory.

2.3. Cheeseboard maze – long-term spatial memory test and perseverative behavioural test

The cheeseboard maze (CB) is a long-term spatial memory test. The CB was chosen as a dry-land version of the MWM to investigate long-term spatial memory. The MWM uses avoidant learning, with mice motivated to learn to avoid drowning. Comparatively, the CB employs positive reinforcement through a food reward. It was chosen to avoid panicked stress as the driver of learning [36] and instead positive reinforcement was used via food reward. There is no mortal threat in the CB with a lack of perseverance, whereas in the MWM the cost of making a false negative error is too high. The CB was constructed by the Behavioural Neuroscience Laboratory according to a previous design by [37] to be a grey painted circular wooden board (94 cm diameter). It was elevated 30 cm off the floor and covered by 32 wells (3.25 cm diameter, 1 cm depth). At each cardinal point a spatial cue was placed. Mice were food-deprived to 85% of their free-feeding body weight throughout testing to provide motivation to search for food reward. Before each trial the maze was cleaned with 10% ethanol and painted with diluted condensed milk (1:4 ratio) to prevent the mice from being distracted or guided by odour cues. Mice were habituated to the maze for two days with two 2 min trials conducted on each day and a 20 min inter-trial interval. Following the habituation trial mice were fed 100 µl of diluted condensed milk. Learning acquisition was then performed over the following 6 days, which included two 2 min trials with a 20 min inter-trial interval per day. All mice began the test from the centre of the maze, under an opaque plastic cover. The test started once the cover was removed and the mouse was free to roam the maze. Mice were placed next to the target well to consume the food reward if they did not locate the target well within the 2 min trial. Latency to find the reward was assessed post-experiment using TopScan version 2.00 and centre body tracking data was analysed. A 2 min probe trial for spatial memory was conducted on day 7. Here there was no baited well and mice explored the maze freely for 2 min. Percentage duration of time

spent in 8 different Cheeseboard zones were assessed in 30 s time bins and over the full 2 min period post-experiment using TopScan version 2.00. Centre body tracking data was analysed. These 8 zones were then grouped into 4 different quadrants (Target, Clockwise, Anti-clockwise, Opposite) for each mouse based on the location of the Target well. The Centre was defined as the region in the middle of the maze that did not contain wells. Its perimeter was the inner radius of wells. The area is a circle with a radius of approximately 13 cm. Increased time spent in the target quadrant within the first 30 s time bin was indicative of intact retrieval memory. Increased time in the target quadrant over the remaining 90 s was indicative of perseverative behaviour.

2.4. Elevated Plus Maze – a test of anxiety

The apparatus was made of light-coloured Perspex consisting of two open arms (5×30 cm) and two enclosed arms ($5 \times 30 \times 14$ cm) extending at 90° angles from a central platform (5×5 cm) forming a plus-shape. The maze was mounted on a base raised 60 cm above the floor. To begin the experiment the mouse was placed in the middle of the maze facing an open arm and tracking was started by a computer keyboard command. Mouse behaviour was recorded for 10 min. Behaviour analysed by TopScan version 2.00 included entries into different arms and time spent in open or closed arms.

2.5. Locomotor – a test of innate activity

Baseline activity level was assessed using locomotor cages ($28.5 \times 28.5 \times 20$ cm). Mouse movement was recognised by infrared sensors and recorded by the automated recording system within the locomotor cells (San Diego Instruments). Behaviours including distance, speed and rearing were recorded over the 1-h test period.

2.6. Immunohistochemistry for co-expression of PV and TrkB

Immunohistochemistry was performed as previously described [30] for male mice. Mice were transcardially perfused with 0.1 M cold phosphate buffered saline (PBS) (~50 ml) followed by 4% paraformaldehyde in cold 0.1 M PBS (~50 ml). Brains were post-fixed in 4% PFA overnight, transferred in a 15% sucrose solution for 24 h and then transferred to a 30% sucrose solution for a further 24 h. Brains were snap frozen and coronal sections were cut on a cryostat at $20 \mu\text{m}$ thickness and collected at 1:6 intervals. Hippocampus was sectioned from -1.94 to -2.92 mm relative to Bregma and mounted on gelatine coated slides. Firstly, sections were washed twice in PBS, incubated in ice-cold 0.3% H_2O_2 methanol for 30 min and then treated with the Vector Mouse on Mouse (M.O.M.) immunodetection kit according to manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Primary antibodies (anti-mouse Parvalbumin, MAB1572, 1:2000, Millipore and anti-rabbit TrkB H181, sc-8316, Santa Cruz, 1:500) were prepared in M.O.M diluent and applied to the sections and left to incubate overnight at 4°C in a humidified chamber. On the second day, sections were washed 3 times in PBS before applying fluorescent secondary antibodies prepared in M.O.M diluent for an incubation period of 1.5 h in the dark. After the incubation period, slides were washed 3 times in PBS and cover slips were secured with VectaShield Mounting Medium for fluorescence with DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, 94010) and left to dry overnight in the dark.

2.7. Stereological cell quantification and image acquisition and analysis

Co-expression of PV and TrkB was determined on images of immunostained brain sections from male PV-Cre and male PV-Cre:Fl^{+/-} mice. 5–6 mice per group were analysed. Stacks of images spanning $20 \mu\text{m}$ in the z-plane were taken in the dorsal hippocampal areas to be analysed. A Nikon C1 Confocal microscope with an Andor Zyla 4.2 sCMOS Camera was used to capture images at 40x magnification and

$3 \mu\text{m}$ step z-stacks. The dorsal hippocampus sections examined related to Bregma co-ordinates -1.42 , -1.82 and -2.18 mm. A maximum projection image was created from each stack using the Nikon C1 Confocal software. Co-expression of markers and area measurements were determined using the FIJI ImageJ 1.52 g software. Cells labelled with DAPI, parvalbumin and TrkB were quantified in serial (1:6) coronal sections in the dorsal hippocampus. The experimenter was blinded to mice numbers when counting and determined brain region boundaries based upon DAPI stain. To determine co-expression, a macro was used to create a mask for the 488-Parvalbumin channel and used to confirm co-expression with 405-DAPI and 594-TrkB. These cells were then counted using the Cell counter plugin.

2.8. Statistical analysis

All data are expressed as the mean \pm the standard error of the mean (SEM). For immunohistochemistry, Elevated Plus Maze and Locomotor Activity all data were normally distributed, thus groups were compared by two-way ANOVA using GraphPad Prism 7.01 with the independent factors being sex (male or female) and genotype (PV-Cre or PV-Cre:Fl^{+/-}), and a total of 4 experimental groups. Three-way ANOVA was performed using SYSTAT 13 software for the Y-maze data with sex, genotype and arm as the independent variables. Here, we uncovered a significant sex \times genotype \times arm interaction, so we split the data by sex and genotype and performed individual unpaired t-tests to compare percentage time spent in the familiar or home arm compared to the novel arm in each genotype and sex. Mauchly's test of Sphericity was screened in the Repeated Measures ANOVA analyses for the CB, and Greenhouse-Geisser correction applied where relevant. Post-hoc comparisons were done with Bonferroni's test. Group differences were considered significant when $p < 0.05$.

3. Results

3.1. Immunohistochemistry co-expression of PV and TrkB results

Percentage co-expression of PV and TrkB in male mice (see Fig. 1 for example stain) was investigated to quantify the extent of knockdown of TrkB receptors on PV-positive cells. Table 1 presents percentage co-expression for the regions CA1 and CA2, which were investigated due to their prominent role in spatial memory [38,39]. Table 2 shows a decrease of -5.83% in the CA1 and -31.01% in the CA2 in PV-TrkB co-expression in the PV-Cre:Fl^{+/-} mice compared to their PV-Cre counterparts.

3.2. Behavioural results

3.2.1. Y-Maze test – Discrimination Index and novel arm preference

Multivariate ANOVA of the DI of time in the Y-Maze arms revealed a significant sex \times genotype \times arm interaction ($F(1, 103) = 6.54$, $p = 0.012$). Based on this interaction we decided to separate the data by sex and genotype and performed individual unpaired t-tests for % time spent in the familiar or home arms compared to the novel arm. Male PV-Cre mice showed a significant preference for the novel arm over the home arm ($p = 0.0079$) and the familiar arm ($p = 0.0017$) (Fig. 2A), while male PV-Cre:Fl^{+/-} mice showed no preference for the novel arm (Fig. 2B). Female PV-Cre mice showed a preference for the novel arm over the home arm ($p = 0.04$), but there was no significant difference between the novel arm and familiar arm (Fig. 2C). Female PV-Cre:Fl^{+/-} mice showed a significant preference for the novel arm over the home arm ($p = 0.01$) and familiar arm ($p < 0.0001$) (Fig. 2D). This indicated a male PV-Cre:Fl^{+/-} specific spatial memory impairment.

3.2.2. Cheeseboard Maze

3.2.2.1. Acquisition - learning to find the baited well. For the latency

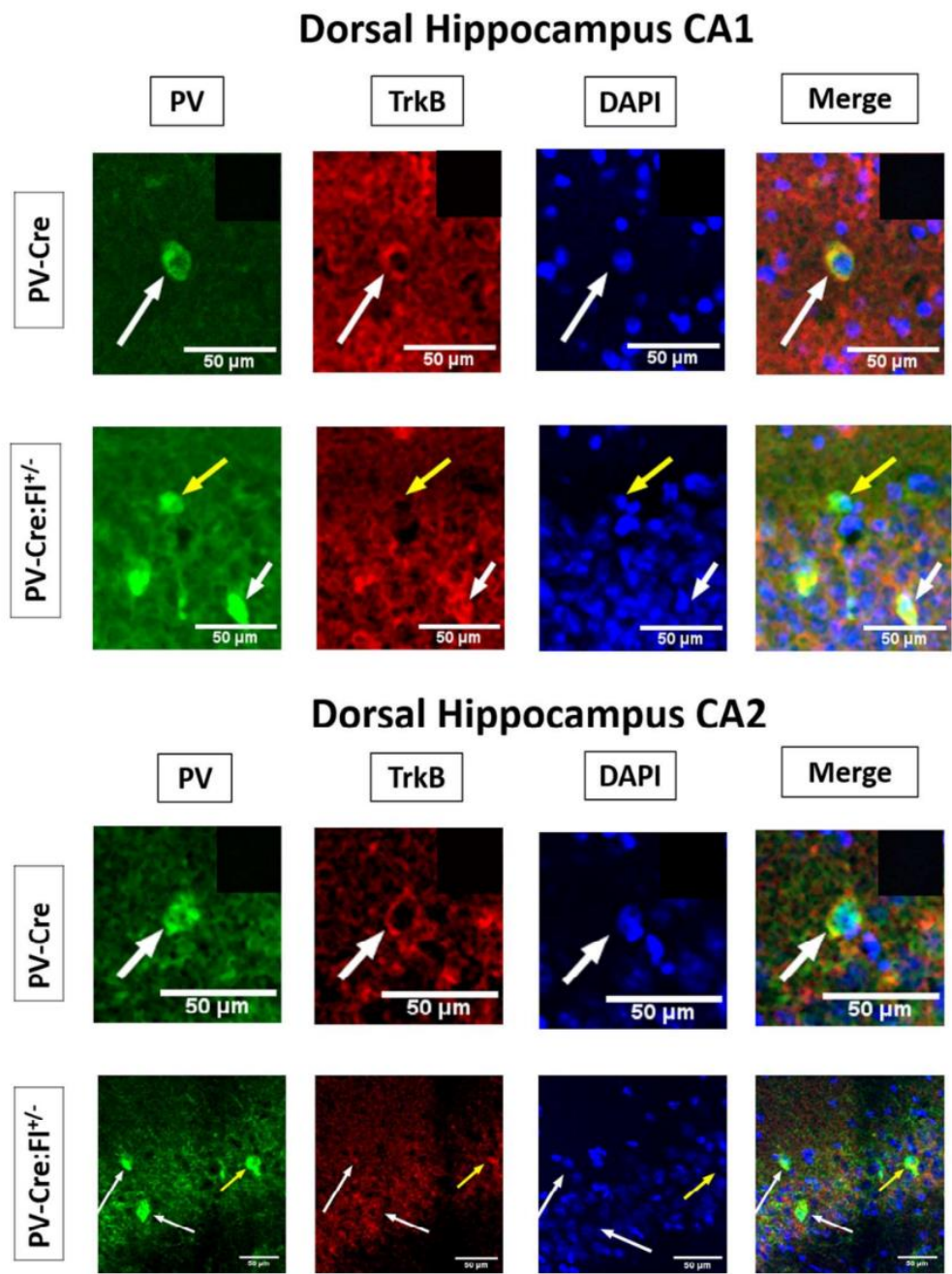


Fig. 1. Example staining in the dorsal hippocampus for co-expression of parvalbumin (green) and TrkB (red) with DAPI (blue) for nuclei staining, in CA1 (Top panel) and CA2 (bottom panel) for PV-Cre control and PV-Cre:Fl^{+/−} mice. Negative controls are shown in the top right corner of PV-Cre panels to ensure specificity of staining. White arrows point to co-expressed cells. Yellow arrows point to PV cells without TrkB expression.

Table 1
Number of mice per group (columns) for each behavioral test (rows).

Behavioural test	Male PV-Cre	Male PV-Cre:Fl ^{+/−}	Female PV-Cre	Female PV-Cre:Fl ^{+/−}
Y-maze	15	10	13	13
EPM	15	13	14	16
Locomotor activity	14	14	14	13
Cheeseboard acquisition	13	12	14	14
Cheeseboard probe trial	6	7	9	13

measurement there was a sex × genotype × day interaction approaching significance ($F(1, 49) = 2.18, p = 0.057$), there was a close to significant main effect of sex ($F(1,39) = 3.61, p = 0.064$), and due to apparent sex effects in other behavioural data sets, the males and females were analysed separately.

3.2.2.2. Male latency to find the baited well. Univariate Repeated Measures Analysis found a significant effect of day ($F(2.89, 66.41) = 7.17, p < 0.0001$, Fig. 3A), whereby the latency to find the reward for both genotypes decreased between Acquisition Day 1 and 6. This indicated that all mice learned the task. There was no significant effect of genotype and no day × genotype interaction, demonstrating that both male genotypes learned the task to the same extent.

3.2.2.3. Female latency to find the baited well. Univariate Repeated

Table 2

Percentage co-expression of PV and TrkB and total PV cell number in the hippocampal subregions CA1 and CA2.

	CA1 % Co-expression	CA2 % Co-expression	Total PV cell Number
PV-Cre n = 5	48.86 ± 5.66	50.11 ± 14.03	82.00 ± 21.66
PV-Cre:Fl ^{+/+} n = 4-5	43.03 ± 6.58	19.10 ± 11.33	82.20 ± 29.96
Difference between genotypes (%)	-5.83	-31.01	0.2

Measures Analysis found a significant effect of day ($F(5, 130) = 9.43$, $p < 0.0001$, Fig. 3B). The effect of day was observed as decreased latency to find the baited well between Acquisition Day 1 and 6. There was no significant effect of genotype or genotype \times day interaction. Like the males, both female genotypes learnt the task to the same extent.

3.2.2.4. Probe Analysis – memory retrieval and perseveration. To analyse memory retrieval and perseveration a 2 min probe trial was performed and separated into four 30 s time bins. A large proportion of mice spent the first minute of the probe trial in the centre of the maze, which distorted the data. The following number of animals spent over 50% of the first 30 s time bin in the center: Male PV-Cre (3), Male PV-Cre: Fl^{+/+} (2), Female PV-Cre (8) and Female PV-Cre: Fl^{+/+} (7). This continued into the second 30 s bin and the following number of animals spent over 50% of the second 20 s time bin in the center: Male PV-Cre (3), Male PV-Cre: Fl^{+/+} (2), Female PV-Cre (8) and Female PV-Cre: Fl^{+/+} (3). Thus, we applied the following equation; duration in quadrant divided by (1-duration in centre multiplied by 0.01).

Following the addition of this equation to our analysis exploration only in the maze quadrants could be compared across mice. Of note, this method changed mouse numbers, since some mice did not leave the centre for the duration of a 30 s time bin and this created an error result. These animals were subsequently not included in that time bin analysis.

Percentage duration of time spent in the assigned target quadrant was assessed for each 30 s time bin. Data were analysed across four 30 s time bins so that memory retrieval as well as perseveration could be

assessed. This is consistent with MWM analysis, whereby the behaviour in the first 20 s of the probe test is thought to reflect spatial-cognitive processes, since the probe test is an extinction trial [40]. It is important to interpret retrieval memory probe behaviour in the context of the previous days' learning, and with the understanding that the well is no longer baited, therefore the mice do not receive a reward. There was a close to significant main effect of sex ($F(1, 39) = 3.61$, $p = 0.064$), and due to apparent sex effects in other behavioural data sets, the males and females were analysed separately.

3.2.2.5. Male memory retrieval probe test. Multivariate tests found that there was a statistically significant time bin \times genotype interaction, $F(3,8) = 4.14$, $p = 0.048$; Wilk's $\Lambda = 0.392$, partial $\eta^2 = 0.608$. Initially, it appears that male PV-Cre control mice spent more time in the target quadrant compared to PV-Cre:Fl^{+/+} mice (Fig. 4A). However, after the first 30 s bin, male PV-Cre control mice spent progressively less time in the target quadrant, demonstrating cognitive flexibility in their adaptation to the lack of food reward. Comparatively, PV-Cre:Fl^{+/+} mice spend approximately 40% of their time in the target quadrant across the entire trial, demonstrating perseverative behaviour. This was confirmed by a significant Mann-Whitney one-tailed t -test, which found a significant difference between the genotypes in time bin 4 ($p = 0.041$).

3.2.2.6. Female memory retrieval probe test. As was performed for the males, the 2 min probe trial was separated into four 30 s bins. No main effect of genotype or time bin was observed. Both female genotypes spent a similar amount of time in the target quadrant across the 2 min probe trial (Fig. 4B). However, female PV-Cre control mice notably

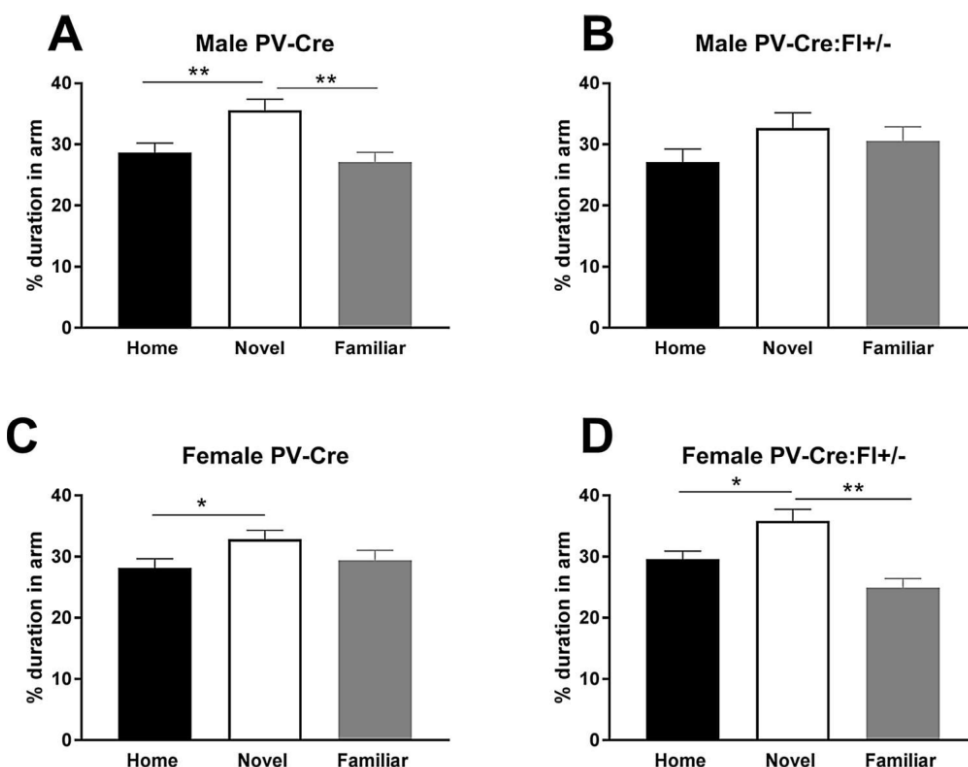


Fig. 2. The Y-Maze was used to assess short-term spatial memory in PV-Cre control and PV-Cre:Fl^{+/+} mice. (A) male PV-Cre control mice show preference for the novel arm (B) male PV-Cre:Fl^{+/+} mice show no preference for the novel arm (impaired spatial memory) (C) Female PV-Cre control mice show a preference for the novel arm (D) female PV-Cre:Fl^{+/+} mice show a preference for the novel arm, demonstrating intact spatial memory. Data expressed as mean + S.E.M, n = 9-15. * denotes $p < 0.05$.

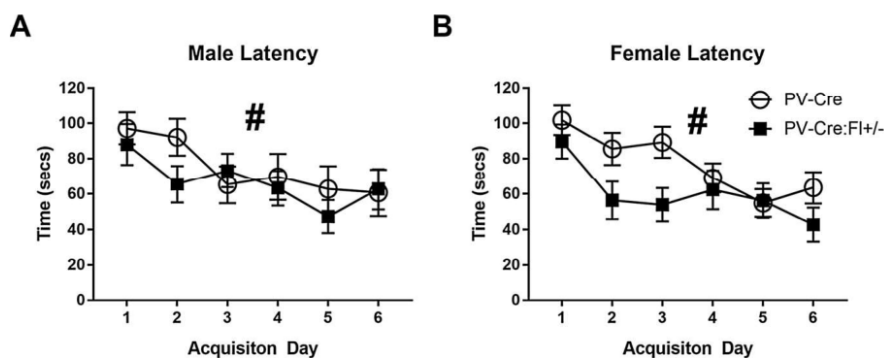


Fig. 3. The Cheeseboard Maze was used to assess long-term spatial learning in PV-Cre control and PV-Cre:Fl^{+/-} mice. (A) both male genotypes show reduced latency to find the baited well over the 6 day training period (B) both female genotypes show reduced latency to find the baited well over the 6 day training period. Data expressed as mean + S.E.M.

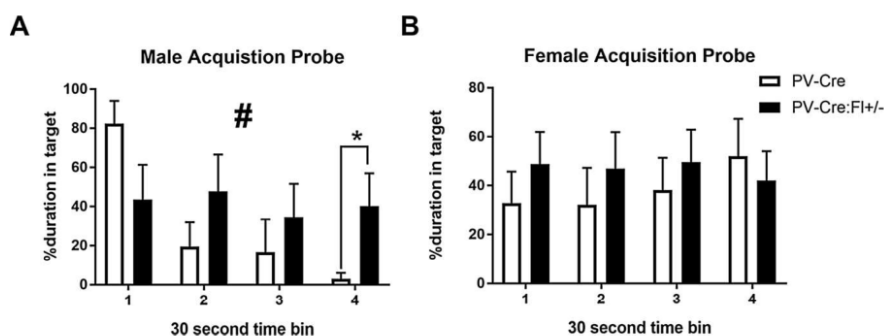


Fig. 4. To assess long-term spatial memory time spent in the target quadrant on probe day 7 was analysed. The two-minute trial was separated into 30second bins, and time spent in the target quadrant across the bins was assessed for each sex. (A) Male mice show a significant time bin \times genotype interaction (#) whereby control mice initially spend more time in the target quadrant, but leave there after – demonstrating cognitive flexibility. PV-CreFl^{+/-} mice, however spend 40% of their time in the target quadrant across the entire trial – demonstrating perseverative behavior. Mann-Whitney one-tailed *t*-test found a significant difference between the genotypes in time bin 4 (*). (B) Female genotypes demonstrated similar behavior across the two-minute trial. Data expressed as mean + S.E.M, $n = 6-13$. * denotes $p < 0.05$.

spent less time in the target quadrant compared to male PV-Cre controls in the first 30 s bin ($p = 0.024$).

3.2.3. Elevated plus maze – measurement of anxiety

There was no effect of sex for time spent in arm ($F(1, 54) = 0.53$, $p = 0.47$) but in the interest of consistency the data was again split by sexes for the EPM analysis. Two-way ANOVA analysis of duration found a significant main effect of arm for males ($F(1, 52) = 259.5$,

$p < 0.0001$, Fig. 5A) and females ($F(1, 56) = 329.1$, $p < 0.0001$, Fig. 5C). For both sexes, both genotypes spent more time in the closed arms compared to the open arms. Two-way ANOVA analysis of entries found a significant main effect of arm for males ($F(1, 52) = 103.7$, $p < 0.0001$, Fig. 5B) and females ($F(1, 56) = 93.14$, $p < 0.0001$, Fig. 5D). For both sexes, both genotypes entered the closed arms more times than the open arms. There was no significant genotype \times arm interaction for either sex, suggesting no significant differences in basal

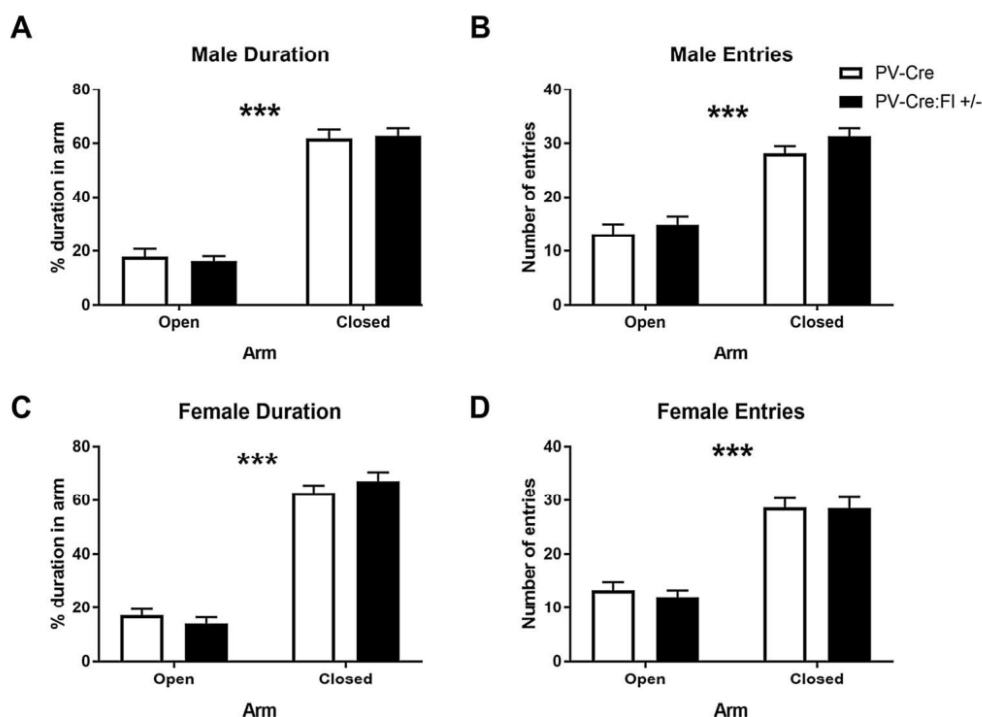


Fig. 5. Basal anxiety levels were measured by the Elevated Plus Maze. (A, B) show that both male genotypes had a preference for the closed arm and (C, D) show that both female genotypes had a preference for the closed arms. Data expressed as mean + S.E.M, $n = 14-16$. **** denotes $p < 0.0001$.

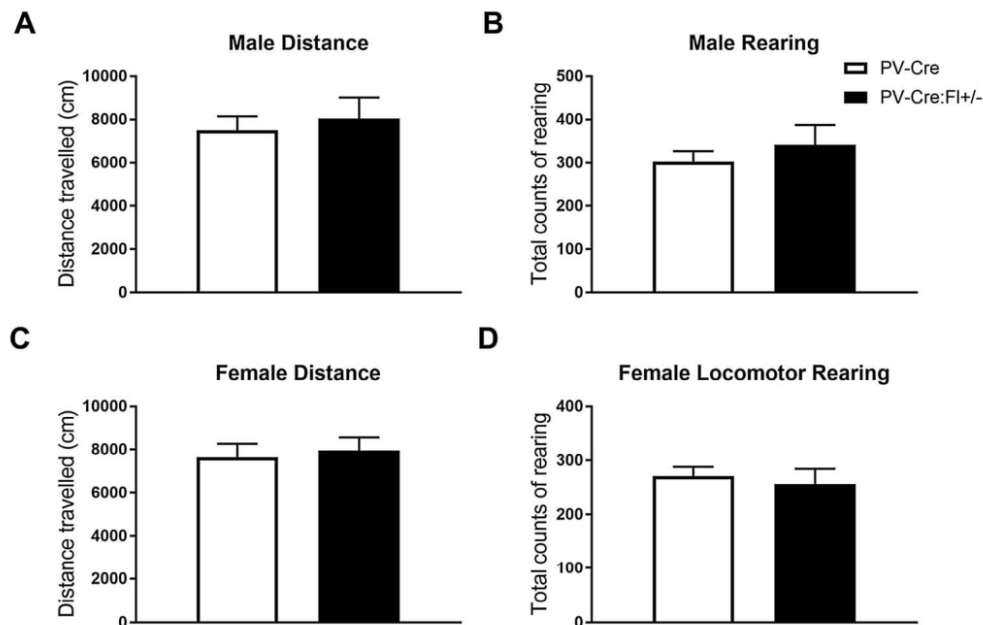


Fig. 6. Basal activity levels were measured by the Locomotor Activity task. (A, B) show no differences in activity parameters between the male genotypes and (C,D) show no differences in activity parameters between the female genotypes. Data expressed as mean + S.E.M, $n = 14$ –16.

anxiety between the genotypes.

3.2.4. Locomotor activity – measurement of activity

There was no effect of sex for distance travelled ($F(1, 54) = 0.73$, $p = 0.40$) or rearing ($F(1, 53) = 2.59$, $p = 0.11$) but in the interest of consistency the data was again split by sexes for the locomotor activity analysis. For males, Univariate ANOVA analysis found no main effect of genotype for distance travelled ($F(1, 26) = 0.35$, $p = 0.56$) or rearing ($F(1, 26) = 2.36$, $p = 0.14$) (Fig. 6A, B respectively). For females, Univariate ANOVA analysis found no main effect of genotype for distance travelled ($F(1, 26) = 0.48$, $p = 0.52$) or rearing ($F(1, 24) = 0.18$, $p = 0.67$) (Fig. 6C, D respectively). Therefore, there were no significant differences in basal activity between the genotypes for both sexes.

4. Discussion

This study aimed to investigate the consequences of disrupted BDNF- TrkB signalling at PV-IN on rodent learning and memory tasks. We have demonstrated that a subtle constitutive knockdown of TrkB on PV-IN had sex-dimorphic effects upon spatial memory in male mice. Our primary result for the Y-Maze was that short-term spatial memory was specifically disrupted in male, but not female, PV-Cre:Fl^{+/−} mice. Our results indicated that PV cell death was not required for spatial memory dysfunction, but rather that disrupted BDNF-TrkB signalling was enough to impair PV-IN function. There were larger differences in PV-TrkB co-expression in the CA2 compared to CA1, however the biological significance of this difference in decreased co-expression is difficult to determine. The CA2 is a much smaller, and more mysterious hippocampal region compared to the CA1. Broad knowledge of differences between the main hippocampal regions for cellular architecture and connections are known [41], with the CA1 having an established pivotal role in hippocampal functions as part of the tri-synaptic circuit [42] and connections to the entorhinal cortex [43]. The authors' hypothesis that it is more likely that the sum of changes to communication between these two areas is contributing to observed behaviour rather than changes in one over the other. It has been established in the literature that both areas contribute to spatial memory [38]. We have found male-specific spatial memory deficits in the Y-maze paradigm. This male-specific effect is like previous work in our laboratory which showed male specific deficits in Y-maze novel arm preference in a two-

hit model of BDNF heterozygosity combined with adolescence exposure to corticosterone [35,44]. Concordantly, male schizophrenia patients have worse cognitive outcomes compared to female patients [45,46] and it has been suggested that estrogen may have a protective role to play here [47–50]. This is reflected in clinical trials that are investigating, and finding positive benefits, of selective estrogen receptor modulators (SERMs) as adjunctive treatments for cognitive symptoms in both male and female schizophrenia patients [51]. It may be in our animals, that the females are protected against altered BDNF-signalling at PV-IN through converging estrogen signalling pathways, with cross-talk and convergence between estrogen and BDNF well established [52–54]. However, we have not counted the PV-TrkB co-expression in female mice and therefore cannot be certain if females do or do not have a similar decrease in co-expression. Or if observed behaviour is due to the convergence of estrogen and BDNF, which subsequently compensates for the loss. Further investigation into this mechanism could be performed through estradiol treatment in this mouse model, to confirm whether male PV-Cre:Fl^{+/−} mice benefit from estrogen receptor activation as an alternative mechanism to support PV cell function. It should be noted here that our Y-maze paradigm had internal cues placed within the maze but did not have distal cues, thus this deficit may be represent more of an object / sign recognition dysfunction than a spatial memory. Here future studies should incorporate tests that include distal cues and also specific tests for object recognition in this model.

We found a male-specific genotype effect in perseverance as measured by the cheeseboard maze (CB) during the probe test. When the memory retrieval data was investigated as an average of the 2 min trial (Supplementary Fig. 1), with no time in centre removed from analysis, male genotypes do not spend significantly more time in the Target Quadrant compared to the other quadrants. In context of the literature, these controls would be considered to have not performed the memory component of the task. Since learning appeared to have taken place, as measured by significant decrease in latency to reward, we analysed the memory retrieval task in 30 s time bins. This approach highlighted that for male PV-Cre control mice the percentage duration spent in the target quadrant of each time bin drastically decreased after time bin 1, suggesting that after not finding the reward in the target quadrant, the mice moved on to explore other quadrants. This was somewhat unexpected, since traditionally percentage duration of time spent in the

target quadrant in both the CB and Morris Water Maze (MWM) is used as a measurement of preserved learning [37,55]. A mouse's innate anxiety and vigilance leads to preference for making false positive errors that help survival [56]. The male PV-Cre:Fl⁺/± mice were more perseverative and remained predominantly in the target quadrant for the duration of the trial, unable to adapt and explore elsewhere. This is potentially indicative of poorer cognitive flexibility compared to the PV-Cre control group. Devan et al [40] suggested that only the first 20 s of a probe test should be analysed, since failure to find the escape platform in the MWM can initiate new learning as the animal responds to its environment [40]. This does not appear to be occurring in the male PV-Cre:Fl⁺/± mice, but a similar scenario may be occurring in the male PV-Cre control mice. The decrease in time spent across the 2 min trial by male PV-Cre mice may be indicative of learning in absence of a reward. Potentially if a mouse has learnt to receive a food reward it may 'forage' elsewhere, once it has realised its food reward is not available. The maze was wiped with condensed milk for this task, and this sensory information in combination with 6 days prior of reward, may have created expectations of a reward and this informed their strategy [57]. Interestingly this appears to be sex-specific, with no differences of time bin or genotype in the females. It should be noted that the latencies achieved of 40–60 seconds suggest that male and female mice may have improved their performance on additional training days. This would have been an ad hoc approach to the methodology, which is why the authors did not do it at the time. However, this has implications for probe trial performance. If more training had been performed, the mice may have had different probe trial behaviour. Another sex-specific finding was that male and female control animals had different memory retrieval performance in the first 30 s time bin. Control animals had a similar latency by Acquisition Day 6 but a sex-dimorphic effect appeared for memory retrieval. There is a mixed literature on sex differences in spatial memory [58]. Studies like Devan et al [40] have found a male advantage in a similar task, while Chow et al [59] found sex differences in acquisition learning in the MWM but not in memory retrieval. Direct comparison between the MWM and CB tasks is not ideal, since the motivator of each task is in direct opposition and could affect cognition differently [36,60]. There is not much evidence for sex differences in the CB. Possibly this is because the task relies on positive reinforcement which may mask subtle memory differences, and/or that there is a much smaller literature compared to the MWM. One consideration is sex differences in response to novelty. A study by Frick et al [61] found that male C57BL/6 mice showed a greater preference for novelty in a spatial and object novelty task than females. Our mice are bred on a C57BL/6 mice background, and potentially this induced a greater urge to explore further in male controls compared to female controls.

It has been reported that food deprivation can enhance spatial cognition in C57BL/6 mice [62], whereas a high stress task such as the MWM can exacerbate spatial memory deficits [63]. The phenotype reported in this paper is subtle, and it may be that food deprivation has dampened the genotype effect. Fear memory has been found to be impaired in these animals [32], and potentially it could follow that more stark learning and memory deficits would emerge following an aversive test such as the MWM.

We found no changes in basal levels of anxiety or activity and no motor impairments were observed in our PV-Cre:Fl⁺/± mice, similar to what has previously been found [32]. Comparatively Xenos et al [31] found their conditional PV Cre × TrkB^{flxed} mice to be less anxious but this is likely due to differences in the behavioural paradigm, with Xenos et al. using open field test as a measure of anxiety [64].

Here we have reported that cre-lox recombination yielded a -5.83% ablation of co-expression of TrkB and PV in the CA1 of the DHP and -31.01% in the CA2. While we have attempted to quantify the percentage co-expression of PV and TrkB, previous groups have confirmed specificity of recombination. There is discord between the studies in extent and efficiency of recombination across the rostral-caudal brain

axis [31,32], and changes in PV cell number [31]. Differences in reporting style and extent of recombination may vary based on mouse background and methods used. One limitation of our approach may be the choice of TrkB antibody, since it appeared to only bind to the membrane whereas other studies had full cell body staining. However we have previously confirmed specificity of this antibody by Western blot analysis [65].

4.1. Limitations

To account for possible non-specific cre activity, a PV-Cre control was included. However, we did not include a wildtype control and this is a limitation of the current study. Importantly, Xenos et al [31] found no behavioural differences between their PV-Cre control mice and wildtype C57/B6 mice in initial pilot experiments, thus we are confident of our control design. An experimental limitation was that only male mice were included in the immunohistochemistry experiment. However, Lucas et al used a similar model and found that recombination was not impacted by sex [32]. Male mice used for the immunohistochemistry were not behaviourally naïve and thus we cannot rule out any potentially neuroplastic changes in response to learning that may have altered PV cell density. Another limitation of the current study is that the motivation of the mice to seek the food reward was not established beyond food deprivation. In future a motivation assay should be applied, since there are limitations to appetitive reinforcers [66]. A potential caveat is the 10 min post-transport acclimatisation to the experimental room, which may have been insufficient. Gerdin et al [67] have previously found that cage transport leads to stress-induced hyperthermia, with an hour of acclimatisation needed to return to basal levels. This would impact the interpretation of the observed behaviour [67]. Finally, this model uses mixed background strains and this may be a caveat when interpreting the behavioural data [60].

4.2. Future directions

A marginal loss of TrkB receptors at PV-positive cells resulted in a sex-specific phenotype, suggesting that the relationship between this neurotrophin receptor and GABAergic IN is important to cognition. Since human mental illness does not result from the dysfunction from one system, it may be useful to utilise this in a 'two-hit' or 'multiple-hit' neurodevelopmental model [65]. The factors investigated here are relevant to neurodevelopmental disorders such as schizophrenia and autism [29,68]. Investigating possible cognitive benefits of estrogen-based strategies in this mouse model would further elucidate possible compensatory roles of estrogen in providing trophic support to PV interneurons.

4.3. Conclusions

Our main finding was male-specific changes to spatial memory and cognitive flexibility/perseverance. This highlights an important relationship between sex, BDNF-TrkB signalling and PV-IN.

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Competing interests

The authors have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bbr.2019.111984>.

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4.3 Supplementary Material Results

4.3.1 Investigation of co-expression of PV and TrkB in the DHP and mPFC

4.3.1.1 No significant differences of co-expression of PV and TrkB between genotypes in the DHP

For total DHP % co-expression, there were no significant differences between PV-Cre and PV-Cre:Fl^{+/-} male mice (Figure 4.1A). There were no significant effects of genotype or region in the DHP for the CA3 and DG in % co-expression (Figure 1B) (Figure 4.2 for example staining). Total number of PV cells (4.1C) and PV density (4.1D) were also unchanged by genotype.

4.3.1.2 No significant differences of co-expression of PV and TrkB between genotypes in the mPFC

In the mPFC co-expression of PV and TrkB was measured in the Cg, PrL and IrL (Figure 4.3). There were no significant differences in co-expression of PV and TrkB in total mPFC (Figure 4.3A) or across sub-regions (Figure 4.3B) (Figure 4.4 for example staining). PV cell number (4.3C) and density (4.3D) were also unchanged.

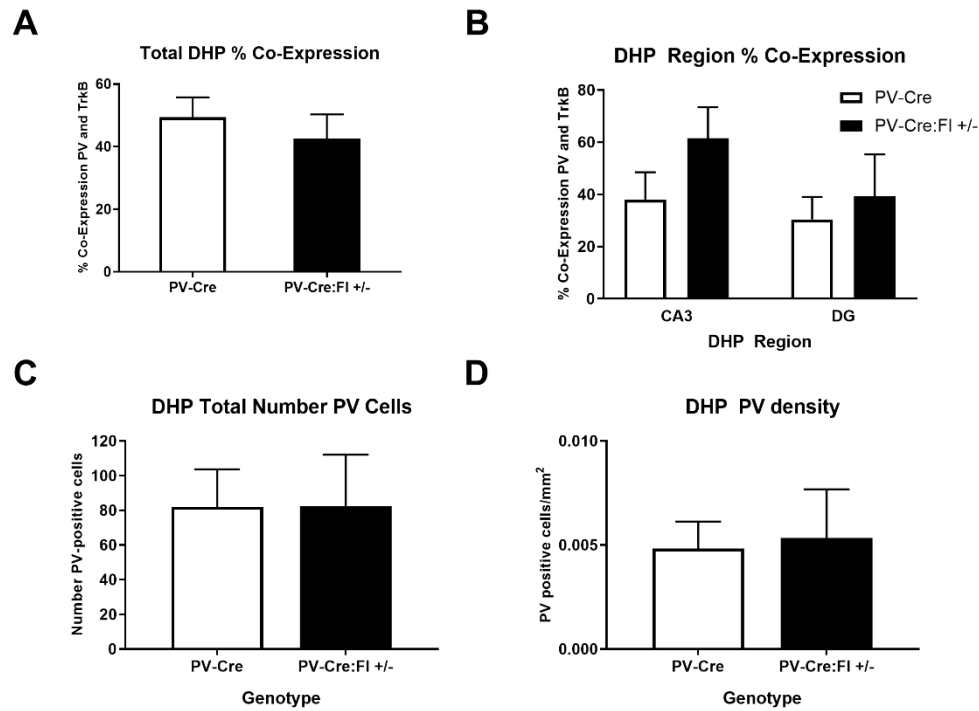
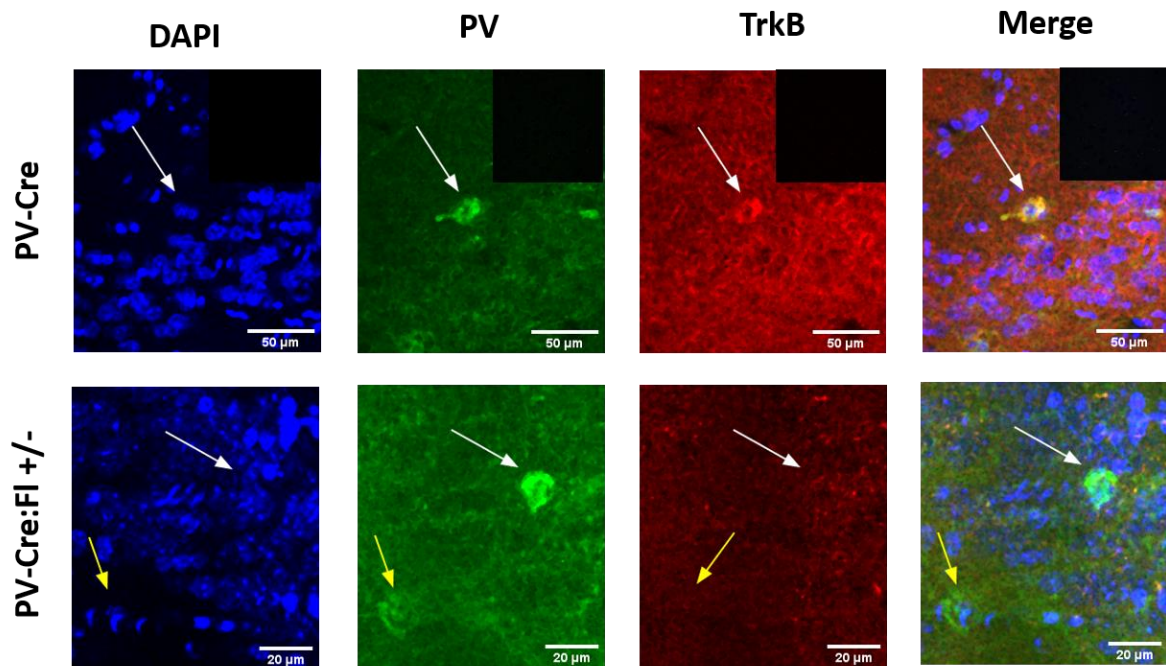


Figure 4.1 - Co-expression of parvalbumin (PV) and TrkB in the male dorsal hippocampus (DHP)

Panel **A** shows a -3.86% difference for co-expression between PV-Cre and PV-TrkB male mice for the total DHP. Panel **B** shows no significant differences in co-expression in the DHP regions CA3 and DG. There were no differences in total number of PV cells (**C**) or PV density (**D**) in the DHP. Data are mean \pm SEM, $n = 5$.

Dorsal Hippocampus CA3



Dorsal Hippocampus Dentate Gyrus

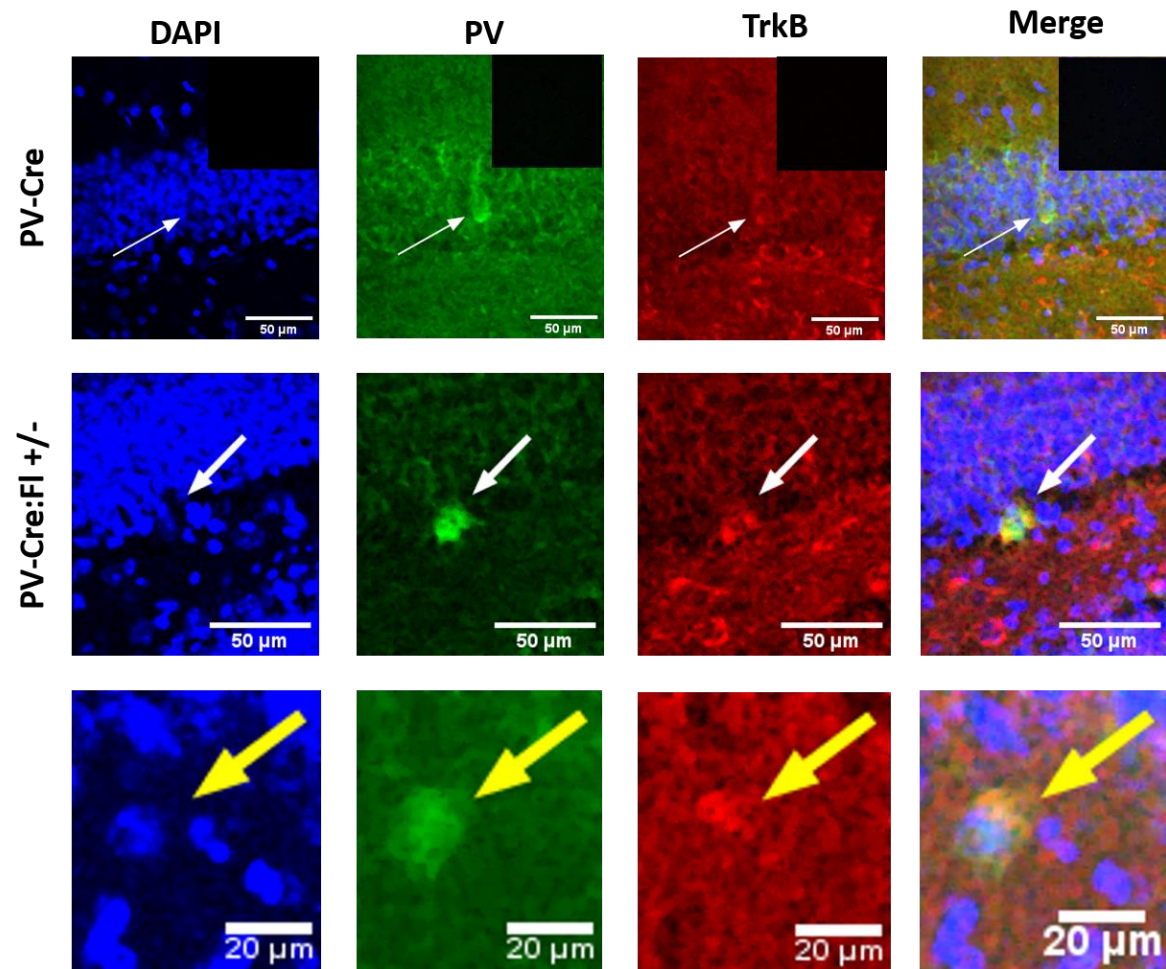


Figure 4.2 - Example staining in CA3 and DG for parvalbumin and TrkB

Example staining in the dorsal hippocampus for co-expression of parvalbumin (green) and TrkB (red) with DAPI (blue) for nuclei staining, in CA3 (Top panel) and dentate gyrus (bottom panel) for PV-Cre control and PV-Cre:Fl^{+/-} mice. Negative controls are shown in the top right corner of PV-Cre panels to ensure specificity of staining. Two controls were performed. 1) Negative control was no staining at all – advised by microscopy staff to ensure lasers were not crossing over each other. 2) Secondary antibodies were added but no primary to ensure that there is no non-specific binding occurring. DAPI is a conjugated stain and we wanted to ensure lasers were not crossing over with each other, this was not added to the negative controls. The control in the figures is the second control described above. White arrows point to co-expressed cells. Yellow arrows point to PV cells without TrkB expression.

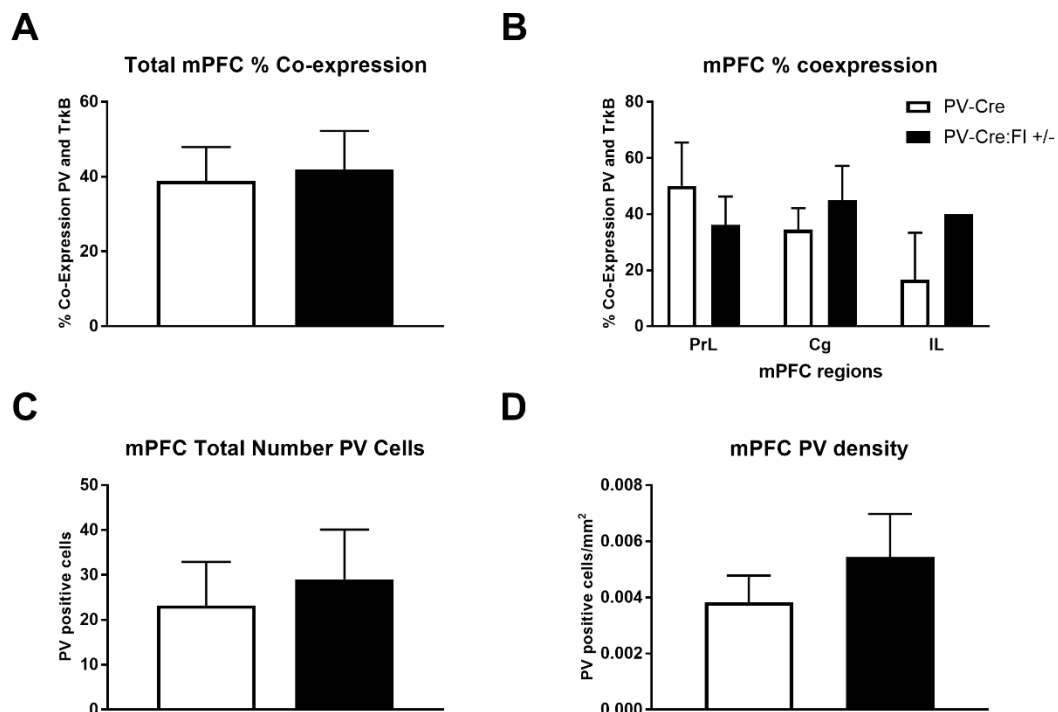


Figure 4.3 – Exploratory investigation of co-expression of parvalbumin (PV) and TrkB in the male medial prefrontal cortex (mPFC)

Panel A shows no qualitative difference for co-expression between PV-Cre and PV-TrkB male mice in the mPFC. Panel B shows no qualitative differences in co-expression in the mPFC regions. There were no qualitative differences in total number of PV cells (C) or PV density (D) in the mPFC. Data are mean \pm SEM, n = 1-6.

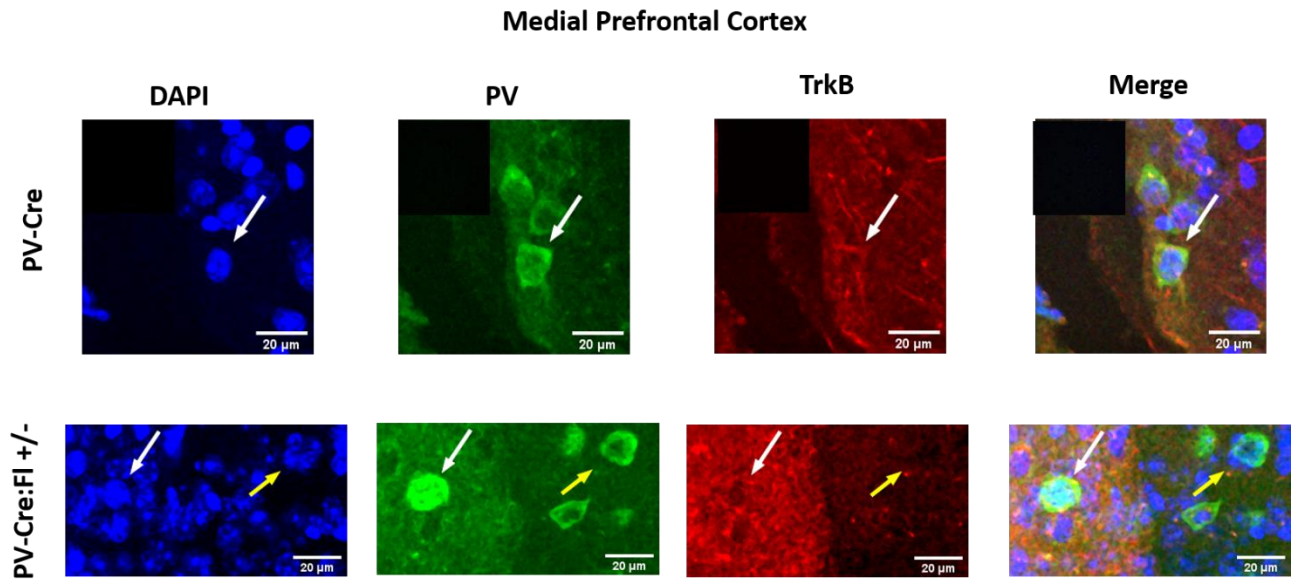


Figure 4.4 - Example staining in mPFC for parvalbumin and TrkB

Example staining in the dorsal hippocampus for co-expression of parvalbumin (green) and TrkB (red) with DAPI (blue) for nuclei staining, in medial prefrontal cortex for PV-Cre control and PV-Cre:Fl^{+/-} mice. Negative controls are shown in the top right corner of PV-Cre panels to ensure specificity of staining. White arrows point to co-expressed cells. Yellow arrows point to PV cells without TrkB expression.

4.3.1.3 No significant differences in co-expression of PV and TrkB based on PV-Cre parentage

Co-expression of PV and TrkB was compared for PV-Cre:Fl +/- offspring from PV-Cre Dams vs PV-Cre studs. Generally, the PV-Cre stud qualitatively has lower co-expression than the PV-Cre dam however the data is quite variable. There were no significant differences found in co-expression based on PV-Cre parentage in DHP (Figure 4.5A) or mPFC (Figure 4.5B).

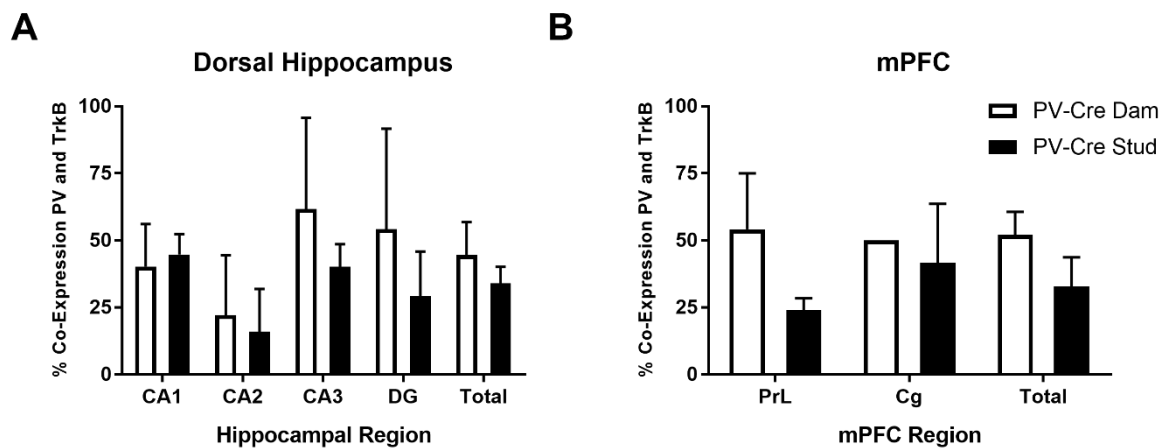


Figure 4.5 - PV-Cre parentage does not affect co-expression of parvalbumin and TrkB

Co-expression of PV and TrkB was compared for PV-Cre:Fl +/- offspring from PV-Cre dams vs PV-Cre studs. There were no significant differences found in co-expression based on PV-Cre parentage in dorsal hippocampus (A) or mPFC (B). Data are mean \pm SEM, n = 2-3.

4.3.2 Behavioural analysis of the PV-Cre:Fl +/- mouse model

4.3.2.1 Investigation of reversal learning a working memory task

4.3.2.1.1 Male latency shows no significant reversal learning

After retrieval memory was assessed on day 7 (see manuscript), we assessed reversal spatial memory. The baited well position was reversed 180° on the Cheeseboard and latency to reach the

baited well was assessed on day 8. For males, student's t-test was performed between trial 1 and trial 2 with no significant difference for PV-Cre ($p = 0.1874$) or PV-Cre:Fl $^{+/-}$ ($p = 0.4888$) latency (Figure 4.6A).

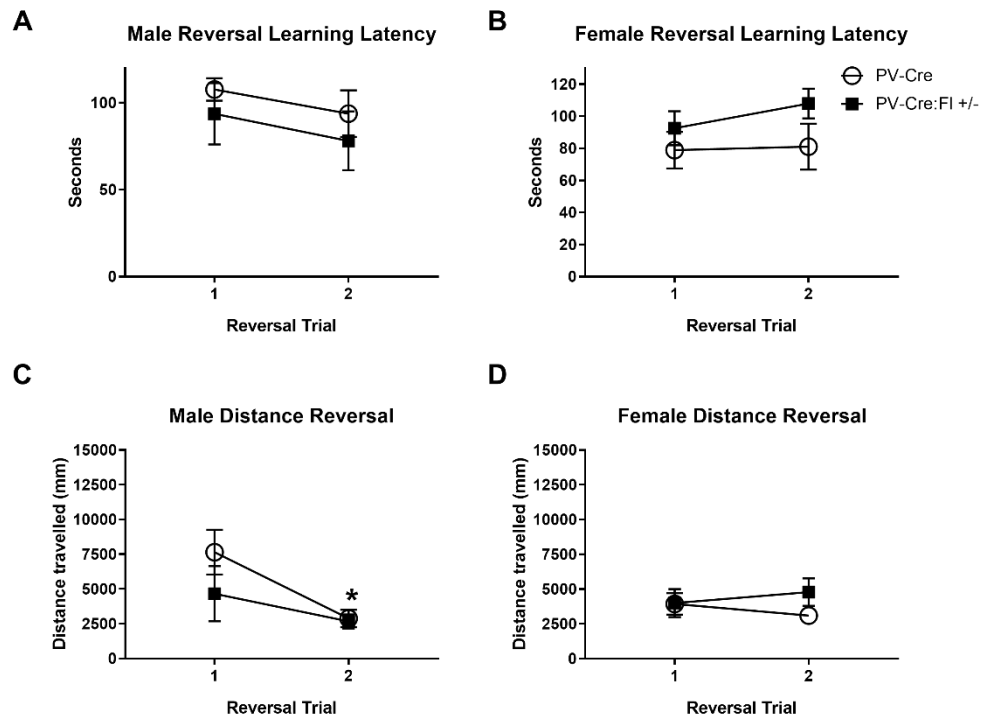


Figure 4.6 - Reversal learning for the Cheeseboard Maze

*Spatial working memory was assessed in these mice by reversing the location of the baited well in the Cheeseboard Maze. Over two trials there were no significant effects of trial or genotype for males (panel A) or females (panel B). Males did decrease their distance travelled over the two trials (panel C) but females did not (panel D). Data expressed as mean + S.E.M, $n = 7-11$, * denotes $p < 0.05$.*

4.3.2.1.2 Trial has a significant effect on male distance travelled

Univariate Repeated Measures Analysis found a significant effect of trial ($F(1,10) = 6.31$, $p = 0.03$), whereby the distance travelled in the second trial was less than the first trial. There was no significant effect of genotype (Figure 4.6C).

4.3.2.1.3 Female latency shows no significant reversal learning

For females, student's t-test was performed between trial 1 and trial 2 with no significant difference for PV-Cre ($p = 0.9141$) or PV-Cre:Fl $^{+/-}$ ($p = 0.2573$) for latency (Figure 4.6B).

4.3.2.1.4 No significant effects for female distance travelled

Univariate Repeated Measures Analysis found no significant effect of genotype or trial for distance travelled (Figure 4.6D).

4.3.2.1.5 No significant effects of time or genotype for learning on probe day

There were no significant differences in time bin or genotype for male (Figure 4.7A) or female (Figure 4.7B) target quadrant duration.

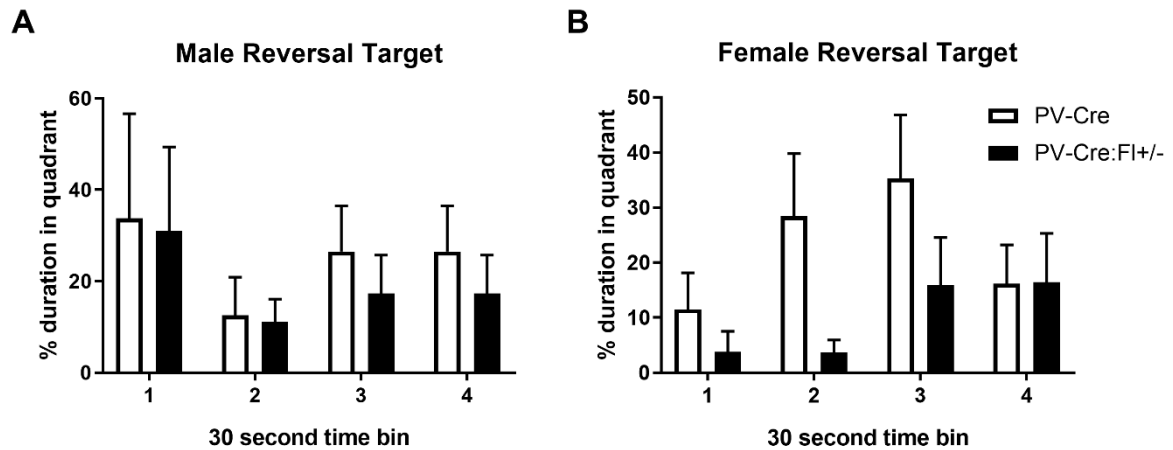


Figure 4.7 - Reversal memory retrieval measured by the Cheeseboard Maze

After the reversal working memory task (day 8), on day 9 a reversal retrieval memory task was performed. No reward was available, and time spent in the target quadrant was assessed in 30 second time bins for the two-minute trial. There were no significant effects of time bin or genotype for males (panel A) or females (panel B). The absence of a significant genotype effect is likely due to the variability in the data. Data expressed as mean + S.E.M, $n = 4-12$.

4.3.2.2 Investigation of egocentric and allocentric navigation

The male Acquisition Probe quadrant analysis (see manuscript) suggested to us that potentially there were differences in search strategies used to navigate the maze. This was investigated in several ways. Initially the number of quadrants crossed was analysed and defined these as Indirect (crossing 4 quadrants), Mid (crossing 2-3) quadrants and Direct (crossing 1 quadrant). However no statistical differences were observed between groups for male (Figure 4.8A) and female (Figure 4.8B) acquisition or male (Figure 4.8C) and female (Figure 4.8D) reversal probe days. Following this, we investigated the traces to qualitatively observe any differences in search strategy, similar to what has been observed and defined in the MWM. For the acquisition probe Focal and Directed searches were grouped as “Focal”; Scanning, Chaining and Thigmotaxis as “Non-focal”; and

Random was a solo group. There were no statistically significant differences between male genotypes (Figure 4.9A) or female genotypes (Figure 4.9B) in search strategies employed. Finally, for the acquisition probe first entry into a quadrant was investigated but again there were no group differences for males (Figure 4.9C) or females (Figure 4.9D) in the first entry into a quadrant being the Target quadrant. These analyses were not performed for the reversal probe as learning did not occur for any group for that task.

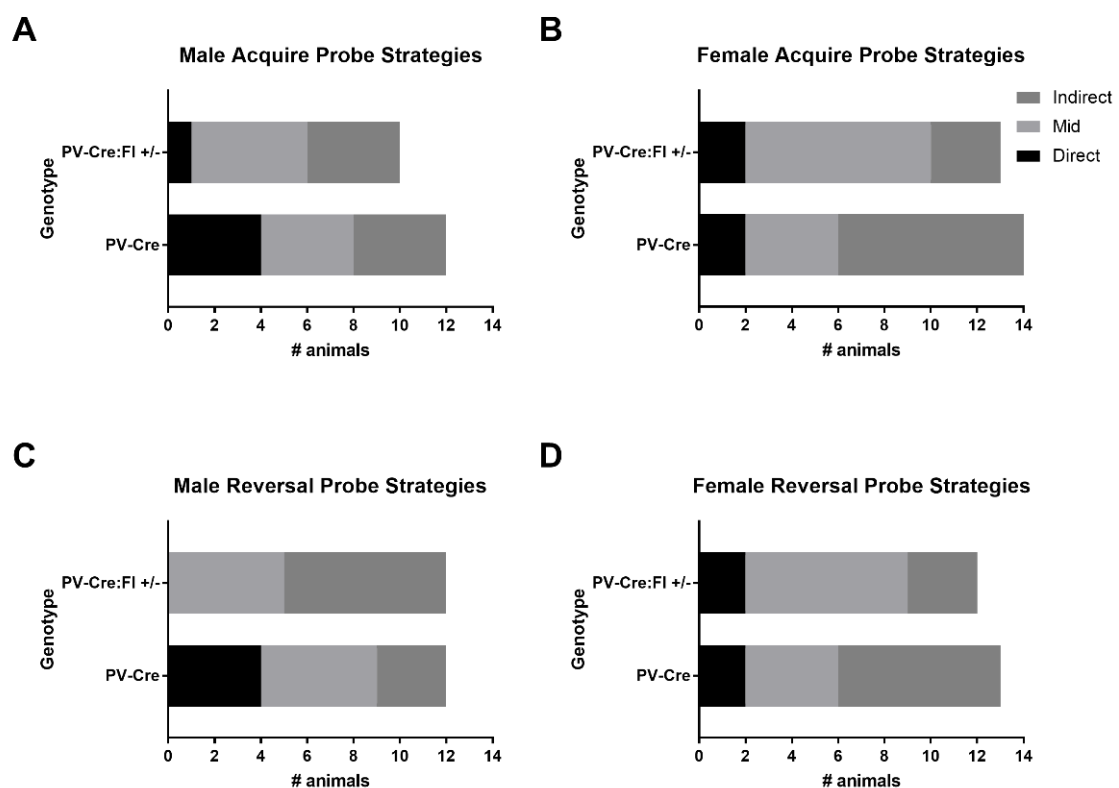


Figure 4.8 - Investigation of search strategies used for the Cheeseboard Maze

Search strategies were defined by the number of quadrants crossed during probe trials. Chi-square calculations found no significant differences were found for acquisition probe strategies for males (A) or females (B). Similar strategies appeared to be used in the reversal probe for male PV-Cre mice (C) and female genotypes (D). Data are mean + SEM, n = 12-14.

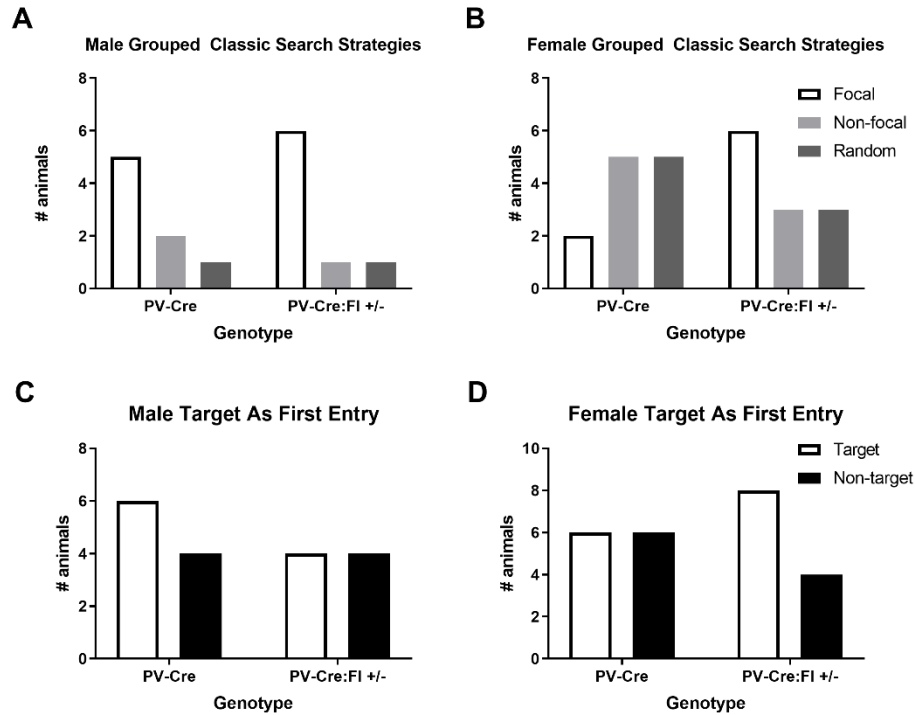


Figure 4.9 - Investigating classic search strategies for the Cheeseboard Maze

Classic Morris Water Maze search strategies were qualitatively defined and grouped accordingly for males (panel A) and females (panel B). Target quadrant as the first quadrant entered was analysed but Chi-square calculations found no significant differences were found for males (panel C) or females (panel D). Data are mean \pm SEM, $n = 8-12$.

4.3.2.3 Investigation of recognition memory using the Novel Object Recognition Task

The first 5 minutes of the Novel Object Recognition Task (NORT) was investigated.

4.3.2.3.1 Analysis of NORT Habituation

Univariate ANOVA analysis found a significant sex \times genotype interaction ($F(1,51) = 11.75$, $p = 0.0012$) and due to this significant sex \times genotype interaction, the sexes were subsequently analysed separately.

4.3.2.3.1.1 Analysis of NORT Habituation Phase Males

Univariate Repeated Measures analysis found a significant effect of genotype ($F(1,24) = 6.620$, $p = 0.0167$), whereby the male PV-Cre:Fl^{+/-} animals spent more time at the objects compared to male PV-Cre (Figure 4.10A). There was no effect of object position and no object position \times genotype interaction.

4.3.2.3.1.2 Analysis of NORT Habituation Phase Females

Univariate Repeated Measures analysis found no significant effect of genotype, object position or object position \times genotype interaction for female mice (Figure 4.10B).

4.3.2.3.2 Analysis of NORT Testing Phase – Recognition Index

There were no differences between male genotypes for Recognition Index (Figure 4.10C). There were no differences between female genotype for Recognition Index (Figure 4.10D). Here it is important to note that neither genotype showed a recognition index above 50% chance level, thus it appears that neither group recognised that the object was novel.

4.3.2.3.2.1 Analysis of NORT Testing Phase – Bouts

The amount of times the objects were approached were analysed. There was a significant genotype effect for males ($F(1, 50) = 5.04$, $p = 0.029$), whereby male PV-Cre:Fl^{+/-} mice approached the objects significantly more often than male PV-Cre mice (Figure 4.10E). However, male PV-Cre:Fl^{+/-} did not approach the novel object more often than the familiar object despite a main effect for object approaching significance ($F(1, 50) = 3.35$, $p = 0.073$). There were no significant differences in genotype or object for female mice (Figure 4.10F).

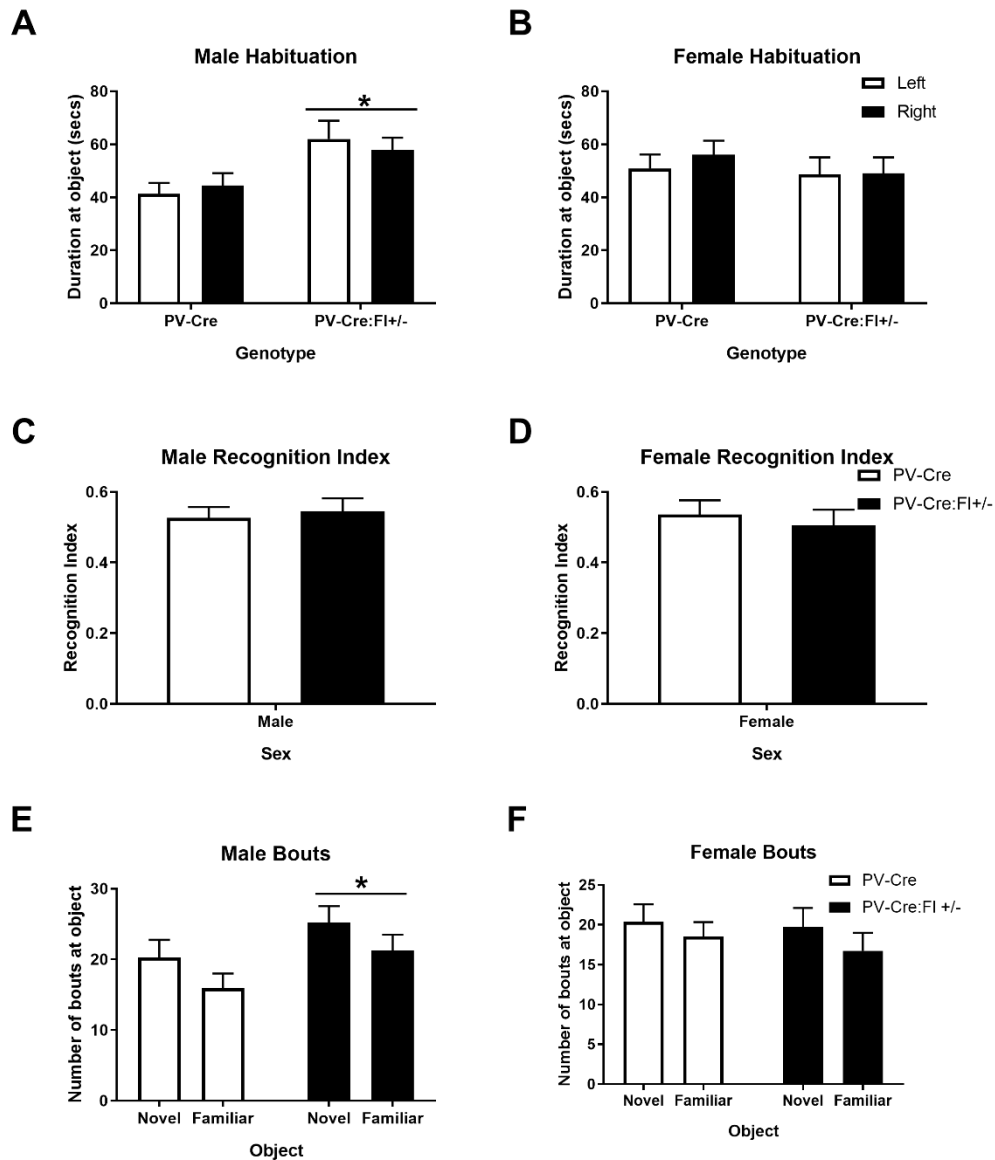


Figure 4.10 - Measurement of object recognition behaviours

Phase one of the Novel Object Recognition Task involves exposing mice to two identical objects. There was a genotype effect for the males (panel A), whereby PV-Cre:Fl+/- mice explored the object for longer the PV-Cre mice. There were no differences observed between female genotypes for time spent exploring the objects in phase one (panel B). No effect of genotype was found for Recognition Index in males (panel C) or females (panel D). However, controls did not demonstrate preference for the novel object. Male PV-Cre:Fl+/- mice

*approached objects significantly more often than male PV-Cre mice (panel E). There were no differences for female mice (panel F). Data are mean \pm SEM, n = 12-14, * p < 0.05.*

4.3.2.3.3 Analysis of exploration of NORT objects – both sexes

Total time spent exploring both objects was compared across trial phase, sex and genotype (Figure 4.11). Univariate Repeated Measures Analysis found a significant sex \times genotype interaction ($F(1,51) = 7.52$, $p = 0.0084$). Bonferroni post hoc comparisons found that during habituation male PV-Cre:Fl $^{+/-}$ spent more time at the objects compared to male PV-Cre ($p = 0.0039$). Comparatively there were no differences in % duration at objects during habituation between female genotypes. There was a main effect of trial phase ($F(1,51) = 35.40$, $p < 0.0001$), whereby all animals spent less time with the objects in the testing phase compared to the habituation phase. There was a close to significant phase \times genotype \times sex interaction ($F(1,51) = 4.02$, $p = 0.05$). Here, Bonferroni post hoc comparisons found that only the male PV-Cre:Fl $^{+/-}$ group significantly differed in time spent at objects ($p < 0.0001$) between phases. Male PV-Cre:Fl $^{+/-}$ mice spent more time exploring the objects in the habituation phase compared to the testing phase. There was no significant main effect of sex or genotype on exploration of objects. Due to the sex interactions described above, further analyses were done investigating each sex separately.

4.3.2.3.3.1 Analysis of NORT Exploration Males

Univariate Repeated Measures analysis found a significant effect of genotype ($F(1,25) = 8.63$, $p = 0.007$). Male PV-Cre:TrkB Fl $^{+/-}$ mice spent more time exploring the objects overall compared to the male PV-Cre group (Figure 4.11A). There was a main effect of phase ($F(1,25)$

= 23.37, $p = 0.0001$). Here, both male genotypes spent more time with the objects in the habituation phase compared to the testing phase.

4.3.2.3.3.2 Analysis of NORT Exploration Females

Univariate Repeated Measures analysis found a significant effect of phase ($F(1,28) = 12.7$, $p = 0.0013$). Both female genotypes spent more time with the objects in the habituation compared to the testing phase (Figure 4.11B). There was no significant effect of genotype.

4.3.2.3.4 Analysis of NORT Distance travelled

There were no differences in distance travelled between genotypes for males (Figure 4.11C) or females (Figure 4.11D).

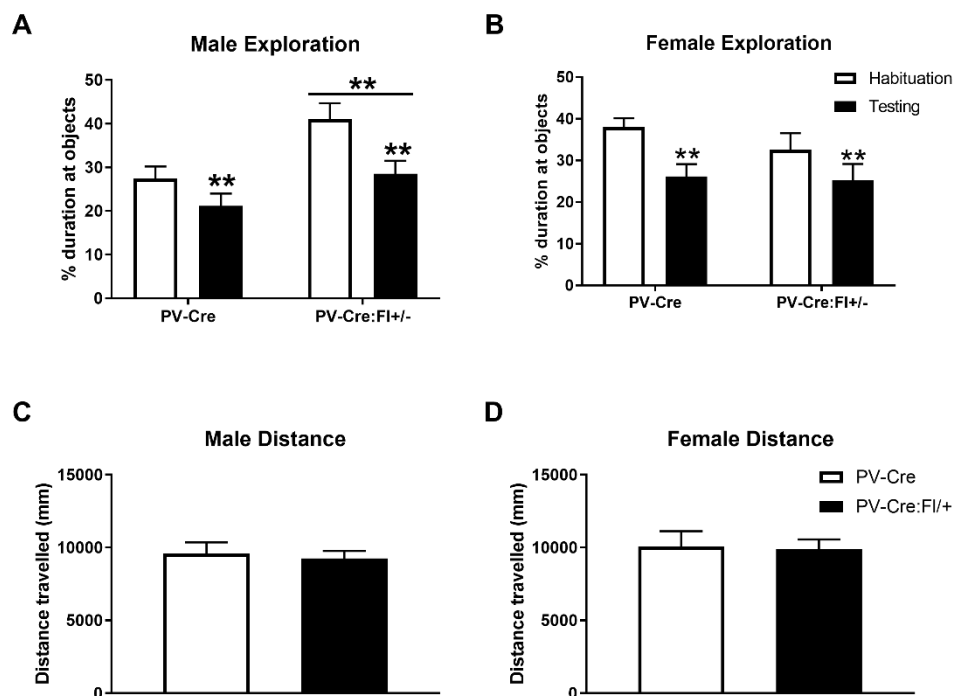


Figure 4.11 - Investigation of exploratory behaviours in NORT

*Exploration between phases was explored, and both male genotypes spent less time exploring the objects in the second phase compared to the first phase (panel A). This was also observed for females, with both genotypes exploring the objects less in the second phase (panel B). There were no differences in distance travelled for males (panel C) or females (panel D). Data are mean + SEM, n = 12-14, * $p < 0.05$, ** $p < 0.005$.*

4.4 Supplementary Material discussion

4.4.1 No significant effects of genotype on co-expression of PV and TrkB

This mouse model had a subtle knockdown of TrkB receptors on PV-positive cells and this was reflected in the subtle memory deficits (see published manuscript). The staining observed appears to be membrane-bound, consistent with the literature (Bartkowska et al., 2014; Helgager et al., 2014). This did make counting harder, and due to the nature of the staining the experimenter had to identify TrkB staining by eye. This may have introduced some biases despite the experimenter being blinded to mouse numbers.

Discussion of this mouse model needs to be in the context of the literature not only on this model, but also on the cre-lox recombination system. Firstly, compared to other reports, we found no significant differences in % knockdown between mPFC and the hippocampus, with total % co-expression at ~40% for both regions (Xenos et al., 2017b). However, other papers using this model have reported only on the level of recombination (Lucas et al., 2014; Xenos et al., 2017b) and did not directly examine co-localisation. We did find variation within brain regions, most strikingly in the DHP, however these were not significant. The low proportions of % co-expression knockdown could be due to our use of TrkB heterozygote floxed animals, and homozygotes would lead to a larger knockdown. However, the use of homozygotes leads to vestibular dysfunction

which creates problems for behavioural testing (Lucas et al., 2014). A limitation here is the small n used for analysis, making this study exploratory in nature. This is the first study to directly analyse co-expression of PV and TrkB in this model. Our results suggest that even subtle knockdown of TrkB in PV cells can generate sex-specific cognitive dysfunction, highlighting the importance to look at both sexes. This supports our previous work where estradiol can mediate PV expression and function (Schroeder et al., 2017; Wu et al., 2014; Wu et al., 2015). It appears that the PV-Cre:TrkB Fl^{+/-} system produces inconsistent KD within the different brain regions, hence the behavioural alterations generated are likely to be idiosyncratic. It is therefore preferential to measure knockdown of co-expression, rather than recombination levels.

Secondly, the cre-recombinase system has some reported limitations. It has been suggested that it can cause non-specific recombination (Vooijs et al., 2001). It has also been reported that the extent of recombination can be dependent on which parent contributes the Cre component (Heffner et al., 2012) and loxP sites sequence homology (Arguello and Moraes, 2015). We attempted to address these issues by comparing % knockdown by parentage, of which there were no differences. Albeit the data here are quite variable and it does look as though offspring arising from Cre-studs show greater knockdown. Complete sequencing of the loxP sites would have served as an additional control measure (Arguello and Moraes, 2015). However since we used heterozygote mice this would have buffered against random transgene insertion that can occur in the absence of a WT chromosome (Jackson Laboratory, 2013). In our hands, even a subtle knockdown caused the emergence of sex-specific spatial memory deficits.

4.4.2 Investigation of reversal learning as measured by the Cheeseboard Maze

4.4.2.1 Absence of reversal learning may be attributable to protocol

Conditional postnatal knockout of TrkB in the forebrain leads to a complete inability to learn the MWM including reversal task (Minichiello et al., 1999) while administration of BDNF has been found to improve reversal learning (Cirulli et al., 2004). The current study did not follow the usual reversal learning paradigm of 4 days of reversal learning and instead investigated spatial working memory. The BDNF-TrkB signaling pathway and PV-IN have been shown to have a role in working memory (Chen et al., 2017b), but whether BDNF-TrkB signaling contributes through its actions at PV-IN (Kim et al., 2016a) does not appear to have been established in the literature. In this study neither sex nor genotype demonstrated behaviour indicative of normal working memory, which may reflect a cognitive flexibility issue. PV-IN have been shown to be part of the network for cognitive flexibility (Murray et al., 2015b) and in this study mice perseverated with the Old Target. It is also likely however that this indicates issues with the protocol. It may have been too complicated a working memory procedure for these mice, with two trials not enough time to learn the new target position. Future studies should follow the usual reversal learning protocol of 4 days to confidently ascertain the cognitive flexibility capabilities of this mouse model. It is highly likely that TrkB knockdown on PV-interneurons would lead to disruptions in cognitive flexibility, if measured with the appropriate task.

4.4.2.2 Search strategies are varied within and between genotype

Allocentric-specific deficits have been observed in schizophrenia (Grech et al., 2018a; Weniger and Irle, 2008) and search strategies were investigated in these mice to determine if this occurs in this model. and sin these mice to determine if this occurs in this model One such classification that was used was judging by exploration across the different quadrants for both ‘acquisition’ and

‘reversal’ probe days. Across both days’ males used more ‘direct’ strategies than females. However, the quantification of crossing quadrants was a basic approach. A more sophisticated approach of investigating search strategies was used for the acquisition probe but not reversal probe, since a preference for the New Target Quadrant was not established. Classic MWM strategies were used to define and then group strategies employed by the mice. Increased BDNF has been associated with successful performance of appropriate search strategies in the MWM (Harvey et al., 2008) but no such inferences were able to be made from this data. Motivation is an important caveat for the Cheeseboard Maze. Compared to the MWM, there may be a lack of pressure to ‘succeed’, as the motivation is not driven by mortality but rather by hunger. Chronic food deprivation as a test motivator has its own caveats, with some evidence pointing to adaptation to food restriction (Rowland, 2007) and improvement of cognitive ability (Fu et al., 2017). If cognitive ability is improved, this may mask subtle differences between the genotypes that perhaps a different motivator, such as provided by the MWM, may emphasise instead.

4.4.3 Absence of Novel Object Recognition Task performance for both genotypes

Initial investigation into NORT found that no group spent significantly more time exploring the novel object compared to the familiar object (data not shown) or approached the novel object more than the familiar. Recognition Index (RI) was then calculated to compare performance between groups. There were no differences in NORT performance between genotypes as measured by RI. It was unusual that the mice had not performed the task, and to further investigate this time at both objects (“Exploration”) was compared for habituation and testing phases. It was found that PV-Cre:Fl^{+/-} spent more time at, and approached more, the objects for both phases compared to male PV-Cre. Anxiety and activity measures indicate that this was not an artefact of underlying affective differences. It is interesting that yet again this is the group that differs from all others.

The published manuscript detailed spatial memory differences that may arise from changes in exploratory drive due to male specific preferences for novelty (Frick and Gresack, 2003). However, in this task, the PV-Cre:Fl +/- is seemingly more interested than the other groups in exploring the objects. Analysis of behaviour showed that this was not driven by novelty, rather there was a higher degree of engagement with the task. Notably, all groups spent less time exploring the objects in the testing phase. It is thought that the lack of exploration in the testing phase may account for NORT not being performed by any group. NORT was the second test performed in the behavioural battery, and due to this early position potentially the mice were stressed. An issue raised in the published manuscript was that the 10-minute post-transport acclimatisation period before testing may not have been sufficient and this may impact the interpretation of results. Gerdin et al (Gerdin et al., 2012) have previously found that cage transport leads to stress-induced hyperthermia, with an hour of acclimatisation needed to return to basal levels.

Disruption to recognition memory because of disrupted BDNF signaling is dependent on the model used. Complete conditional deletion of BDNF in the DHP disrupts NORT performance in male mice (Heldt et al., 2007). Comparatively, male BDNF HET mice are reported to have intact recognition memory but it is thought that BDNF HET mice may be compensated by other neurotrophins (Carretón et al., 2012).

6.1.1 Male specific genotype divergences in spatial memory performance and novelty seeking

The aim of this project was to characterize the effects of TrkB receptor ablation on PV-positive cells on cognition. The main finding from this project was that subtle knockdown of TrkB on PV-

positive cells in the CA1 and CA2 regions of the dorsal hippocampus led to male-specific genotype divergences in spatial memory tasks. Specifically, for the Y-Maze only male PV-Cre:Fl^{+/-} mice showed no preference for the novel arm. Other studies using a similar model have observed sex-dimorphic effects on fear memory (Lucas et al., 2014), and here males had impaired extinction consolidation. The Cheeseboard maze was used as a long-term spatial memory task, and here again there was a male specific genotype difference in memory retrieval in the Cheeseboard Maze. Specifically, male PV-Cre:Fl^{+/-} mice were more perseverative in their learning than male PV-Cre control mice. Concordantly, unpublished data found that for “Exploration” activity in the Novel Object Recognition Task (NORT), PV-Cre:Fl^{+/-} spent more time at, and approached more, the objects for both the habituation and testing phases compared to male PV-Cre. Again, it would appear that the male PV-Cre:Fl^{+/-} mice are more perseverative in their behaviour than the male PV-Cre control group. This may reflect a loss of novelty seeking in the male PV-Cre:Fl^{+/-} group, which has not previously been reported in this model (Lucas et al., 2014; Xenos et al., 2017b).

Overall, the evidence from this study suggests that sex had a developmental influence on this constitutive model. Male spatial memory was altered by the disruption to BDNF-TrkB signaling at PV-IN. Most encouragingly, this aligns with human males showing more severe cognitive dysfunction in schizophrenia.

4.5 Summary and Prelude to Val66Met paper

This study provided insight into the relationship between the BDNF-TrkB signaling pathway and PV-positive cells. The cre-lox recombination model caused a subtle knockdown that appeared to have impact on novelty seeking in males.

The literature demonstrates a relationship between BDNF-TrkB signaling pathway and other IN. However, it was not appropriate to knockout the TrkB receptor on each IN subtype, due to the differences in TrkB expression across the inhibitory subtypes. Instead, the following chapter will investigate IN expression in a humanized BDNF^{Val66Met} mouse model. This mutation of a valine to methionine at codon 66 in the BDNF gene leads to decreased activity-dependent secretion of BDNF. This is a common mutation in humans that has been associated with a range of psychiatric disorders, cognitive functions, and hippocampal changes. The consequences of this constitutive mutation on hippocampal IN densities was characterised.

Chapter 5. BDNF Val66Met genotype selectively alters dorsal hippocampal somatostatin cell density

5.1. Chapter 5 Introduction

Chapter 4 presented evidence for a relationship between sex, BDNF-TrkB signaling and IN function, with spatial memory of male mice altered by a loss of TrkB signaling at parvalbumin-⁺INs. To further develop an understanding of the relationship between sex, BDNF-TrkB signaling and IN a more clinically relevant model was chosen.

The BDNF^{Val66Met} mutation involves the substitution of a valine for a methionine at codon 66 in the BDNF gene. This results in subsequently decreased activity-dependent secretion of BDNF. The BDNF^{Val66Met} mutation is common in the human population, and has been suggested to moderate psychiatric disorders, cognitive functions and associated hippocampal structural changes. Additionally, the effects of this polymorphism are sensitive to stress exposure. It is widely accepted that it is not just one risk factor that leads to the development of a mental illness but rather the interaction between multiple risk factors. The cumulative effect of the BDNF^{Val66Met} mutation and chronic stress on interneuron density has not yet been characterized, despite all of these factors being implicated in the pathophysiology of cognitive symptoms of schizophrenia.

The aim of the following chapter was to measure cell density of multiple IN subtypes, using confocal microscopy, in a humanized BDNF^{Val66Met} mouse model exposed to chronic corticosterone. This included analysis of percentage co-expression of SST and CAL-positive cells in the dorsal and ventral hippocampus. Co-expression of these inhibitory markers was investigated due to previous reports in the literature in other mouse models (Xu et al., 2006), which found that SST⁺/CR⁺ cells had increased horizontally extended dendritic fields and primary process compared to SST⁺/CR⁻ cells. This had functional consequences, with SST⁺/CR⁺ cells exhibiting slower afterhyperpolarization than SST⁺/CR⁻ cells (Xu et al., 2006). The further subgrouping of IN in this manner could reflect divergences in Ca²⁺ buffering capacity, an important regulator of dendritic Ca²⁺ signal distribution (Goldberg and Yuste, 2005) and K⁺ channels (Halabisky et al.,

2006). Therefore, profiling of these markers co-expression of SST and CAL was investigated. While the significance of the co-expression of these inhibitory markers has not been fully elucidated, functional consequences have been demonstrated as described above by (Xu et al., 2006) and therefore, this is an important consideration for future classification of these interneurons.

The following hypothesis was submitted to the *Journal of Neurochemistry*:

- 1) It was hypothesised that the BDNF^{Val66Met} polymorphism with a history of stress hormone exposure would decrease GABAergic inhibitory neuron cell density in the hippocampus of male and female mice.

The following hypotheses were investigated as supplementary data:

- 1) It was hypothesised that Met/Met mice would have decreased co-expression of SST- and CAL-positive cells compared to Val/Val mice.
- 2) It was hypothesised that there would be a sex difference, with females having higher co-expression than males.

5.2. Submitted Manuscript

Brain-Derived Neurotrophic Factor Val66Met polymorphism is associated with selective reduction of somatostatin interneuron density in the dorsal hippocampus of mice

Adrienne Mary Grech^{1,2}, Michael J. Notaras^{2,3}, Mauricio Sepulveda^{2,4}, Maarten van den Buuse^{4,5,6} and Rachel Anne Hill^{1,2}

1. Department of Psychiatry, School of Clinical Sciences, Monash University, Monash Medical Centre, Clayton, VIC, Australia
2. The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC, Australia.
3. Weill Cornell Medical College of Cornell University, Center for Neurogenetics, Brain & Mind Research Institute, New York City, New York, USA
4. School of Psychology and Public Health, La Trobe University, Melbourne, VIC, Australia
5. Department of Pharmacology, University of Melbourne, VIC, Australia
6. The College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia

Introduction

Brain-Derived Neurotrophic Factor (BDNF) is imperative for normal development and maturation of the brain (Ernfors et al., 1994; Thoenen, 1995). The common BDNF Val66Met polymorphism leads to decreased activity-dependent secretion of BDNF into the synapse (Chen et al., 2006; Notaras et al., 2015b) and has been associated with a range of symptom categories and psychiatric illnesses (Chen et al., 2006; Notaras et al., 2015a). In humans, Met/Met carriers show poor episodic memory (Egan et al., 2003), reduced hippocampal volume (Chen et al., 2006) and poorer memory-related hippocampal activity. In Val66Met mice, long-term potentiation and depression were disrupted in the hippocampus of BDNF^{Met/Met} mice compared to BDNF^{Val/Val} control mice (Bath et al., 2012a; Ninan et al., 2010b). BDNF^{Met/Met} mice furthermore show disruptions in spatial memory (Notaras et al., 2016b), deficits in extinction learning (Dincheva et al., 2012) and a depression-like phenotype (Notaras et al., 2017a).

BDNF supports the development and regulates the activity of GABAergic inhibitory interneurons. Signalling via BDNF and its receptor, Tropomyosin-related kinase B (TrkB), directs inhibitory synapse assembly (Chen et al., 2011; Rico et al., 2002), and moderates GABAergic synaptic plasticity (Park and Poo, 2013) and neurotransmission (Jovanovic et al., 2004b). In mice, BDNF heterozygosity alters inhibitory cell density and protein expression (Du et al., 2018) and disruptions to BDNF-TrkB signalling at inhibitory interneurons can lead to cognitive dysfunction (Grech et al., 2019a; Lucas et al., 2014; Xenos et al., 2017a). Animal studies have furthermore found that disruptions to inhibitory interneuron development lead to cognitive disruptions in adulthood (Cho et al., 2015) and that silencing of inhibitory interneurons disrupts higher order functioning (Murray et al., 2015a). The Val66Met polymorphism in humans is associated with alterations to excitation/inhibition balance (Wiegand et al., 2016) and with hyperexcitability, representing impaired inhibitory regulatory processes (Strube et al., 2015b).

There are over 20 different subtypes of GABAergic interneurons in the hippocampus, which are classified based on their unique morphology, specific targets (e.g. pyramidal cell or other interneurons), innervation and electrophysiological output, as well as specific molecular markers such as calcium-binding proteins or peptide expression profiles (Kelsom and Lu, 2013a). Human post-mortem studies have found altered expression and cell density of parvalbumin (PV) and somatostatin (SST)-positive GABAergic neurons in prefrontal cortex and hippocampal regions in schizophrenia and bipolar disorder (Fung et al., 2014; Konradi et al., 2011).

Chronic mild stress (involving intermittent sensory and environmental stressors) has previously been shown to alter hippocampal GABAergic interneuron cell density in rats (Czeh et al., 2015) and a history of stress hormone exposure modified behavioural outcomes in hBDNF^{Val66Met} mice, including memory function and sensorimotor gating (Notaras et al., 2016b; Notaras et al., 2017c). (Notaras et al., 2016b; Notaras et al., 2017c). However, little is known on whether the interaction of chronic stress and Val66Met genotype on behaviour is associated with changes in interneuron density and phenotype in the brain. In the present study we therefore compared the density of PV, SST and Calretinin (CAL)-positive interneurons in mice with the BDNF^{Val66Met} polymorphism and treated with chronic corticosterone (CORT) treatment during adolescence to simulate the effects of chronic stress. We chose to focus on the hippocampus because of its prominent role in learning and memory, and as a region previously found to be highly impacted by the Val66Met substitution (Dincheva et al., 2012). Dorsal and ventral hippocampus were analysed separately because of their distinct roles in specific behaviours and functional connections to other brain regions (Fanselow and Dong, 2010). Broadly, the DHP is strongly implicated in spatial memory (Klur et al., 2009) while the VHP is involved in emotional and social processing (Felix-Ortiz et al., 2013; Felix-Ortiz and Tye, 2014). Subregions of the dorsal and ventral hippocampus including the CA1-3 and DG were assessed as these discrete areas differentially respond to environmental factors such as stress (Zhang et al., 2011) and have

different expression of neurochemical markers (Coultrap et al., 2005; Silva et al., 2001). PV and SST were selected because of their association with schizophrenia (Fung et al., 2014; Morris et al., 2008) and their reliance upon functional BDNF-TrkB signalling during development (Du et al., 2018; Hashimoto et al., 2005b), while CAL was chosen as a control. CAL-IN is generally spared in the pathophysiology of schizophrenia (Banasr et al., 2017; Brisch et al., 2015; Eyles et al., 2002; Knable et al., 2004) and does not have a strong relationship with the BDNF-TrkB signaling pathway, likely due to only 30% of CAL-IN co-expressing with the TrkB receptor, compared to 80% and 50% of PV- and SST-IN respectively (Gorba and Wahle, 1999). Therefore, CAL-IN present as a good control for the analysis of the between BDNF-TrkB signaling pathways and IN. Additionally, because some GABAergic subtypes have been shown to be influenced by sex hormones (Wu et al., 2014), we also included both male and female mice in this study.

Methods

Animals

Male and female hBDNF^{Val66Met} mice were generated as previously described (Cao et al., 2007) and offspring used in the current study were obtained from a breeding colony at the Florey Institute, Melbourne, Australia. All mice were on a C57Bl/6 background and hBDNF^{Val/Met} × hBDNF^{Val/Met} breeding pairs were used to generate Val/Val and Met/Met littermates. There were 8 groups in this study comprising 2 genotypes (hBDNF^{Val/Val} and hBDNF^{Met/Met}), 2 treatments (water and corticosterone) and 2 sexes (male and female). The number of mice per group are shown in the timeline below.

Mice were pseudorandomized into the above treatment groups. Males and females were group-housed separately ($n = 2-6$ per box) in individually-ventilated cages (IVC; Tecniplast). This ensures that social stress doesn't impact animal welfare, e.g. males fighting over females (Kappel

et al., 2017) and unwanted reproduction. The animals had *ad libitum* access to food and water in a temperature-controlled room maintained at approximately 22 °C and on a 12/12-hour light/dark cycle. From 6 to 8 weeks of age mice were exposed to 25mg/L of CORT-hemisuccinate (Q1662-000 Steraloids Inc, United States) in their drinking water, while control groups received water without CORT (a total of 3 weeks). This is a more ethical approach to inducing stress hormones compared to non-physiological stress paradigms such as restraint stress, as the mouse is not made to feel fear or stress.

All mice were anaesthetised with a single intraperitoneal injection of 100 µl pentobarbitone diluted 1 in 10 (Virbac, NSW, Australia) at week 15 in the morning (between 9am and 12pm) before transcardial perfusion. This choice of anaesthetic is optimal for transcardial perfusions. All procedures were performed according to guidelines set by the National Health and Medical Research Council of Australia and approved by the Florey Institute for Neuroscience and Mental Health Animal Ethics Committee (16-056) and comply with the ARRIVE guidelines. The study was not pre-registered. Timeline of experimental events with animal numbers is shown below:

Mice day 0	Week 6	Week 7	Week 8	Week 15
Male Val/Val (N = 5)	Water			Animals perfused fixed and brains collected
Male Val/Val (N = 5)	CORT in drinking water (25mg/L)			
Male Met/Met (N = 4)	Water			
Male Met/Met (N = 4)	CORT in drinking water (25mg/L)			
Female Val/Val (N = 5)	Water			
Female Val/Val (N = 5)	CORT in drinking water (25mg/L)			
Female Met/Met (N = 4)	Water			
Female Met/Met (N = 4)	CORT in drinking water (25mg/L)			
Total mice = 38				

Immunohistochemistry

Mice were transcardially perfused with 0.1M ice-cold phosphate-buffered saline (PBS) (~50 ml) followed by 4% paraformaldehyde in ice-cold 0.1M PBS (~50 ml). Brains were post-fixed in 4% PFA overnight, transferred to a 15% sucrose solution for 24 hours, and then transferred to a 30% sucrose solution for a further 24 hours. Brains were snap-frozen and 20 µm coronal sections were cut on a cryostat. Free-floating tissue sections were stored in cryoprotectant (300ml ethylene glycol, 150g sucrose, 275ml dH₂O, 275ml 0.1 PB pH 7.4) in 24-well cell culture plates. Staining was done in these plates (Costar 3524, Corning Incorporated, Corning, NY, USA) to cause minimal disruption to tissue.

Free-floating tissue sections were sorted to find representative sections of Bregma -1.46, -1.82, -2.18, -2.54, -2.80 and -3.16 mm, to image throughout the dorsal and ventral hippocampus. On Day 1 sections were washed in PB for 10 minutes on a shaker. PB wash buffer was removed and blocking solution (10% Normal Donkey Serum (NDS) + 1% Triton-X100 in PB) added. Samples were incubated in blocking solution for 1 hour at room temperature. Blocking solution was removed and primary antibody solution (primary antibodies + 2% NDS + 0.3% Triton-X100 in PB) added after which the sections were incubated overnight on a rocker at room temperature. On Day 2 samples were washed in PB 3 x 5 minutes. Samples were then incubated in secondary antibody solution (secondary antibodies + 2% NDS in PB) for 3 hours. Following this, samples were washed in PB 3 x 5 minutes.

Primary antibodies used were: rat anti-SST (1:100, Merck, MAB354), mouse anti-PV (1:1000, Sigma-Aldrich, Sig P30881), rabbit anti-CAL (1:1500, Swant, 7691), and guinea pig anti-NeuN (1:1000, Merck, ABN90). Secondary antibodies used were: donkey anti-rat AlexaFluor 647 (1:200, Jackson ImmunoResearch), donkey anti-mouse AlexaFluor 488 (1:400, Jackson

Immunoresearch), donkey anti-rabbit AlexaFluor 594 (1:400, Jackson Immunoresearch), and donkey anti-guinea pig Dylight 405 (1:200, Jackson Immunoresearch).

Tissue sections were mounted on glass slides, cover-slipped using Dako Fluorescence Mounting Medium (Dako, North America Inc., CA, USA), and edges sealed with nail polish. Sections were stored at -20°C. As shown in Figure 1, this method resulted in successful co-labelling of the three interneuron markers; SST (aqua), PV (green), CAL (red), as well as NeuN (blue) (Figure 1). Negative controls included omitting each primary antibody while all secondary antibodies were included (Supplementary Figure 1B-E).

Stereological Cell Quantification and Image Acquisition and Analysis

Stacks of images spanning 20 µm in the z-plane were taken in the hippocampal areas to be analysed. DHP samples related to bregma coordinates -1.42, - 1.82 and -2.18mm, whereas VHP samples related to bregma coordinates -2.54mm, -2.80mm and -3.16mm. A Nikon C1 confocal microscope with an Andor Zyla 4.2 sCMOS camera was used to capture images at 20x magnification and 3µm step z-stacks. A maximum projection image was created from each stack using the Nikon C1 confocal software. Expression of markers and area measurements were determined using the FIJI ImageJ 1.52g software. The experimenter was blinded to mice numbers when counting and determined brain region boundaries based upon NeuN stain. To determine marker expression, a macro was designed for efficient and objective counting. The macro created an individual mask for the 488-PV channel, 647-SST channel and 594-CAL channel. Each of these masks was applied to the 405-NeuN channel to identify co-expression of NeuN with each marker, confirming that the observed immunofluorescence was a neuronal cell. The macro then automatically counted particles between 50-300 microns.

Statistical analysis

All data are presented as mean \pm the standard error of the mean (SEM). Outliers were identified using the ROUT test with Q=1% (GraphPad PRISM software) and removed if sections were identified to be of poor quality (e.g. torn or damaged section precluding appropriate cell density counts). D'Agostino-Pearson tests were performed to confirm data are normally distributed. Cell density was firstly assessed across the whole dorsal or ventral hippocampus. Groups were compared by univariate ANOVA with the independent factors being sex (male or female), genotype (hBDNF^{Val/Val} or hBDNF^{Met/Met}) and treatment (CORT or water), using SPSS Statistics 24 software. To assess the effects in different hippocampus subregions (CA1, CA2, CA3, DG), a repeated measures ANOVA was used. If region interacted with genotype, sex or treatment, data were split accordingly and analysed by two-way ANOVAs using GraphPad PRISM software with Sidak's multiple comparison where justified. Group differences were considered significant when $p < 0.05$.

Results

Dorsal hippocampus

Examining the influence of val/met genotype and CORT treatment on interneuron density in the whole DHP

Analysis of SST cell density in whole DHP showed a significant main effect of genotype ($F(1,37) = 4.46$, $P = 0.043$) with hBDNF^{Met/Met} mice showing reduced cell density compared to hBDNF^{Val/Val} (Figure 2A). There was also a significant main effect of sex ($F(1,37) = 4.73$, $P = 0.038$) with females showing higher SST cell density than males (Figure 2A). No significant effect of CORT treatment on SST cell density was found and there were no significant interactions. No significant effects or interactions were found for PV (Figure 2B) or CAL (Figure 2C) cell density in the whole DHP. One animal was considered an outlier in the female met/met + saline group. Figure 2D-G shows example staining from male and female Val/Val and Met/Met water groups.

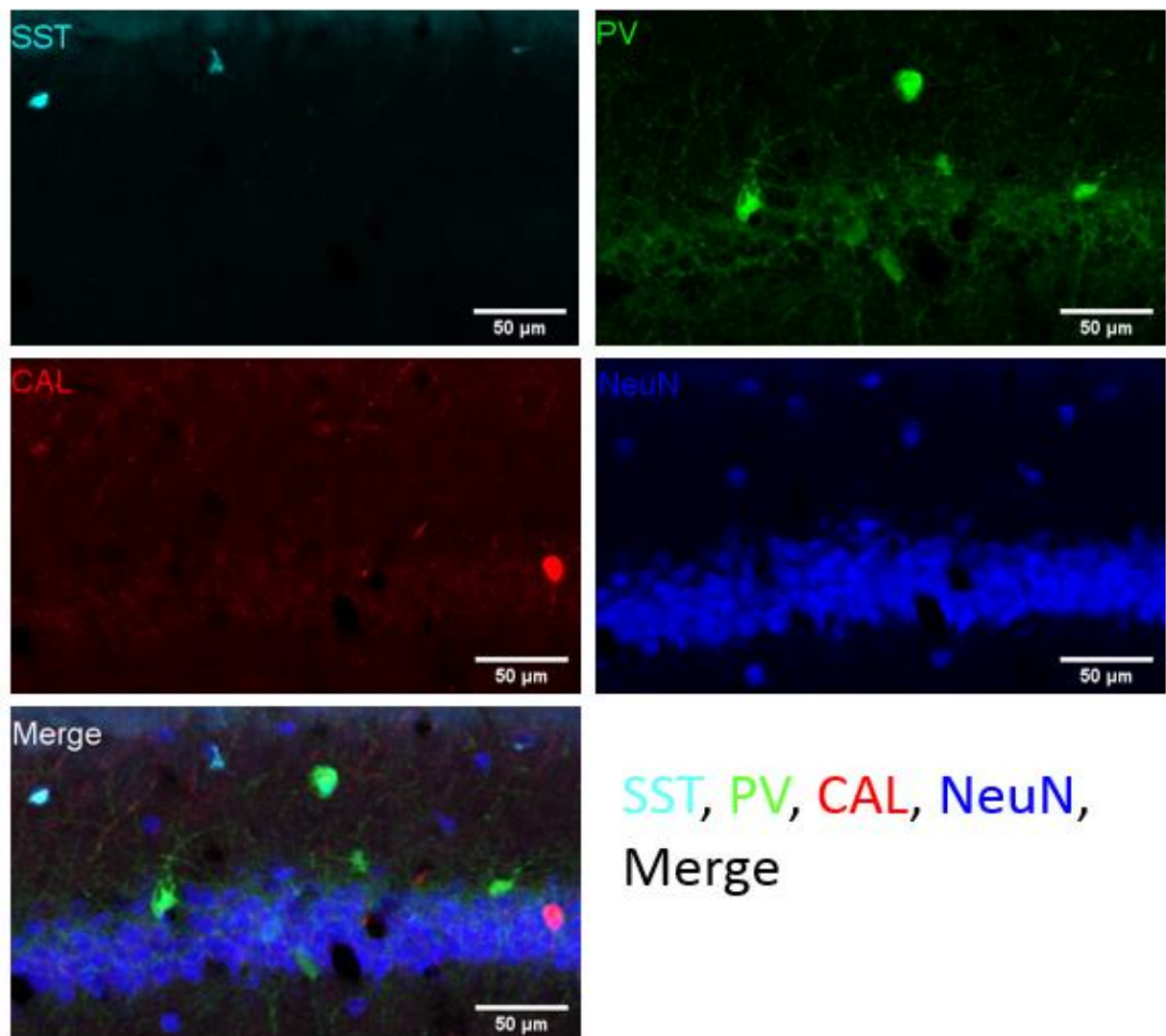


Figure 1. Example images of 4 x fluorescent immunohistochemistry. Panel (A) shows section stained with SST. Panel (B) shows staining with PV. Panel (C) shows staining with CAL. Panel (D) shows staining with NeuN. Panel (E) shows merged image with all 4 markers.

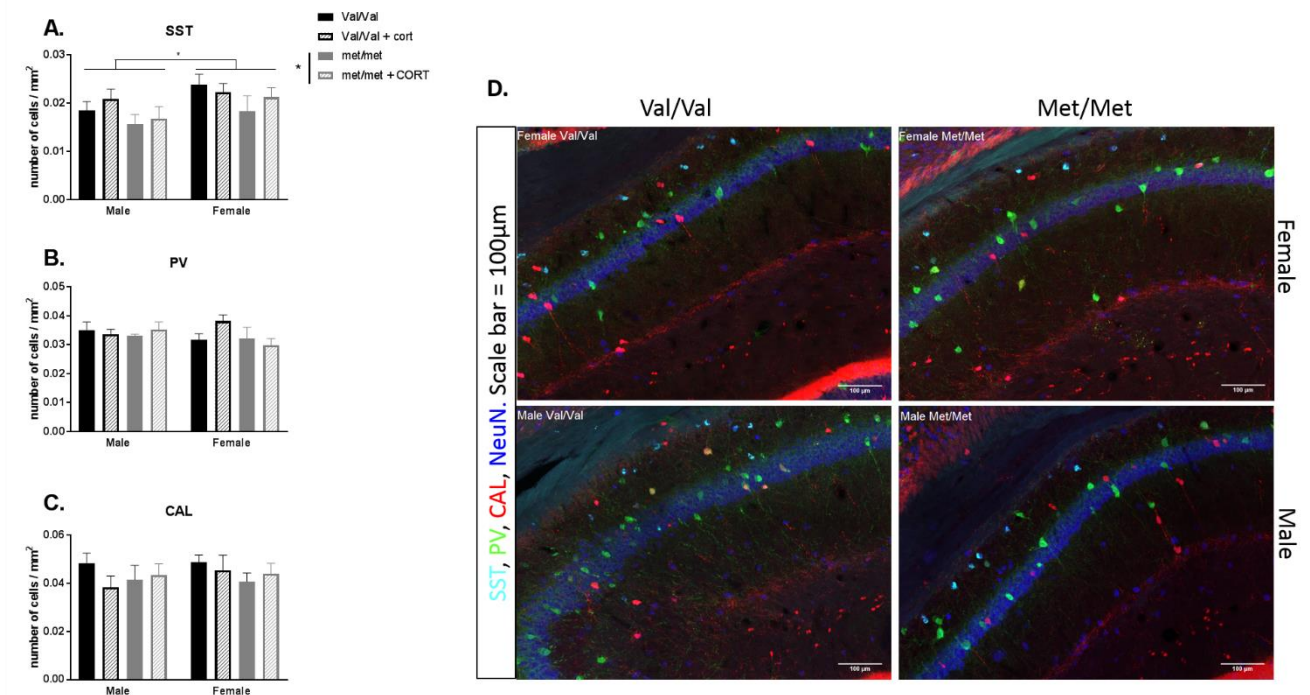


Figure 2. Average interneuron cell density across the whole DHP in male and female $hBDNF^{Val/Val}$ and $hBDNF^{Met/Met}$ mice that were either control or CORT-treated. **A)** SST cell density is significantly lower in $hBDNF^{Met/Met}$ compared to $hBDNF^{Val/Val}$ mice and higher in females than in males. **B)** PV cell density is unchanged by genotype, treatment or sex, **C)** CAL cell density is unchanged by genotype, treatment or sex. * next to met/met legend indicates significant main effect of genotype ($p < 0.05$), * over lines indicates significant main effect of sex ($p < 0.05$). $N = 4-5/\text{group}$. Panel **D)** show representative images of 3 interneurons + NeuN stain in DHP of male and female Val/Val and Met/Met mice, scale bar = 100μm.

Examining the influence of val/met genotype and CORT treatment on interneuron density in subregions of the DHP

Analysis of the effect of genotype, treatment and sex on SST, PV and CAL cell density within subregions of the DHP showed a significant region \times sex \times genotype interaction for SST cell density ($F(9,133) = 3.70$, $P = 0.024$). We then split the data by region to further investigate this

interaction and merged the treatment groups as no effect of treatment and no interaction with treatment was found here. In the CA1 we found a significant effect of genotype ($F(1,33) = 4.59$, $P = 0.039$) and a genotype \times sex interaction ($F(1,33) = 5.33$, $P = 0.027$). Sidak's multiple comparisons tests showed significantly reduced cell density in male $hBDNF^{Met/Met}$ compared to $hBDNF^{Val/Val}$ mice ($P = 0.010$) but no significant genotype effect in females (Figure 3A). No significant effects of genotype or sex were found in the CA2 (Figure 3B). In both the CA1 and CA2 one animal in the male val/val group was considered an outlier. However, in the CA3 there was a significant effect of genotype ($F(1,34) = 7.62$, $P = 0.009$) and of sex ($F(1,34) = 4.31$, $p = 0.045$). $hBDNF^{Met/Met}$ mice had reduced SST cell density compared to $hBDNF^{Val/Val}$, and females showed higher cell density than males (Figure 3C) No significant genotype \times sex interaction was found in the CA3. In the DG there was a significant effect of genotype ($F(1,34) = 7.17$, $P = 0.011$) and sex ($F(1,34) = 4.79$, $P = 0.035$). Once again, $hBDNF^{Met/Met}$ mice showed reduced SST cell density compared to $hBDNF^{Val/Val}$, and females had overall higher cell density than males (Figure 3D). No significant sex \times genotype interactions were found in the DG. Figure 3E shows representative images of SST staining in the CA3 of male and female $hBDNF^{Val/Val}$ and $hBDNF^{Met/Met}$ mice, with Met/Met mice showing reduced SST cell density.

No significant interactions between region, Val66Met genotype, CORT treatment or sex were found for PV and CAL interneuron density in the DHP (data not shown).

Ventral Hippocampus

Examining the influence of val/met genotype and CORT treatment on interneuron density in the whole VHP

There were no significant main effects of genotype, sex or treatment on SST cell density in the whole VHP, however there was a significant sex \times genotype \times treatment interaction ($F(1,28) = 4.59$, $P = 0.041$; Figure 4A). While post-hoc analysis found no significant changes, this interaction appears to be driven by the fact that male CORT-treated $hBDNF^{Met/Met}$ mice had the lowest SST cell density. No significant effects of sex, genotype or treatment and no significant interactions were found for PV and CAL in the whole VHP (Figure 4B and 4C). Figure 4D shows a representative VHP image of all 3 interneurons and NeuN in the male Met/Met + CORT and female Met/Met + CORT. No outliers were identified in the VHP.

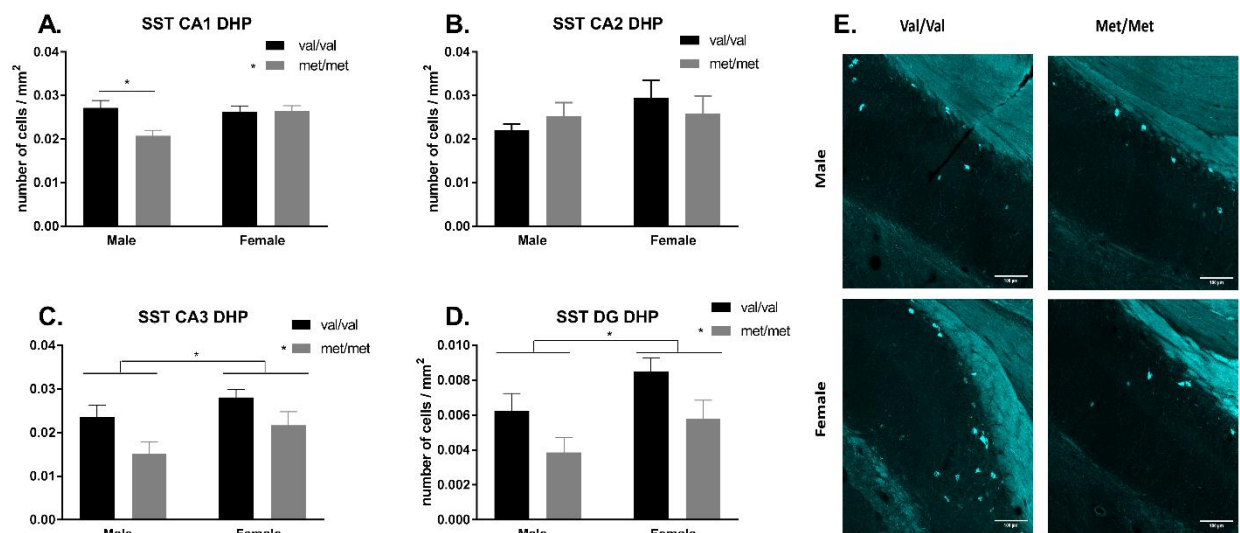


Figure 3. SST cell density in subregions of the DHP in male and female $hBDNF^{Val/Val}$ and $hBDNF^{Met/Met}$. **A)** CA1: male $hBDNF^{Met/Met}$ mice show significantly lower cell density compared to male $hBDNF^{Val/Val}$. **B)** CA2: no effect of genotype or sex was found. **C)** CA3: $hBDNF^{Met/Met}$ mice show significantly lower cell density compared to male $hBDNF^{Val/Val}$ and females show higher cell density than males. **D)** Dentate gyrus: $hBDNF^{Met/Met}$ mice show significantly lower cell density than male $hBDNF^{Val/Val}$ and females show higher cell density than males. **E)** Representative images of SST cell density in male and female Val/Val and Met/Met CA3. * next to grey Met/Met square indicates significant main effect of genotype ($p < 0.05$), * over lines connecting males and

*females indicates significant main effect of sex ($p < 0.05$), * over line connecting male $hBDNF^{Val/Val}$ to male $hBDNF^{Met/Met}$ indicates post-hoc male-specific genotype effect ($p < 0.05$). $N = 4-5$ per group.*

Examining the influence of val/met genotype and CORT treatment on interneuron density in subregions of the DHP

Analysis of the effect of genotype, treatment and sex on SST, PV and CAL cell density within subregions of the VHP showed no significant interactions with Val66Met genotype, CORT treatment or sex for SST, PV or CAL cell density (data not shown).

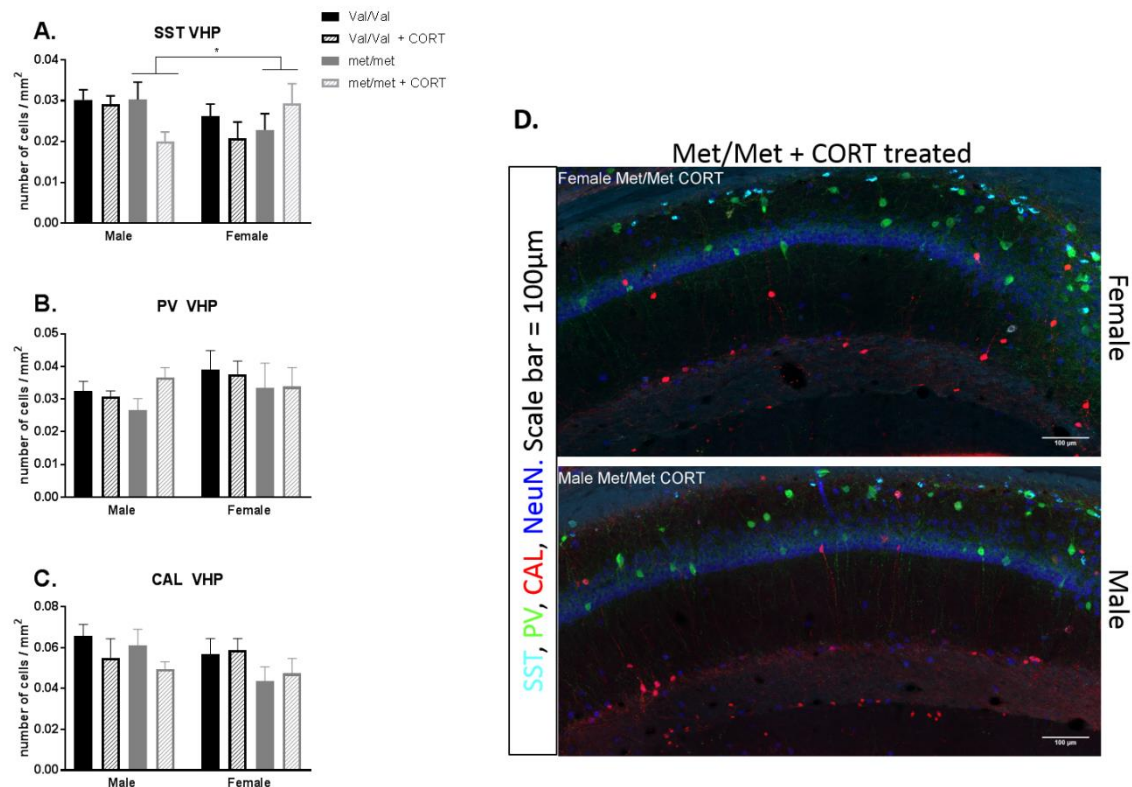


Figure 4. Average interneuron cell density across the whole VHP in male and female $hBDNF^{Val/Val}$ and $hBDNF^{Met/Met}$ mice that were control or CORT-treated. **A)** SST cell density is lowest in male $hBDNF^{Met/Met}$ mice treated with CORT, **B)** PV cell density is unchanged by genotype, treatment or sex, **C)** CAL cell density is unchanged by genotype, treatment or sex. * over lines indicates significant sex \times genotype \times treatment interaction. $N = 4-5/\text{group}$. **D)** Representative image of 3 interneurons + NeuN stain in VHP of female and male Met/Met + CORT mice. Scale bar = 100µm.

Discussion

This study investigated the effects of Val66Met genotype, sex, and a history of adolescent stress hormone exposure on inhibitory interneuron density in the dorsal and ventral hippocampus. Our

main findings were that in most subregions of the DHP, but not the VHP, hBDNF^{Met/Met} mice showed reduced SST cell density, while females had a higher SST cell density than males. These effects were selective for SST as, in contrast, neither Val66Met genotype nor sex of the animals had any effect on PV and CAL cell density. There were also no effects of prior CORT exposure on SST, PV or CAL cell density in the DHP.

We have previously shown deficits in spatial memory (Y-maze) and context and cue-dependent fear memory in hBDNF^{Met/Met} compared to hBDNF^{Val/Val} mice, behaviours which are strongly reliant on intact dorsal hippocampus functioning (Notaras et al., 2016b). SST, but not PV interneurons, have been shown to be an important part of CA1 inhibitory circuits that govern contextual fear conditioning (Lovett-Barron et al., 2014). Optogenetic inhibition of SST, but not PV interneurons, disrupted spatial encoding and reduced hippocampal-prefrontal synchrony (Abbas et al., 2018b). Optogenetic inhibition of SST, but not PV interneurons, disrupted spatial encoding and reduced hippocampal-prefrontal synchrony (Abbas et al., 2018b). SST interneurons in the dentate gyrus have also been reported to play an essential role in the formation of granule cell assemblies during memory acquisition, particularly in the context of spatial information (Yuan et al., 2017). Our study suggests that spatial and context-dependent memory deficits in hBDNF^{Met/Met} mice may be due to the reduction in SST cell density in the CA1, CA3 and DG of the DHP.

In the CA1, we found a significant genotype x sex interaction where only male hBDNF^{Met/Met} mice showed reduced SST cell density, while females did not. However, when comparing to behavioural results in separate cohorts of this model, this (Notaras et al., 2017a; Notaras et al., 2016b; Notaras et al., 2017c). Previous studies have reported a neuroprotective role for estradiol on SST-positive neurons of the hippocampus (Azcoitia et al., 1998), which may explain why females were not affected by genotype in our study. However, this sex-specific effect was only

observed in the CA1. Both estrogen receptor α and β are expressed in the CA1 - albeit only on fibers for ER β - however, they are both also expressed within the DG and CA3 in mice (Mitra et al., 2003). Future studies should assess whether estrogen receptors co-localize with SST interneurons and whether regional and sex differences in this co-localization occur.

BDNF has previously been shown to be important in regulating SST gene expression in the prefrontal cortex (Glorioso et al., 2006a) and BDNF treatment in striatal (Mizuno et al., 1994) and hippocampal cultures (Marty and Onteniente, 1999) increases SST levels and the number of SST-immunoreactive neurons, respectively. Recently we reported that both male and female BDNF heterozygous mice show decreased mPFC SST density at 12 weeks compared to 4 weeks, indicating developmental interactions with BDNF genotype in the PFC (Du et al., 2018). These findings support the notion that BDNF is important for the development of normal SST cell density across forebrain regions.

Previous studies using BDNF knockout (BDNF $^{-/-}$) mice found markedly reduced immunoreactive staining of several cell markers in the hippocampus and frontal cortex, including neuropeptide-Y, SST and PV, but an increase in the number of CAL-positive cells (Grosse et al., 2005). Such findings are in line with our previous findings of altered density of PV-positive cells in the prefrontal cortex of BDNF heterozygous (BDNF $^{+/-}$) mice (Du et al., 2018). Several other studies have also demonstrated a key role for the BDNF-TrkB signalling in PV interneuron development (Grech et al., 2019a; Hashimoto et al., 2005b; Lucas et al., 2014; Xenos et al., 2017a). (Grech et al., 2019a; Hashimoto et al., 2005b; Lucas et al., 2014; Xenos et al., 2017a). While we expected a more subtle phenotype in the hBDNF^{Val66Met} model, it was surprising that there was *no* effect of genotype on PV interneuron density. Future studies should extend the present study to the effects of the hBDNF^{Val66Met} genotype on inhibitory interneurons in the prefrontal cortex to determine whether there is a more profound effect of reduced BDNF secretion there.

While previous studies have reported that chronic stress alters hippocampal interneuron density (Czeh et al., 2015) in rodents, our model of chronic CORT treatment during adolescence showed no effects on PV, SST or CAL cell density in the DHP. It is important to note that the chronic stress model by (Czeh et al., 2015) subjected rats to 9 weeks of daily stress including intermittent illumination, stroboscopic light, soiled cage and 45° cage tilting while our model was 3 weeks of CORT exposure during adolescence/early adulthood. Circulating CORT was not measured in this study, however previous studies by our laboratory have found that at a CORT concentration of 25mg/L, mice will ingest a dosage of 6-8mg/kg/day (Klug et al., 2012). Comparatively at 50 mg/L concentration this has been found to be a dosage between 10 and 20 mg/kg/day (Grech et al., 2018b). Our laboratory has also investigated faecal boli for CORT levels, reporting that at a concentration of 50 mg/L, this results in CORT levels of ~600 pg/mL for males and ~1500 pg/mL for females (Schroeder et al., 2018). Other stress paradigms report varied CORT levels, with repeated social stress reported to results in ~300 ng/mL CORT levels (a three-fold increase) (Hanke et al., 2012) and chronic unpredictable stress administered for 8 weeks results in 150 ng/mL CORT levels (Monteiro et al., 2015).

In the VHP we did find a significant sex \times genotype \times treatment interaction for SST density, which appeared to be driven by male hBDNF^{Met/Met} + CORT exposed mice showing the lowest density. Once again, this male-specific vulnerability may be attributed to neuroprotection afforded to females by higher circulating levels of estradiol. Indeed, estradiol and BDNF downstream signalling pathways converge, suggesting one may compensate for the other (Hill et al., 2012a). Once again, this subtle sex-specific effect does not appear to correspond to any sex-specific behavioural phenotypes shown in this model so far. Similarly the study reported by (Czeh et al., 2015) reported that most behavioural phenotypes did not align with the effects of stress on interneuron density, however, they do note that SST (as well as NPY and CAL) density was reduced in anhedonic rats. Previously, male BDNF^{+/-met} mice exposed to 7 days of restraint stress

exhibited a selective induction of anhedonia in the sucrose preference test (Yu et al., 2012b). Given the role of SST and VHP in affective-related behaviour, it would therefore be interesting to now extend this work by comparing phenotypes between male and female hBDNF^{met/met} mice to tease apart sex-specificity in the induction of anhedonia by stress and the role that SST may play in the VHP to mediate this.

A limitation of this study is that we did not stain for glutamate decarboxylase (GAD), the enzyme that synthesizes GABA (Pinal and Tobin, 1998a) and is generally used as marker for the inhibitory interneuron system (Engel et al., 2001b). (Engel et al., 2001b). We were limited by how many channels could be imaged by the confocal equipment and chose to use NeuN as a cell-body marker. However, the literature indicates that the majority of hippocampal interneurons expressing our chosen markers are inhibitory (DeFelipe et al., 2013; Freund and Buzsáki, 1996; Gonchar and Burkhalter, 1997). Moreover, PV has been found to be limited to GABA-expressing interneurons in the rat cerebral cortex (Celio, 1986) and hippocampus (Aika et al., 1994; Kosaka et al., 1987; Zaletel et al., 2016), and in the majority of mouse hippocampal interneurons (Fukuda et al., 1997). It has been suggested that the proportion of GABAergic PV interneurons is similar between mouse and rat (Jinno et al., 1998). Similarly, SST has been found to be limited to inhibitory interneurons in the rat (Esclapez and Houser, 1995; Köhler and Chan-Palay, 1982; Kubota et al., 1994; Morrison et al., 1982), with 91% of SST-positive cells in the rat hippocampus reported to co-express with GAD (Kosaka et al., 1988). Additionally, the majority of CAL-positive cells have been found to co-express with GABA in rats (Gonchar and Burkhalter, 1997) and have characteristic properties of GABAergic interneurons (Gulyás et al., 1992). Therefore, it can be assumed that the PV-, SST- and CAL-positive neurons in our study represent GABAergic interneurons. Another limitation is the small n used for this study and interpretation of results must be done carefully. Future work should investigate markers of stress in the hippocampus to

determine how stressed the brains of these mice were, including glucocorticoid receptor and FKBP5 expression (Adzic et al., 2019).

In conclusion, our results show that hBDNF^{Val/Met} genotype is a critical modifier of SST cell density, but importantly, does not appear to impact on PV or CAL cell density. Furthermore, chronic CORT exposure from 6-8 weeks of age had no effect of interneuron cell density in the DHP in adulthood and only a subtle effect on SST density in the VHP of male hBDNF^{Met/Met} mice, suggesting the combination of being male and a Met/Met carrier increases vulnerability to chronic stress hormone exposure. This could be contributing to the earlier onset of schizophrenia observed in males compared to females (Markham, 2012; Ochoa et al., 2012) and the association between depression and Met allele, specific to males (Verhagen et al., 2010). The hBDNF^{Val66Met} genotype has been implicated as a risk factor in a broad range of psychiatric disorders including bipolar disorder (Mandolini et al., 2019), PTSD (Mühlberger et al., 2014), susceptibility to anxiety (Notaras et al., 2015a) and is as a modifier of psychiatric phenotype (Chao et al., 2008b). Our data shed new light on the functional relevance of this common polymorphism, and the mechanism by which it may alter behaviours related to the above psychiatric disorders.

Author Contributions and Acknowledgments

AMG performed the immunohistochemistry and confocal microscopy studies and initial data analysis and wrote the first draft of the paper. MJN performed all *in vivo* experiments, including mouse CORT treatments, and perfuse-fixations and edited the manuscript. MS sectioned and pre-treated the brains. MvdB and RH funded and co-designed the project and edited the manuscript. The authors wish to acknowledge the help of Dr. Shane Cheung, Monash Micro Imaging – MHTP, The Hudson Institute of Medical Research, for development of the macro used for immunohistochemistry analysis. The authors would like to acknowledge One in Five

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5.3. Supplementary Material Results

5.3.1. The percentage of calretinin-positive cells that are double positive with somatostatin

5.3.1.1. CA1

Figure 5.1A shows that for DHP CA1 there was a sex \times genotype \times treatment interaction approaching significance ($F(1, 30) = 3.70$, $p = 0.064$). This suggested that CORT increased percentage co-expression of calretinin-positive (CAL+) cells with somatostatin (SST) in male Val/Val mice but decreased in female Val/Val mice. Figure 5.1B shows there was no significant effects for VHP CA1.

5.3.1.2. CA2

For DHP CA2 there was a significant main effect of genotype ($F(1, 30) = 5.24$, $p = 0.029$). Here, Met/Met groups had a lower percentage co-expression in CAL+ cells (Figure 5.1C). For VHP CA2 there was a significant sex \times genotype \times treatment interaction $F(1, 22) = 8.9$, $p = 0.007$). Sidak post hoc comparisons found that male Met/Met control had a significantly higher co-expression in CAL+ cells compared to female Val/Val CORT groups ($p = 0.044$) (Figure 5.1D). There was a marked difference in percentage co-expression; male Met/Met control had 20% co-expression while female Val/Val CORT had 0% co-expression. Overall, here it appears that CORT decreased percentage co-expression for CAL+ cells for male Met/Met mice but female Met/Met were unchanged. Concurrently, CORT increased percentage co-expression for CAL+ cells for male Met/Met mice but there was no percentage co-expression for female Met/Met mice.

5.3.1.3. CA3

There were no significant effects of sex, genotype or treatment on percentage co-expression for CAL+ cells in the CA3 (Figure 5.1E and Figure 5.1F) or total hippocampus (Figure 5.3A and Figure 5.3C).

5.3.1.4. DG

For DHP DG, there was a close to significant effect of sex ($F(1, 30) = 3.74$, $p = 0.063$), which suggested that females had a higher co-expression percentage for CAL+ cells than males (Figure 5.1G). There were no significant effects for VHP DG (Figure 5.1H).

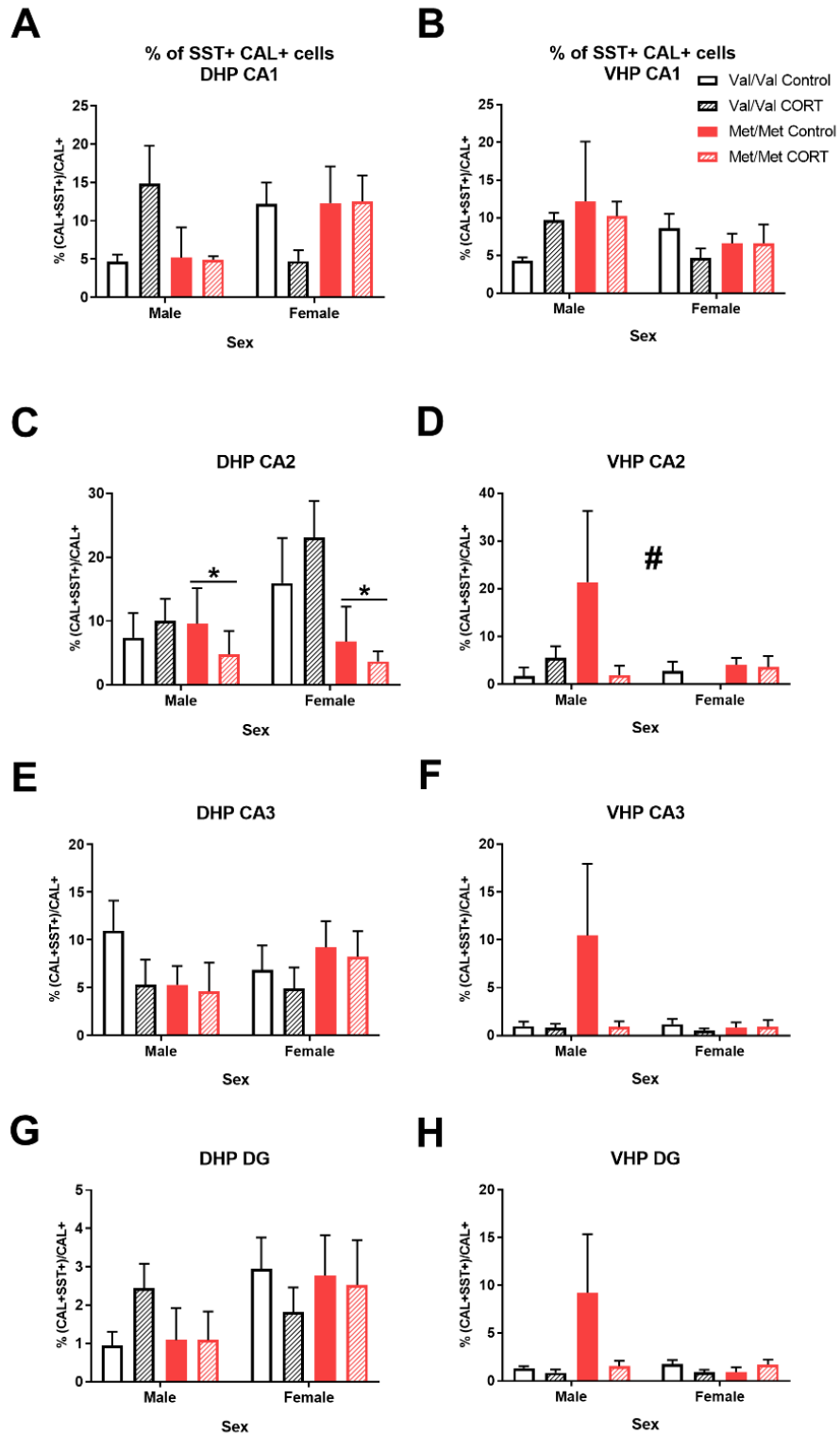


Figure 5.1 - The percentage of calretinin-positive cells that are double positive with somatostatin

*Panel A and B show no significant effects for co-expression percentage. Panel C shows a main effect of genotype for co-expression percentage. Panel D shows a significant sex × genotype × treatment interaction for co-expression percentage. Panel E, F, G and H show no significant effects for co-expression percentage. Data are mean ± SEM, n = 3-5, # p < 0.05, * p < 0.05.*

5.3.1.5. Hippocampal region comparison

For DHP there was a significant region × sex × genotype interaction ($F(3, 87) = 4.70$, $p = 0.005$), with significant differences emerging between the DG and CA1 ($p < 0.0001$), CA2 ($p < 0.0001$) and CA3 ($p < 0.0001$). Here, Met/Met genotype had significantly lower co-expression in the CA2 but not in other hippocampal subregions. Notably, the DG had the lowest co-expression amongst all subregions. Val/Val genotype appears to interact with sex and CORT in the CA1 and DG, but not in the CA2 and CA3. For VHP there was a significant region × genotype interaction ($F(3, 39) = 3.3$, $p = 0.03$), with significant differences emerging between the CA2 and CA1 ($p < 0.0001$) and DG ($p < 0.0001$). In the VHP, the Met/Met male group appears to have the largest co-expression by far, except for in the CA1.

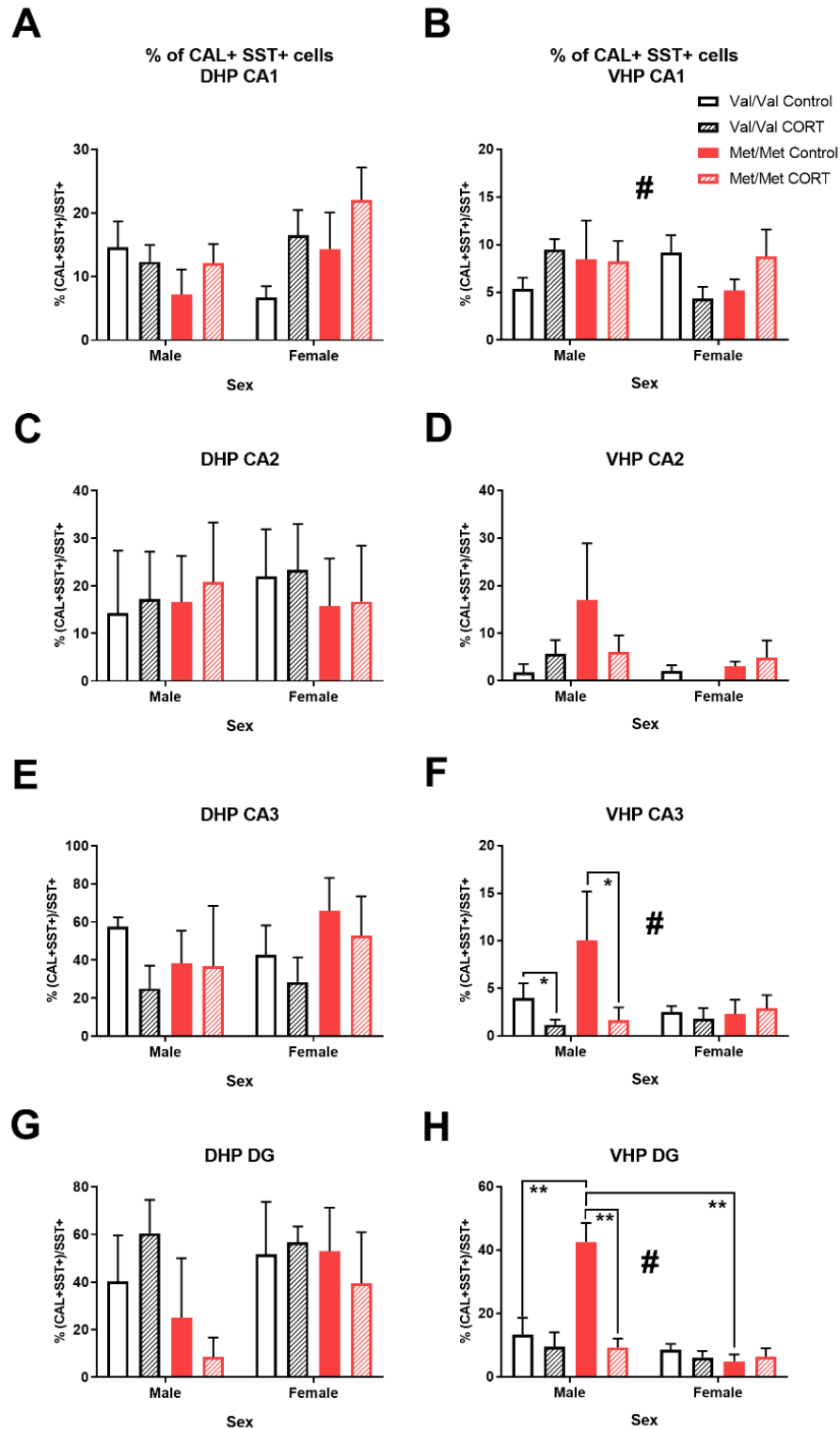


Figure 5.2 - The percentage of somatostatin-positive cells that are double positive with calretinin

*Panel A shows no significant effects for co-expression percentage. Panel B shows a significant sex × genotype × treatment interaction for co-expression of CAL in SST+ cells. Panel C, D and E show no significant effects for co-expression percentage. Panel F shows a significant sex × treatment interaction and significant post hoc comparisons. Panel G shows no significant effects for co-expression percentage. Panel H shows a significant sex × genotype × treatment interaction and significant post hoc comparisons. Data are mean + SEM, n = 3-5, interaction denoted by # $p < 0.005$, Sidak post hoc comparisons * $p < 0.05$, ** $p < 0.001$.*

5.3.2. Percentage of somatostatin-positive cells that are double positive with calretinin

5.3.2.1. CA1

Figure 5.3A shows no significant effects for percentage co-expression in DHP CA1 for SST+ cells. For VHP CA1 (Figure 5.3B) there was a significant sex × genotype × treatment interaction ($F(1, 27) = 4.83, p = 0.037$), with no significant post hoc comparisons. Here, it appears that CORT increased percentage co-expression for SST+ cells in male Val/Val but decreased for female Val/Val. Concurrently, CORT increased percentage co-expression for SST+ cells in female Met/Met but male Met/Met were unchanged.

5.3.2.2. CA2

Figure 5.3C shows no significant effects for percentage co-expression in DHP CA2 for SST+ cells. For VHP CA2 (Figure 5.3D) there was a close to significant effect of genotype ($F(1, 24) = 4.12, p = 0.054$), which suggested that Met/Met mice had higher co-expression percentage for SST+ cells than Val/Val mice.

5.3.2.3. CA3

Figure 5.3E shows no significant effects for percentage co-expression in DHP CA3 for SST+ cells. For VHP CA3 (Figure 5.3F) there was a significant sex \times treatment interaction ($F(1, 24) = 4.96$, $p = 0.036$). Sidak post hoc comparisons found that male CORT groups had significantly higher percentage co-expression for SST+ cells than male control groups ($p = 0.023$). There were no differences found between female groups.

5.3.2.4. DG

Figure 5.3G shows no significant effects for percentage co-expression in DHP DG for SST+ cells. For VHP DG (Figure 5.3H) there was a sex \times genotype \times treatment interaction ($F(1, 26) = 11.16$, $p = 0.003$). Sidak post hoc comparisons found that percentage co-expression for SST+ cells was significantly higher in the Met/Met control group compared to male Val/Val control ($p < 0.001$) and female Met/Met control ($p < 0.001$). There were no differences found between female groups.

5.3.2.5. Total Hippocampus

For total DHP (Figure 5.5B) there was a significant effect of sex ($F(1, 29) = 5.62$, $p = 0.025$), here females had a higher co-expression percentage for SST+ cells. When sex was analysed separately males had a close to significant genotype effect ($F(1, 13) = 4.67$, $p = 0.05$), suggesting that male Met/Met mice had lower co-expression percentage for SST+ cells compared to male Val/Val. There were no significant effects for female mice. There were no significant effects for co-expression percentage for SST+ cells in total VHP (Figure 5.5D).

5.3.2.6. Hippocampal region comparison

For DHP there was a significant effect of region ($F(3, 57) = 11.14$, $p < 0.001$), CA1 appeared to have the lowest overall percentage co-expression for SST+ cells (Figure 5.3A, C, E and G). For

VHP there was a significant region \times sex \times treatment interaction ($F(3, 57) = 7.81, p < 0.001$). It seems that within each hippocampal region CORT will have differential effects dependent on sex. Notably, in VHP CA3 and DG CORT decreased percentage co-expression in SST+ cells for males but females generally were unchanged by CORT. In the VHP there was also a significant region \times sex \times genotype interaction ($F(3, 57) = 5.50, p = 0.002$). It seems that within each hippocampal region, genotype will have differential effects dependent on sex. Specifically, male genotypes did not differ in VHP CA1 but do across the other hippocampal regions. Comparatively, female genotypes only differed in VHP CA1 and had similar co-expression percentages across the other hippocampal regions.

SST- and CAL-positive cells did not co-label with PV-positive cells.

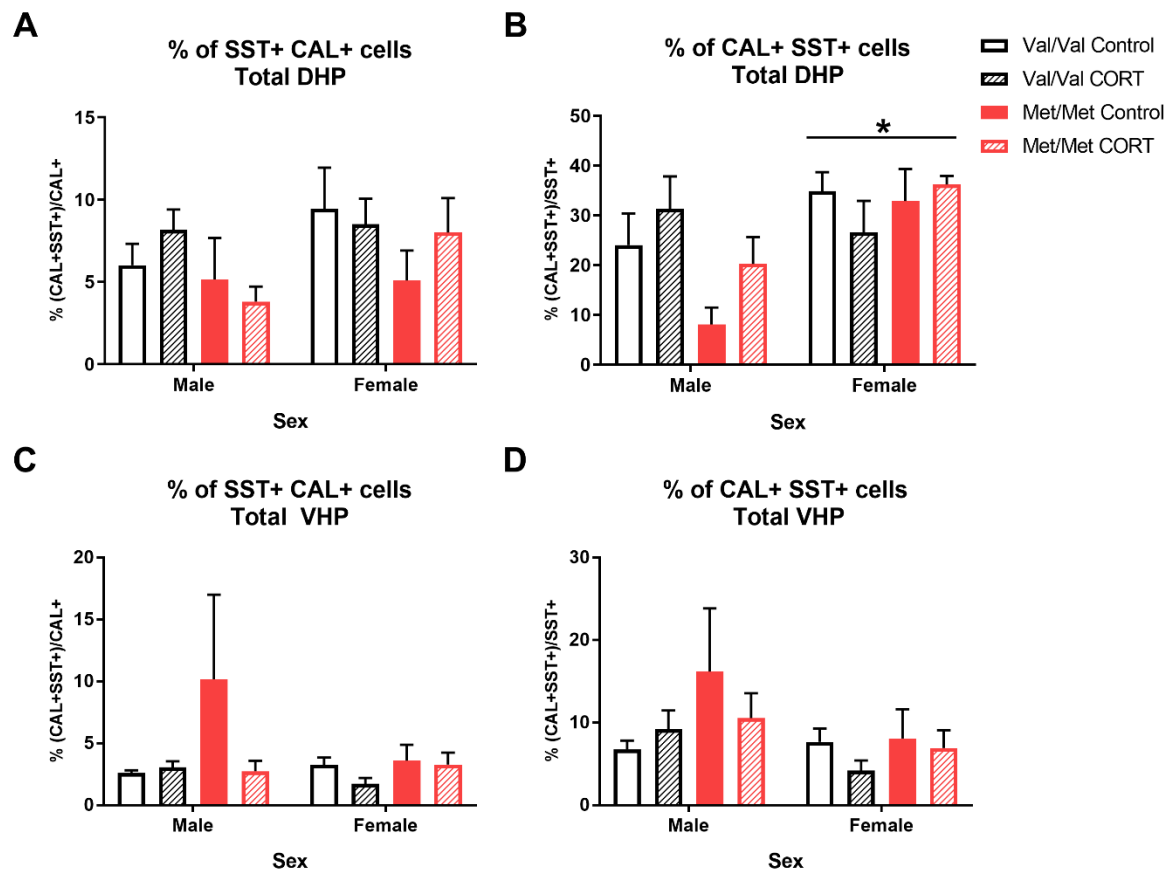


Figure 5.3 - The total percentage double positive cells in dorsal and ventral hippocampus

*Panels A shows no significant effects for co-expression percentage. Panel B shows a significant sex effect for co-expression percentage. Panel C and D show no significant effects of co-expression percentage. Data are mean \pm SEM, $n = 3-5$, * $p < 0.05$.*

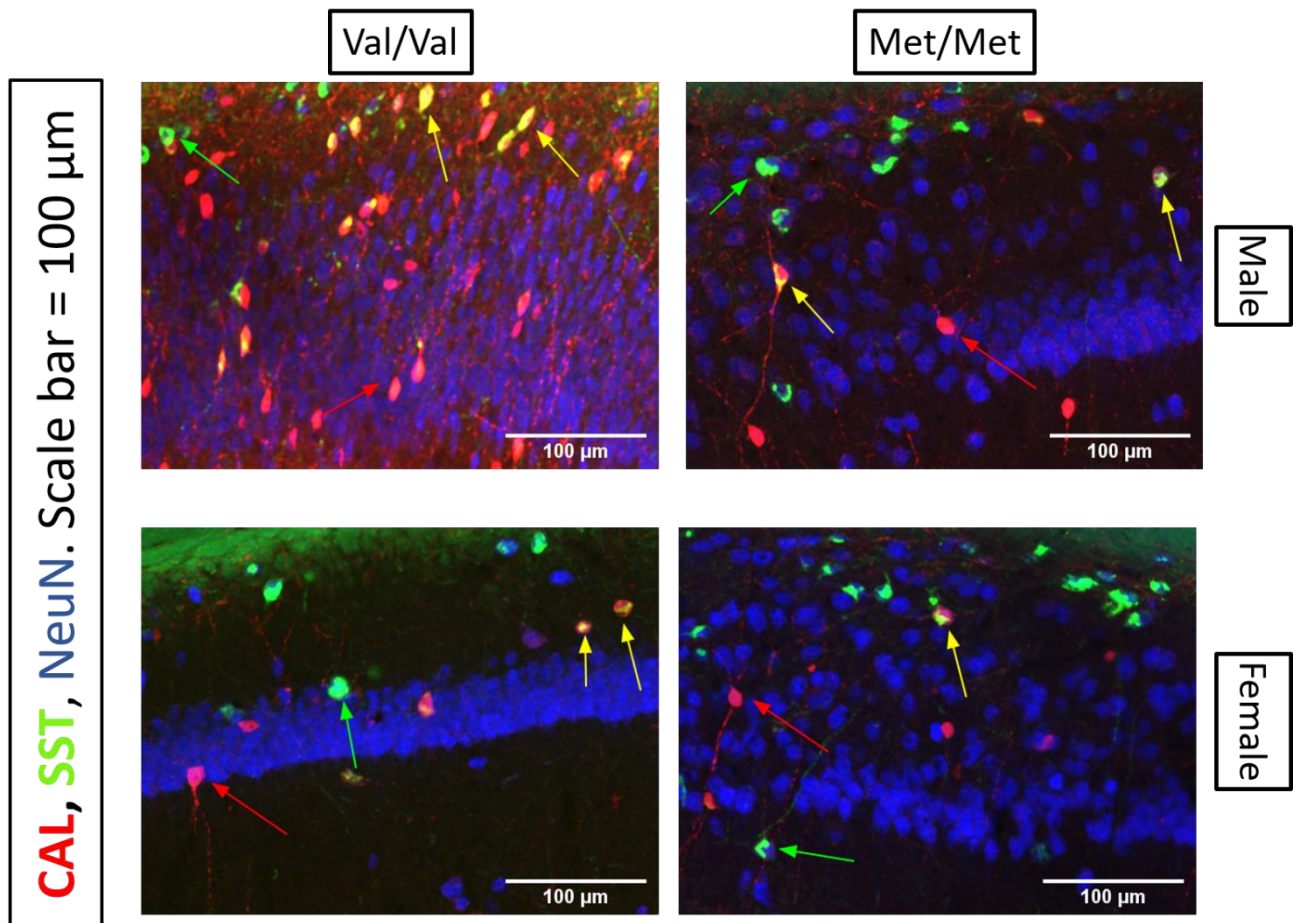


Figure 5.4 - Example staining of co-expression of calretinin and somatostatin

Example staining of co-expression of calretinin and somatostatin. Yellow arrows point to calretinin and somatostatin positive cells. Red arrows point to calretinin-positive cells. Green arrows point to somatostatin-positive cells.

5.4. Supplementary Material Discussion

This novel study investigated percentage co-expression of SST and CAL in the hippocampus of hBDNF^{Val66Met}, to characterise these mice in context of literature that indicates these inhibitory markers co-express. The main finding was that sex and genotype interacted across hippocampal subregions for percentage co-expression of SST and CAL.

Sex and genotype interact for co-expression percentage of SST and CAL

Mouse cerebral cortex studies have found that CAL and SST can co-express (Gonchar et al., 2008), while others have failed to report co-expression between SST and CAL (Oliva et al., 2000). However, none have reported co-expression data in the hippocampus for the hBDNF^{Val66Met} model. Oliva et al. (Oliva et al., 2000) investigated hippocampal GABAergic interneurons using transgenic mice that were generated to express enhanced green fluorescent protein (EGFP) in subpopulations of GABAergic neurons. EGFP was found to specifically express in hippocampal SST-IN but immunohistochemistry did not find co-expression with CAL (Oliva et al., 2000). Comparatively, in the same mouse model SST and CAL were found to co-express in ~40% of SST-IN in the sensorimotor cortex (Halabisky et al., 2006). Concordantly, Xu et al. (Xu et al., 2006) reported co-expression of CAL and SST in the frontal cortex (FC), Primary Somatosensory Cortex (S1), and Primary Visual Cortex (V1). In the wildtype-like mouse, the total % of SST+ cells that co-expressed CAL was 20.0% in FC, 23.4% for S1 and 43.5% in V1. CAL-positive cells had higher total co-expression % with SST+ cells in the FC and S1 with 41.3% and 29.6% respectively. However in V1 there was lower co-expression at 27.5%. (Xu et al., 2006). CAL-positive cells may have higher co-expression in FC and S1 since they are more likely to co-express with other neuropeptides, compared to other IN (Cauli et al., 2014). Xu et al. (Xu et al., 2006) did not report any sex differences, while we found co-expression percentage was dictated by the

interaction between sex and genotype. The Miyoshi et al. paper (Miyoshi et al., 2007) investigated fate-mapped cortical interneurons grouped by overlapping IN signature. They reported that measurement at P21 after tamoxifen administration at E15.5 ~10% of somatosensory barrel cortex interneurons co-expressed SST and CAL. Our study investigated the dorsal and ventral hippocampi and here we reported that in control hBDNF^{Val66Met} mice between up to ~35% co-expression percentage in SST+ cells in total DHP and VHP, and up to ~10% co-expression percentage in CAL+ cells (see Figure 5.4 for example co-expression). This is in the range of the Xu et al. (Xu et al., 2006) and Miyoshi et al. papers (Miyoshi et al., 2007). The discrepancy between studies is most likely due to the different brain regions investigated, animal models and/or methods used.

Notably, genotype did not influence the percentage of co-expression of CAL+ and SST+ cells alone, rather it generally interacted with sex. This was surprising, since BDNF-TrkB signaling plays a key role in the development and maintenance of IN. Estradiol has been identified as a key regulatory factor in expression of both SST (Panda et al., 2018) and CAL (Fan et al., 2006) in the hippocampus. This suggests that co-expression of these factors may be influenced by sex. These markers would not necessarily be changed in parallel, as they may be regulated through different mechanisms. In particular, CAL expression was reduced by absence of estrogen receptor β (Fan et al., 2006), while deprivation of estrogen led to an increase of SST-IN in preterm babies (Panda et al., 2018). Importantly, estradiol converges and synergises with the BDNF-TrkB signaling (Scharfman and MacLusky, 2005) and this may contribute to the sex \times genotype interactions. Additionally, Chapter 3 suggested that female sex may buffer against stressors and a similar estrogen-protective action may be occurring here. Female sex has specifically been found to buffer against CORT in a similar model (Klug et al., 2012). CAL was chosen as a control due to its

weaker relationship with the BDNF-TrkB signaling pathway, but these results indicate it may not have been the ideal control, since it was also affected by sex. A better control would be something independent of both BDNF-TrkB signaling and sex influences, however this would be hard to identify since both these factors are ubiquitous in their influence across brain structure and function. A limitation of this study is the small n value, and this should be a consideration for analysis of results. Ultimately, the interaction between genotype and sex across cell types and hippocampal regions illustrates a potential modulatory role sex plays in the density of these IN.

5.5. Summary and prelude to chapter 6

The major result from the submitted manuscript was that that hBDNF^{Val/Met} genotype is a critical modifier of SST cell density, but importantly, does not appear to impact on PV or CAL cell density. This was surprising since PV and TrkB co-express more so than SST and TrkB, implying a stronger functional relationship between PV and TrkB. This may suggest that the model for Chapter 4 should have been a knockdown of TrkB on SST interneurons. However, with approximately 50% SST-IN expressing the TrkB receptor compared to 80% PV-IN (Gorba and Wahle, 1999) it is quite surprising that SST-IN density was affected. This is a constitutive mutation and potentially BDNF is especially important for SST-IN development, while other TrkB agonists compensated for BDNF for development of PV-IN.

Interneurons can be differentiated through a range of criteria, one being the calcium binding protein or neuropeptide they express. This is complicated by the fact that IN can co-express multiple neurofactors. In mice, CAL-IN and SST-IN are known to co-express, and here we find that co-expression % is generally defined by an interaction between genotype and sex. Currently, the biological relevance of co-expression of CAL and SST is unknown, but it demonstrates the complexity of the IN system and its subtypes. More research is necessary to elucidate the

functional differences between IN subtypes that do and do not co-express CAL and SST. This is important to know in preclinical mental health research when linking structure to function, since this information can inform therapeutic approaches.

Following on from this, chapter 6 will be a discussion of the main findings of this thesis and the new knowledge surrounding the relationship between BDNF-TrkB signaling, INs and the cognitive symptoms of schizophrenia.

Chapter 6. General Discussion

6.1 Introduction

Currently there are no targeted treatments or approaches for cognitive symptoms of schizophrenia and as the underlying neurobiology is not comprehensively understood, this thesis has focused on understanding the functional relevance of some promising potential targets. One such promising lead is that cognitive symptoms are thought to arise from the dysfunction of several key neural systems, including the BDNF-TrkB signaling system (Mohammadi et al., 2018) and the GABAergic inhibitory system (Xu and Wong, 2018). Both the BDNF signaling (Hill et al., 2012b) and the GABAergic inhibitory system show protracted development into adolescence (an important neurodevelopmental period) (Donato et al., 2013; Wu et al., 2014) and BDNF-TrkB signaling plays a role here for the normal development of the inhibitory system throughout brain development (Fiorentino et al., 2009). The hippocampus is known to be dysfunctional in schizophrenia and BDNF and GABAergic pathways play a critical role in the hippocampus in modulating learning and memory. In this thesis, three different mouse models of disrupted BDNF-TrkB signaling have been used to investigate the dynamics between this signaling pathway, IN and cognition. In all three models, significant sex and genotype differences were found for behavioural, molecular, and cellular data.

Firstly, in a two-hit model of BDNF heterozygosity and chronic CORT treatment, sex dictated the molecular and behavioural response to EE. In the second model, a subtle knockdown of TrkB at PV-positive cells had a male-specific genotype effect for spatial cognition. Finally, in the hBDNF^{Val/Met} mouse model both sex and genotype effects emerged, whereby females had higher SST density and Met/Met mice had lower SST density in the hippocampus. By using different mouse models to investigate the BDNF-TrkB signaling pathway at different levels of the

molecular hierarchy, comprehensive support for the involvement of these systems in the pathophysiology of cognitive symptoms of schizophrenia has emerged.

6.2 Female BDNF HET respond to preventative environmental enrichment through restoration of excitatory/inhibitory protein expression

6.2.1 EE protocol provides an additional stress unless previously inoculated

A main finding for cohort 1 was that EE was a statistically significant preventative for CORT-induced spatial memory impairment only for female two-hit mice. Two-hit mice were BDNF HET that CORT treatment via water during adolescence. CORT has previously been established to impair spatial memory specifically in male BDNF HET mice at a dosage of 25 mg/L (Klug et al., 2012). For this study the CORT dosage was increased to 50 mg/L due to a loss of male phenotype after moving institutes. This was attributed to the new institute being much more sterile, with less ambient noise and animals being kept in IVC cages instead of open top. However, this meant females also experienced a CORT-induced spatial memory impairment. It is interesting to note that it took a larger dose of CORT across studies for females to experience a spatial memory deficit, and that subsequently they had a more robust response to preventative treatment. Recent unpublished work presented at the IBNS 28th Annual Meeting, Cairns, Australia by Iremonger et al., that suggested the sexes metabolize CORT differently (Iremonger et al., 2019). At a dosage of 25 µg/ml, CORT failed to inhibit neural excitability in females and this correlated with non-elevated plasma CORT levels (Iremonger et al., 2019). Potentially this increased metabolism of CORT is also occurring in our two-hit female mice, which explains the lack of spatial memory at 25 mg/L (Klug et al., 2012) but at 50 mg/L this is enough to reach the brain and impact female cognition. Other possible variables include that sex steroids are differentially affected post-stress with females more likely to have higher expression of steroids (Sze and Brunton, 2019) and that

the estrous cycle can regulate glucocorticoid levels in females (Buckingham et al., 1978; Kitay, 1963; Nichols and Chevins, 1981). Future studies should radiolabel CORT and assess how much enters the brain in males compared to females.

EE protocols vary in their inclusions of objects, running wheels and frequency of change. Generally, EE has been found to improve a range of cognitive and medical conditions in mice (Garofalo et al., 2015; Novkovic et al., 2015; Yuan et al., 2012). Our EE protocol involved novel objects being changed weekly for 3 weeks. Unexpectedly, in WT animals EE alone was detrimental to spatial memory performance. This was primarily attributed to the absence of a running wheel. EE has been shown in multiple circumstances to improve spatial cognition, but it is likely due to the presence of a running wheel, rather than sensory and cognitive stimulation provided by novel objects (Rogers et al., 2016). This is likely due in part to the direct increase of BDNF induced by exercise (Szuhany et al., 2015). A running wheel was not used in this study to strictly investigate possible benefits of a novel environment, rather than exercise. There is evidence of benefits of novel objects on cognition in the literature (He et al., 2017; Zeleznikow-Johnston et al., 2017). There is a lot of discrepancy in approach, e.g. He et al. (He et al., 2017) had a longer protocol (4 weeks versus 3 weeks) and changed objects more often (every 3 days versus once per week) (He et al., 2017). Comparatively, while Zeleznikow-Johnston et al. only changed objects once per week, their protocol ran for 20 weeks (Zeleznikow-Johnston et al., 2017). The difference in benefits between exercise and novel objects is likely attributable to how much BDNF is secreted in an activity-dependent manner and period of exposure. Cognitive benefits were observed when exposure to novel objects occurred over a longer period of time (Zeleznikow-Johnston et al., 2017). Importantly, our EE protocol timeline ran for 3 weeks during adolescence, with **mice then moved back to standard housing for 1 week before undergoing**

behavioural testing (Figure 2.1). Here, having experienced EE and then returning to standard housing seems to have been detrimental. If so, the stress experience of female two-hit mice appeared to inoculate them against this “third hit”. That females presented with a greater resilience, and indeed positive response to EE, indicates a potential contribution by female sex. To molecularly determine how female sex may be neuroprotective, Western Blot experiments were performed to analyse the protein expression of key markers. The results of these experiments are discussed below.

6.2.2 Molecular profiles

6.2.2.1 Excitatory markers – GluN subunit expression profile

The molecular foundations of observed behaviour were comprehensively investigated through Western Blot analysis of protein expression of excitatory and inhibitory markers. Protein expression of NMDA receptor subunits (GluN) were found to parallel Y-Maze behaviour. Specifically, CORT increased male BDNF HET GluN2B protein expression to probable excitotoxic levels (well above physiological) to impair Y-maze performance. This is consistent with previous research by our lab (Klug et al., 2012), which found that CORT treatment increased NR2B protein levels in male BDNF HET mice and paralleled impaired spatial memory as measured by the Y-maze (Klug et al., 2012). This probable excitotoxic protein expression was not observed in female two-hit mice.

6.2.2.2 Inhibitory markers protein expression

Unpublished investigation of key inhibitory markers found that a genotype \times CORT interaction was common to all inhibitory markers' protein expression, but the direction of this interaction was sex and region dimorphic. Notably, only male BDNF HET CORT mice had changed CAL protein

expression in the DHP. CAL-IN have a disinhibitory role (Cauli et al., 2014). While they do not directly mediate learning and memory processes, they are an important regulator of the circuits that do. This may be contributing to the excitotoxic imbalance presumed in male mice. Comparatively, increased SST expression in female WT mice correlated with a lack of CORT induced spatial memory deficits. SST-IN have a role in spatial memory (Cao et al., 2005) and potentially decreased inhibition provided by SST-IN contributed to the spatial memory deficits observed in female BDNF HET CORT mice. Finally, female BDNF HET CORT mice had decreased PV protein expression and functionally these mice had impaired spatial memory, as determined by a DI value of 1. PV-IN are strongly associated with hippocampal dependent memory processes (Murray et al., 2011). It is notable here that female BDNF HET CORT mice have decreased expression of two inhibitory markers that have been found to interact with each other (Scheytlens and Arckens, 2016). This provides further support that the potential disrupted function of these markers, as implied by reduced protein expression, is contributing to the spatial memory deficits in this group. However, EE did not restore the protein expression of these markers in female BDNF HET CORT mice and instead it is likely the increased expression of the glutamatergic marker GluN1 that is driving the restoration of the excitatory/inhibitory balance in these mice. Sex hormones can affect a broad range of pathways that are involved in learning and memory. Estrogen can facilitate neurogenesis (Tibrewal et al., 2018) and has common downstream signaling pathways with BDNF including MAPK (ERK1/2), PI3K and PLC pathways (Scharfman and MacLusky, 2005; Scharfman and MacLusky, 2006). These processes may also be contributing to the restoration of spatial memory in female two-hit mice exposed to EE.

6.2.2.3 *Learning and memory protein markers*

BDNF-TrkB signaling and ERK1/2 signaling makers were measured as a possible correlate to learning and memory behaviour. However, a caveat here is that hippocampal lysates were measured 5 weeks post-behavioural testing and 7 weeks after EE/CORT treatments. No acute effects were measured on the parameters investigated in this thesis. This study found that CORT and EE protocols differentially altered the activation of discrete TrkB residues. In the literature, chronic CORT treatment disrupts the interaction between glucocorticoid receptors and TrkB with subsequent dampening of the PLC and ERK signaling pathways in cell lines at 3 days and 7 days (Numakawa et al., 2013; Odaka et al., 2016). Inhibiting TrkB expression in addition to a chronic combinatory stress paradigm in adolescence results in sex- and stress-specific behavioural effects (Azogu et al., 2018). Specifically, females displayed heightened sensitivity to chronic stress. In this study, CORT did not have a consistent effect upon TrkB residue activation. While the Y816 ratio was collectively increased, the Y515 ratio was increased only in male WT mice exposed to CORT. This demonstrates that these residues are discrete and have individual regulation in response to a common factor. Similarly, EE activated Y816 in a sex-dimorphic manner, whereby EE increased Y816 ratio in males but decreased it in females. These individual residues are linked to different signaling pathways, which have different downstream effects and ultimately divergence in functional outcomes. This is outlined in detail in section 1.5.2.1. In brief, both Y515 and Y816 contribute to the ERK 1/2 and PLC γ 1 pathways (Ambjørn et al., 2013; Atwal et al., 2000; Minichiello et al., 2002) that are responsible for a broad range of cellular processes including cell proliferation (Cargnello and Roux, 2011), cell survival and axon elongation (Atwal et al., 2000; Ming et al., 1999). Point mutation studies are able to demonstrate the discrete roles of these residues. Point mutation of Y816 has been shown to impair phosphorylation of a range of signalling cascades but excludes MAPK and PI3K that are activated through Y515 (Minichiello

et al., 2002) and additionally demonstrating that the PLC γ docking site is imperative to TrkB-dependent LTP functions (Minichiello et al., 2002).

This study provided a comprehensive molecular map of how glucocorticoids and the BDNF-TrkB signaling pathway dynamically interact.

The ERK1/2 signaling pathway was chosen as a measurement of residue downstream activity. ERK1/2 protein expression was primarily dictated by EE. This is likely due to the multiple systems that are influenced by EE and converge onto this pathway, including the glutamatergic system. Indeed, both Y515 and Y816 residues contribute to the production of ERK1/2 (Ambjørn et al., 2013; Atwal et al., 2000). It is a common downstream signaling cascade for estrogen and BDNF-TrkB signaling (Singh et al., 1999), which may explain sex-dimorphic molecular responses.

The above results should be interpreted carefully, as BDNF is secreted in an activity-dependent manner (Hashimoto et al., 2000) and 7 weeks post treatment it is unlikely that this is the same BDNF. This study is instead demonstrating the long-term consequences of the treatments. The activity-dependent secretion of BDNF is significantly contributed to by Bdnf exon IV (Lu, 2003). GC agonists decrease Bdnf exon IV activity (Chen et al., 2017a) while EE increases it (Dong et al., 2018). Bdnf exon IV has been implicated in the beneficial effects of EE in a range of disease models including Huntington's Disease (Zajac et al., 2010). This is the most likely Bdnf exon to be impacted in this study, but no work has been done to investigate this in this study.

6.2.3 Clinical Implications of EE for neurodevelopmental and mental health disorders

Holistic health and preventative treatments for neurodevelopmental and mental illness disorders have a precedent in the human literature, with evidence suggesting that these approaches can create resilience and help prevent the onset of mental health issues (Arango et al., 2018). EE has been explored as a potential preventative approach in animal models of a range of psychiatric

disorders including depression and anxiety, with promising results (Jha et al., 2011; Urakawa et al., 2013).

A personalised approach for the treatment of schizophrenia is an emerging focus of the field, since there are multiple factors that contribute and intersect for the precipitation of the disorder making it difficult for a standardised treatment to be developed. Digital phenotyping is one approach to creating a profile of a patient (Torous et al., 2018), for the development of individualised treatment plans based on metadata analysis to estimate cognitive ability and monitor disease progression. Adjunctive treatments that could be individualised based on patient sex and disease state include exercise (Firth et al., 2016), estrogen treatments (Searles et al., 2017) and cognitive behavioural therapy (Stafford et al., 2013). This is still an emerging area of research but promises to complement basic research for a broader view of the disorder (Buckley and Miller, 2017).

Chapter 3 of this thesis provides further support for a stronger focus on developing preventative interventions to promote good mental health and resilience in adolescence for at risk individuals. General approaches include proper nutrition and regular exercise, which can promote resilience to mental health vulnerability (Arango et al., 2018; Strong et al., 2005), with exercise shown to improve cognitive symptoms in individuals with schizophrenia (Falkai et al., 2017; Firth et al., 2016). Sex has been shown to moderate the effect of exercise on BDNF levels, with smaller effects for females (Szuhany et al., 2015). Other interventions can include cognitive behavioural therapy and family therapy, which have been shown to be efficacious for the long-term reduction of attenuated psychotic symptoms (Devoe et al., 2018). Successful early interventions for schizophrenia include Assertive Community Treatment, which encourages treatment of a patient within the community has been shown to reduce time in hospital (Randall et al., 2015) and delay of first-episode onset through a combination of antipsychotic medication and cognitive therapy (McGorry et al., 2002). Management of cognitive symptoms could also be

achieved through healthy diet including avoidance of a high-fat, refined-sugar diet (Molteni et al., 2002), intake of flavonoids (Yang et al., 2014) and polyphenols (Gomez-Pinilla and Nguyen, 2012), which all increase BDNF levels. Additionally, it may be that these kind of environmental interventions are only effective for the ‘deteriorated group’ identified by (Wells et al., 2015) and described in section 1.1.2, which experience a decrease in cognitive abilities as their schizophrenia progresses presenting an opportunity for early intervention. Alternatively, there may be lower efficacy for the ‘compromised’ group, which has severe cognitive impairment across development (Wells et al., 2015).

Adolescence is a pivotal neurodevelopmental period and it is during this period that individuals are particularly vulnerable to the onset of mental health issues and disorders. Chapter 3 reported that for the EE protocol using only novel toys, this had a sex-dimorphic effect on spatial memory. While there were caveats to this protocol, it reflected that there are sex-dimorphic aspects to neurodevelopmental disorders such as schizophrenia due to underlying biology. As discussed above, this can have impact on cognitive outcomes. Future research into treatment design should accommodate sex as a factor, to ensure any sex-dimorphic responses to early interventions or related environmental approaches are elucidated.

6.3 Behavioural characterization of conditional TrkB deletion on parvalbumin-expressing interneurons

6.3.1 Cre-lox recombination as a method to knockdown TrkB receptors on PV-cells

To the best of our knowledge, this is the first published study to directly analyse co-expression of PV and TrkB in this model rather than measuring recombination. This study indicated that cre-lox recombination did not significantly knockdown TrkB receptors on PV-positive cells in the mPFC and dorsal hippocampus. Instead, cre-lox recombination produced inconsistent knockdown

of TrkB within the different brain regions. Therefore, the behavioural alterations generated are likely to be idiosyncratic. For future work using this model, the author strongly suggests that it is preferential to measure knockdown of co-expression, rather than recombination levels. Additionally, this is a constitutive model, which provides opportunity for compensatory mechanisms to be activated throughout development. In context of the above, even a subtle knockdown caused the emergence of sex-specific spatial memory deficits. This reflects the fact that each PV-IN influences hundreds of neurons in the local brain region. Increasing the knockdown rate through alternative methods such as the similar FLP-FRT system and delivery through recombinant adeno-associated viruses (rAAVs) (Tang et al., 2017) or the CRISPR-Cas9 system (Singh et al., 2015) may augment the behavioural results observed here, but the subtle knockdown may be more biologically relevant. Hippocampal BDNF mRNA has been reported to be decreased by up to 2.15-fold in schizophrenia patients compared to healthy controls (Reinhart et al., 2015) and decreased hippocampal TrkB mRNA levels of 36% has been shown in schizophrenia patients (Thompson Ray et al., 2011). Mouse models are designed to generate insights into human conditions and here it would seem that a more subtle knockdown, rather than a substantial ablation, is a more robust option for understanding the pathophysiology of cognitive symptoms of schizophrenia.

6.4 BDNF Val66Met genotype selectively alters hippocampal somatostatin cell density in mice

6.4.1 Met/Met genotype specifically decreased density of SST-IN

This project aimed to determine the effect of Val66Met genotype and chronic CORT treatment on the density of hippocampal IN. A specific Met/Met genotype effect was found, whereby Met/Met mice had decreased hippocampal SST-IN density. BDNF regulates SST gene expression

(Glorioso et al., 2006b) and can increase SST expression (Marty and Onteniente, 1999). Here, it would appear that the decreased synaptic release of BDNF throughout development has led to a decreased density of SST-IN in the hippocampus. This aligns with previous research from our laboratory, whereby both male and female BDNF HET mice show decreased mPFC SST density at 12 weeks compared to 4 weeks, indicating developmental interactions with BDNF genotype in the PFC (Du et al., 2018). BDNF is developmentally important for most IN, so the finding that there is a specific genotype effect for SST is interesting. Literature does not indicate that this subtype would be especially vulnerable to disrupted BDNF-TrkB signaling and rather it points to PV-IN being the most vulnerable (Grech et al., 2019b; Hashimoto et al., 2005a). Future work should focus on this question, as to why SST-IN would be most vulnerable to this mutation. This could be explored through comprehensive profiling of these systems through proteomics and genetic studies. SST-IN sensitivity to Met/Met genotype may be region sensitive and perhaps BDNF has a more important role to play in PV interneurons of the PFC.

6.4.2 Female sex mitigates Met/Met genotype effect

Female sex interacted with both genotype and CORT treatment to maintain SST-IN density specifically within the CA1 region of the DHP. Estradiol has been found to regulate SST expression (Panda et al., 2018) and estrogen receptors are expressed in DHP CA1 (Mitra et al., 2003). There is the potential for compensatory signaling to be protecting females, since estradiol and BDNF converge upon similar pathways (Singh et al., 1999). Female mice had higher SST density in the dorsal hippocampus, and our laboratory has previously reported differences in IN development (Wu et al., 2014). It is interesting that both female BDNF genotypes had higher densities, but whether this is the normal expression has not been established in this study. Future work comparing these genotypes to a wildtype-like mouse would be informative as to whether

this high female SST density is ‘normal’ or still a reduction. Sex hormones play a key role in neurodevelopment and differences in psychiatric epidemiology between the sexes (Hill, 2016). This result here indicates that sex hormones can produce structural differences, however this did not emerge as sex-dimorphic behaviour (Notaras et al., 2017b; Notaras et al., 2016a; Notaras et al., 2017d). Comparatively, the supplementary data found that sex and genotype interacted for co-expression of SST and CAL. The main observation was that the male Met/Met group had the highest level of co-expression percentage in the VHP. Again, sex is a main driver of IN results highlighting that this factor should be investigated across behavioural and molecular studies.

6.4.3 Clinical implications for Val66Met model and inhibitory interneurons

This mutation is clinically relevant for a range of mental illness and health disorders, including schizophrenia (Notaras et al., 2015a). The results of this project provide mechanistic insight into the neurobiology that may underlie that association between this polymorphism and behavioural outcomes. While there were sex effects for the density of IN, this did not emerge in previously published behaviour studies using this model (Notaras et al., 2016a; Notaras et al., 2017d). Additionally, CORT alone did not have an effect upon IN density but instead interacted with sex and genotype. Post-mortem studies of schizophrenia patients have found altered BDNF genotype and BDNF signaling has been associated with altered SST expression (Fee et al., 2017; Mellios et al., 2009) but to the best of the author’s knowledge, there are no post-mortem studies specifically investigating IN expression and BDNF^{Val66Met} genotype. The Met allele has been associated with cortical disinhibition in humans, indicating that the excitatory/inhibitory balance is disrupted (Morin-Moncet et al., 2018; Strube et al., 2015a). In clinical terms, this mutation interacts with other risk factors that then moderate psychiatric phenotype (Chao et al., 2008a; Gatt et al., 2009; Shalev et al., 2009). Potentially, the best clinical approach is preventative (Arango et

al., 2018) and encouraging upregulation of BDNF activity through exercise (Hung et al., 2018) and healthy living from a young age (Arango et al., 2018) may reduce the risks of developing schizophrenia. In summary, this project highlights the complexity of IN interaction with BDNF-TrkB signaling and encourages further investigation of this relationship in context of cognitive symptoms of schizophrenia.

6.5 Caveats and future directions

The work presented in this thesis supports and extends current knowledge surrounding cognitive symptoms of schizophrenia, by investigating the interaction between the BDNF-TrkB signaling pathway and INs primarily in the hippocampus. However, animal research comes with a range of limitations that must be addressed and considered.

6.5.1 Environmental Enrichment in a Developmental “Two-Hit” Mouse Model Combining BDNF Haploinsufficiency and Chronic Glucocorticoid Stimulation

The main caveat of this project was the protein measurement of key markers was done in hippocampal lysates collected 5 weeks post behavioural testing and . This does not allow for direct correlation between markers and reported behaviour. Nevertheless, it provides insight into long-term neural activity changes. For more acute information, future work should endeavour to euthanize research animals immediately after key behavioural tests and measure all signaling pathways downstream of the major TrkB residues. The use of homogenised tissue does not allow for subregion or cell-type specific changes, which was reported in project 3 and which may have been masked in project 1. Future work should use methods such as proteomic analysis to investigate possible subregion differences in functional outputs.

A study design limitation is the EE protocol, which only used novel objects and no running wheel. An improvement to this study design would be to have different EE protocols including: 1) novel objects 2) running wheel and 3) novel objects and running wheel so that comparison between key protocol components would be possible. There is much debate around how much novel objects vs. running wheel contributes to the benefits of EE. This study aimed to investigate the potential of EE as a preventative treatment, and therefore elucidating which component, or more likely what combination of components, is therapeutically beneficial is important to distinguish. Additionally, it may be that the time between undertaking EE, returning to standard housing and then beginning behavioural testing was detrimental. Future studies should investigate whether administering EE up until the end of experiments has a positive effect on behavioural measurements in this model.

6.5.2 Using cre-lox recombination for conditional TrkB deletion on parvalbumin-expressing interneurons

A design limitation of this project is that immunohistochemistry to confirm knockdown of TrkB receptors on PV-positive cells was not performed prior to behavioural testing. This may have influenced co-expression through neuroplasticity. Regardless, a male-specific phenotype emerged from the subtle knockdown indicating that this could be used in a multi-hit neurodevelopmental model. Neurodevelopmental disorders such as schizophrenia are likely to emerge from the accumulation and compounding of multiple risk factors. It would be interesting to pair this model with another risk factor such as stress and observe how a chronic stress treatment in addition to impaired BDNF-TrkB signaling at PV-IN would impact performance on a behavioural battery. Subsequently, there may have been motivation issues in the Cheeseboard Maze. Future work should investigate this model using the MWM, as the fear and the survival drive may be better at bringing out cognitive impairments (Lucas et al., 2014).

6.5.3 Using confocal microscopy to measure inhibitory interneurons in a hBDNF^{Val66Met} mouse model

The main limitation of this project was that IN density was investigated only in the hippocampus, however the mPFC and amygdala is also significantly impacted by the Val66Met mutation. Future work should investigate IN density in the mPFC and amygdala to confirm if the Met/Met genotype effect is hippocampal specific (Yu et al., 2012a). Due to microscope capability restrictions, confocal microscopy did not include staining for GAD to confirm that measured cells were GABAergic. An ideal experiment would include GAD and investigate other calcium binding proteins and neuropeptides that are part of the inhibitory family. It would also be informative to measure excitatory markers to compare with inhibitory markers, to confirm if the Val66Met mutation has any effect on the density of excitatory cells.

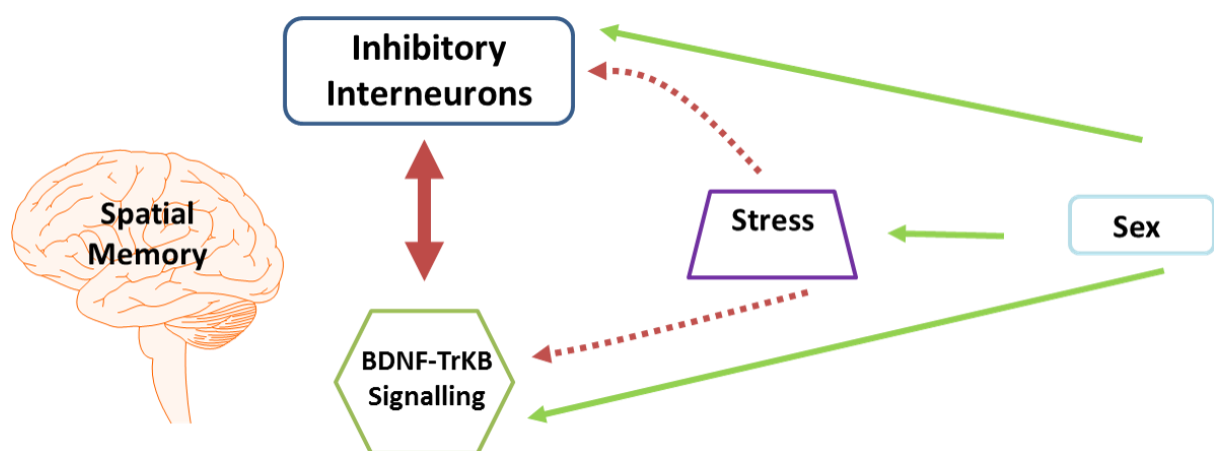


Figure 6.1 - The interactions between key neural systems and risk factors that ultimately dictate cognitive phenotype

6.6 Conclusions

What the results above indicate is that sex is a major mediator of risks of mental illness, such as BDNF genotype. This thesis supports the literature by reporting that alterations to BDNF-TrkB signaling and exposure to different environments leads to sex-dimorphic effects on molecular expression and subsequently learning and memory behaviours. This was confirmed at three different hierarchical levels of the BDNF-TrkB signaling pathway including: genetically decreased availability of BDNF and its interaction with different environments, constitutive genetic knockdown of altered BDNF-TrkB signaling at PV-IN, and the effects of decreased BDNF secretion and stress on hippocampal IN density. It is apparent that in the hippocampus the BDNF-TrkB signaling pathway and IN interact, with the work presented in this thesis providing further support for alterations to the BDNF-TrkB signaling pathway and IN to be contributing to the cognitive symptoms of schizophrenia. Here, stress is a modulator of BDNF-TrkB signaling and IN. This animal model research provides support for clinical research to target this pathway through preventative measures or to augment BDNF activity, with specific attention to be given to male adolescents given their vulnerability. Specifically, the detected sex effects in these mouse models and reported epidemiological data suggest that future human treatment strategies should be in part informed by sex of the patient. For example, this thesis suggests that the BDNF-TrkB signalling pathway and its relationship with parvalbumin cells affects male novelty seeking. It may be that this molecular relationship needs to be exploited through early interventions such as exercise and BDNF mimetic supplements in young male adolescents, whereas later interventions may be more relevant to the pathophysiology of female schizophrenia patients including SERM interventions. However, while there is promising evidence that find improvements to cognition in response to SERM treatment (Huerta-Ramos et al., 2014; Usall et al., 2011) there are also studies that find no cognitive improvements (Kulkarni et al., 2016; Weiser et al., 2017).

Ultimately, this thesis provides evidence that sex is a key modulator in the dynamic relationship between BDNF-TrkB signaling, stress, IN and cognition. Work from thesis has contributed towards the Behavioural Neuroscience Laboratory and Translational Molecular Psychiatry laboratory initiating a collaboration with Keqiang Ye from Emory University for future clinical trials of the BDNF mimetic R13. This may offer a targeted treatment for cognitive symptoms of schizophrenia and is a necessary area of research for this disorder.

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Appendices

Appendices A and B are manuscripts that I authored or contributed to during my candidature but were inappropriate to include as chapters.

Appendix A

Grech, A.M., Nakamura, J.P. and Hill, R.A. *The importance of distinguishing allocentric and egocentric search strategies in rodent hippocampal-dependent spatial memory paradigms - getting more out of your data*. 2018. The Hippocampus Ales Stuchlik, IntechOpen, DOI: 10.5772/intechopen.76603.

Appendix B

Du, X., Serena, K., Hwang, W.J., **Grech, AM**, Wu, Y.C., Schroeder, A. and Hill, RA. *Prefrontal cortical parvalbumin and somatostatin expression and cell density increase during adolescence and are modified by BDNF and sex*. Mol Cell Neurosci. 2018 Apr;88:177-188. doi: 10.1016/j.mcn.2018.02.001.

Appendix A

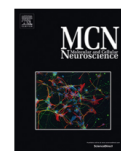
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Prefrontal cortical parvalbumin and somatostatin expression and cell density increase during adolescence and are modified by BDNF and sex

X. Du^{a,*}, K. Serena^b, W. Hwang^b, A.M. Grech^a, Y.W.C. Wu^b, A. Schroeder^b, R.A. Hill^a

^a Behavioural Neuroscience Laboratory, Department of Psychiatry, Monash University, Clayton, Victoria 3168, Australia

^b Psychoneuroendocrinology Laboratory, Florey Institute for Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria 3010, Australia

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is known to play a critical role early in the development of cortical GABAergic interneurons. Recently our laboratory and others have shown protracted development of specific subpopulations of GABAergic interneurons extending into adolescence. BDNF expression also changes significantly across adolescent development. However the role of BDNF in regulating GABAergic changes across adolescence remains unclear. Here, we performed a week-by-week analysis of the protein expression and cell density of three major GABAergic interneurons, parvalbumin (PV), somatostatin (SST) and calretinin (Cal) in the medial prefrontal cortex from prepubescence (week 3) to adulthood (week 12). In order to assess how BDNF and sex might influence the adolescent trajectory of GABAergic interneurons we compared WT as well as BDNF heterozygous (+/–) male and female mice. In both males and females PV expression increases during adolescent development in the mPFC. Compared to wild-types, PV expression was reduced in male but not female BDNF +/– mice throughout adolescent development. This reduction in protein expression corresponded with reduced cell density, specifically within the infralimbic prefrontal cortex. SST expression increased in early adolescent WT females and this upregulation was delayed in BDNF +/–. SST cell density also increased in early adolescent mPFC of WT female mice, with BDNF +/– again showing a reduced pattern of expression. Cal protein expression was also sex-dependently altered across adolescence with WT males showing a steady decline but that of BDNF +/– remaining unaltered. Reduced cell density in on the other hand was observed particularly in male BDNF +/– mice. In females, Cal protein expression and cell density remained largely stable. Our results show that PV, SST and calretinin interneurons are indeed still developing into early adolescence in the mPFC and that BDNF plays a critical, sex-specific role in mediating expression and cell density.

1. Introduction

Cognitive deficits are a key feature of schizophrenia and are highly predictive of functional outcome (Green et al., 2004). Dysfunction of the inhibitory circuits in the cerebral cortex is a major contributor to cognitive deficits (Volk and Lewis, 2002).

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter and GABAergic interneurons are the main inhibitory neurons in the cerebral cortex. While the classification of GABAergic interneurons is an ongoing area of scientific refinement (DeFelipe et al., 2013; Petilla Interneuron Nomenclature et al., 2008), interneurons have traditionally been divided into subpopulations based on their morphology, targets (e.g. pyramidal cell or other interneuron), innervation, electrophysiological output, and specific molecular markers such as neuropeptides, Ca²⁺ proteins, ionic channels, receptors and transporters (Batista-Brito and Fishell, 2009; Varga et al., 2014).

GABAergic interneurons that are labelled by the molecular markers parvalbumin (PV) and somatostatin (SST) have both been shown to be reduced in the dorsolateral prefrontal cortex of subjects with schizophrenia (Fung et al., 2010; Hashimoto et al., 2008b). An important role played by these interneurons is to safeguard synchronized network oscillations by which activities of pyramidal neuron assemblies are orchestrated and controlled (Beierlein et al., 2000). This function is disrupted in schizophrenia (Moran and Hong, 2011; Uhlhaas and Singer, 2010) resulting in impaired cortical circuit operation. Hence perturbations in the GABAergic system have been associated with common cognitive deficits in schizophrenia, such as deficits in pre-pulse inhibition (Freedman et al., 2000; Javitt, 2009) and working memory (Chen et al., 2014; Haenschel et al., 2009).

Rodent studies show that adolescence is a period of dynamic alterations in interneuron expression (Caballero et al., 2014; Wu et al., 2014). Adolescence and early adulthood is also the vulnerable period

* Corresponding author at: Behavioural Neuroscience Laboratory, Department of Psychiatry, Monash University, Monash Medical Centre, Clayton, Victoria 3168, Australia.
E-mail address: xin.du@monash.edu (X. Du).

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when schizophrenia predominantly manifests. Moreover, schizophrenia displays robust sex-dimorphism with females having later onset and lower prevalence than males – suggestive of protective effects of estrogen, the level of which rises sharply in adolescence (Elsabagh et al., 2009; Hafner, 2003; Zhang et al., 2012). Despite this, the adolescent developmental trajectory of GABAergic interneurons is not well characterised.

Evidence suggest that the protective effects of estrogen is partly mediated through its ability to increase the expression of brain-derived neurotrophic factor (BDNF) (Matsuki et al., 2014; Podfigurna-Stopa et al., 2013; Wu et al., 2013), an essential neurotrophin whose depletion is consistently implicated in cognitive deficits and other symptoms in schizophrenia as well as in many other neurological disorders (Du and Hill, 2015; Notaras et al., 2015; Thompson Ray et al., 2011). BDNF has been shown to be critical for GABAergic interneuron network development and function (Fiorentino et al., 2009; Sakata et al., 2009; Shinoda et al., 2011; Zheng et al., 2011) and prefrontal deficits of GABAergic gene expressions in post-mortem schizophrenia subjects correlate with reductions of BDNF and its receptor TrkB (Hashimoto et al., 2005; Mellios et al., 2009). In the BDNF heterozygous (BDNF +/–) mouse model, reduced BDNF to ~50% of wild-type (WT) level resulted in attenuated GABAergic and glutamatergic synaptic transmissions in thalamic circuits (Laudes et al., 2012) and impaired GABAergic inhibition in the visual cortex (Abidin et al., 2008).

Previously, our laboratory reported sex-differences in the expression of BDNF and TrkB during adolescence. Male but not female mice show significant increases in BDNF expression in the medial prefrontal (mPFC) cortex, while female, but not male mice show a significant rise in BDNF and PV expression in the hippocampus across adolescence (Hill et al., 2012; Wu et al., 2014). Furthermore, beneficial effects of estradiol on hippocampal PV expression and cell density and hippocampal-dependent spatial memory were found to be mediated by BDNF (Wu et al., 2015). PV interneurons are critical regulators of high-frequency gamma oscillations (Sohal et al., 2009) which may be altered by changing levels of estradiol (Schroeder et al., 2017) and BDNF (Zheng et al., 2011). While PV levels have been shown to increase during adolescence in the prefrontal cortex (PFC) of rats (Caballero et al., 2014), adolescent expression and cell density of PV in the mouse mPFC has not been thoroughly explored, and furthermore, it is not established whether BDNF plays a role here, and whether adolescent changes in PV are sex-specific. SST interneurons are responsible for the generation of theta oscillations, which are critical to cognitive functions such as working memory and synaptic plasticity (Raghavachari et al., 2006). Schizophrenia patients exhibit perturbed theta oscillations during working memory tasks (Schmiedt et al., 2005) and SST mRNA expression is consistently reduced in the dorsal lateral prefrontal cortex of schizophrenia patients (Fung et al., 2010; Hashimoto et al., 2008a; Morris et al., 2008). Like PV, SST gene expression is influenced by BDNF signalling. Both BDNF (Glorioso et al., 2006; Guilloux et al., 2012; Tripp et al., 2012) and TrkB-knockdown mice (Morris et al., 2008) show reduced SST mRNA expression in the frontal cortex and amygdala. However, little is known about the regulation of SST expression across adolescence in the mPFC.

We hypothesise that BDNF signalling is required for normal GABAergic interneuron development in the mPFC that extends into adolescence and this may be sex-dependent. Here, we analysed the protein expression of three interneuron markers PV, SST and calretinin (Cal) in both male and female, WT and BDNF +/– mice weekly from a pre-pubertal age (week 3) until adulthood (week 12). This was followed by immunofluorescence and cell density analysis of the three subtypes of neurons within the infralimbic (Irl), prelimbic (PrL) and cingulate cortex (Cg) at prepubescence (4 weeks), adolescence (6 weeks) and adulthood (12 weeks) in male and female, WT and BDNF +/– mice. Previous studies have shown robust reductions in BDNF mRNA levels in various brain regions of BDNF +/– mice (Ernfors et al., 1994; Kerner et al., 2000). Our group has shown that in the BDNF +/– model, both

male and female mice exhibit ~40–50% reduction of BDNF protein in the prefrontal cortex (Hill and van den Buuse, 2011), ensuring this is a model of BDNF insufficiency. We have focused on the mPFC, a region vital for high order cognitive functioning. Dysfunction in the human mPFC is specifically observed in schizophrenia, both pre-clinically and in diagnosed patients (Sakurai et al., 2015). While how far the homology between rodent and human mPFC may be asserted is debatable, the concept of homologous structures with similar functions may apply in providing clues to the aetiology of neurodevelopmental anomalies (Schubert et al., 2015).

2. Materials and methods

2.1. Animals

Male and female BDNF +/– and wild-type mice on a C57Bl/6 background were derived from a breeding colony at the Florey Institute of Neuroscience and Mental Health. Animals were housed under standard conditions with ad libitum access to water and mouse chow. To assess the developmental trajectories of different markers during the adolescent period, 8 mice per group from weeks 3–12 of age (ten groups) were utilised to conduct a week by week protein expression analysis. For immunohistochemistry studies 5 mice per group from weeks 4, 6 and 12 were used to assess prepubescence, adolescence and adulthood. In female mice, cycle stage was assessed by collecting vaginal smears and further staining them with methylene blue for microscopic analysis. Female mice were sacrificed when they were in the proestrus (high E2) stage of the cycle. Proestrus was defined by nucleated and non-nucleated epithelial cells (Mettus and Rane, 2003). Mice were culled by cervical dislocation between 10:00 h and 12:00 h and brains were snap-frozen for future dissection. All experimental procedures were approved by the Animals Experimentation Ethics Committee of the Florey Institute, University of Melbourne, Australia.

2.2. Brain dissections

To collect fresh frozen brains, mice were culled by cervical dislocation and the brains removed within 1 min of death. Brains are then placed onto one layer of aluminium foil which is placed flat onto dry-ice and allowed to freeze. Collected brains were micro-dissected into multiple regions using a mouse brain mould. The frozen brains, were placed inverted on an ice-cold mouse brain mould (–8 °C) with 1 mm inserts. Three 2 mm coronal slices were taken from the forebrain. The second slice (Bregma 3.08–1.18 mm) contained the medial prefrontal cortex, which was dissected with a surgical blade (mPFC). Samples were snap-frozen in dry ice and stored at –80 °C until further use.

2.3. Protein extraction

Frozen tissue samples were weighed and appropriate amounts of lysis buffer containing 150 mM sodium chloride (ChemSupply, AU), 1.0% Triton-X-100 (Sigma Aldrich), 0.1% sodium dodecyl sulphate (Sigma Aldrich) and 50 mM Tris pH 8.0 (Trizma Base, Sigma Aldrich), protease inhibitor cocktail set III (dilution 1:200) and the phosphatase inhibitor cocktail set IV (dilution 1:50) (Merck; Kilsyth, Vic., Australia) were added according to the weight (1000 µl per 100 µg). Tissue samples were homogenized and were left on ice for 10 min. Samples were then left to rotate for 1 h at 4 °C, followed by 15 min on the centrifuge at 14000 g and 4 °C. The supernatant was then extracted and 3 µl of the supernatant protein stock was used for a bicinchoninic acid (BCA) protein assay to determine total protein levels. The remaining stock protein was then stored at –80 °C.

2.4. Western blot analysis

Western blot analysis was performed using 50 µg sample/ animal.

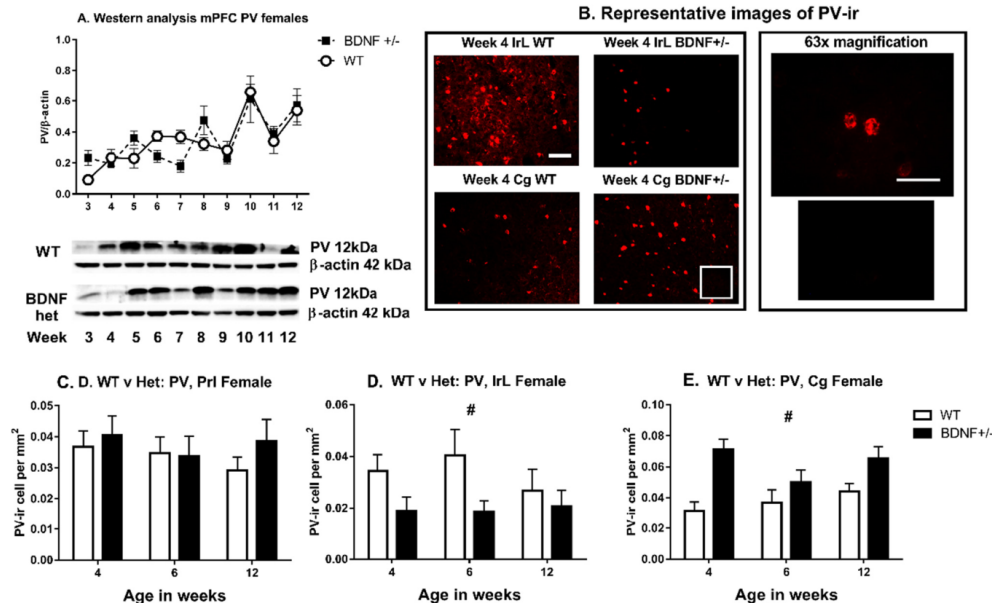


Fig. 1. Female PV protein expression and cell density in the mPFC. Female mice of both genotypes show a significant increase in PV protein levels with age but no genotype effect (A). Cell density in the PrL is comparable (C) but in the IrL is reduced in BDNF +/- compared to WT (D). It is however increased in BDNF +/- compared to WT in the Cg region (E). Representative images (B) in left box (10× magnification) show the decreased PV-ir density in the IrL and the increased PV-ir density in the Cg of the BDNF +/- compared to WT at 4 weeks. Negative control minus primary antibody is displayed in the small box situated in the right corner. Top image in the right box (63× magnification) shows PV-ir staining in PrL of WT mice; bottom image shows lack of auto-fluorescence in the green channel. *N* = 5–7 per group, # signify overall effect of genotype. Scale bar = 100 μm for 10× magnification and 60 μm for 63× magnification.

First, an equal volume of loading buffer (0.4 M Tris, pH 6.8, 37.5% glycerol, 10% SDS, 1% 2-mercaptoethanol, 0.5% bromophenol blue, dH₂O) was added to each sample (~10 μl) and samples were denatured for 10 min at 95 °C on the heat block before SDS-polyacrylamide gel electrophoresis (15% acrylamide gel, 120 V, 1.5 h) for separation of the denatured sample proteins. Protein samples were then transferred to a nitrocellulose membrane for 1.5 h at 120 V, 4 °C. To reduce non-specific antibody binding, the membranes were blocked with 5% milk in TBST for 1 h at room temperature. The membranes were then incubated overnight with primary antibody (in 5% bovine serum albumin (BSA) in TBST) at 4 °C.

The primary antibodies used were mouse anti-PV (dilution 1:1000; 12 kDa; Merk-Millipore; MAB1572; Billerica, MA, USA), rat anti-SST (dilution 1:2000; 14 kDa; Merk-Millipore; MAB354; Billerica, MA, USA), rabbit anti-Cal (dilution 1:2000; 29 kDa; Swant; CR7697; Bellinzona, Switzerland), rabbit anti-mBDNF (dilution 1:500; 13 kDa; Santa Cruz; H117; Dallas, Texas, USA) and mouse anti-β-actin (dilution 1:10,000; 42 kDa; Sigma-Aldrich; A5316; Castle Hill, NSW, Australia).

The next day, the membranes were washed twice for 15 min in TBST then applied with matching secondary antibodies of anti-rabbit (dilution 1:2000; Cell signalling; Danvers, MA, USA), anti-mouse (dilution 1:2000; Cell signalling; Danvers, MA, USA) or anti-rat IgG HRP-linked antibodies (dilution 1:2000; Merk-Millipore; Billerica, MA, USA). The blots were imaged by Luminescence Image Analyzer (LAS-4000; FujiFilm Life Science, Stamford, CT, USA) and were further analysed using Image Quant software (GE Healthcare, Milwaukee, WI, USA). PV, Cal, SST and mBDNF were normalised against the level of the control gene β-actin.

2.5. Immunohistochemistry

Mice for immunohistochemical analyses were transcardially perfused with 0.1 M cold phosphate buffered saline (PBS) (~50 ml) followed by 4% paraformaldehyde in cold 0.1 M PBS. Brains were removed and post-fixed in 4% PFA overnight. For cryoprotection, brains were first transferred in a 15% sucrose solution for 24 h and then transferred to a 30% sucrose solution for a further 24 h. Brains were then snap frozen and sections were cut on a cryostat at 20 μm thickness and collected at 1:6 intervals. The coronal sections of the prefrontal cortex were sectioned from 2.80 to 1.72 relative to Bregma and mounted on gelatine coated slides (approximately 10 sections/slide, 1:6 interval spanning entire mPFC). Every 6th section of the mPFC was collected and stained, amounting to approximately 10 sections/animal across the entire medial prefrontal cortex. Sections were washed in PBS and incubated in ice-cold methanol for 30 min before being treated with the Vector Mouse on Mouse (M.O.M.) immunodetection kit according to manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Primary antibodies diluted in M.O.M diluent were then applied to the slides and left to incubate overnight at 4 °C in a humidified chamber. The next day, sections were washed for 2 × 5 min in PBS and fluorescent secondary antibodies diluted in M.O.M diluent were added to the sections and left to incubate in the dark at room temperature for 1.5 h in a humidified chamber. Antibody specificity was examined using negative controls that did not have primary antibody applied. Following application of secondaries, sections were washed for 2 × 5 mins in PBS and cover slips were placed on with the use of DAKO Fluorescent Mounting Medium (Dako, North America Inc., CA, USA) and left to dry overnight in the dark. Imaging was undertaken using a Leica -DM2500 fluorescence microscope with a Leica DFC310FX camera,

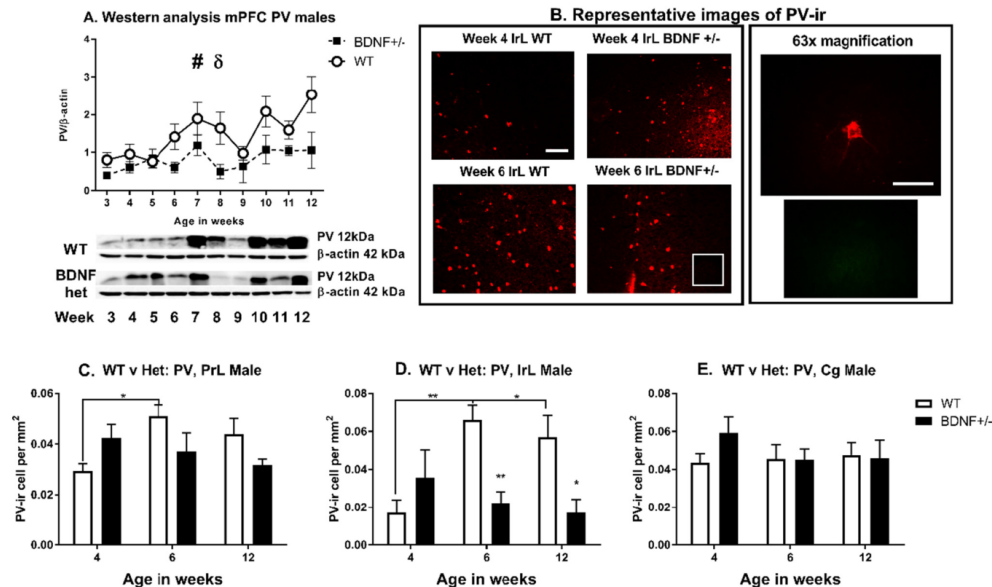


Fig. 2. Male PV protein expression and cell density in the mPFC. In male mice, WT mice show a significant increase in PV protein level with age whereas BDNF^{+/-} exhibit a consistently flat expression that is significantly lower than WT (A). WT mice also show an increase in PV-ir density in the PrL (C) and IRL (D), which is absent in BDNF^{+/-}. No changes in cell density were detected in the Cg (E). Representative images (B) in the left box (10× magnification) show the increases in PV-ir density in WT IRL from week 4–6 but relative unaltered density in BDNF^{+/-}. Negative control minus primary antibody is displayed in the small box situated in the right corner. Top image in the right box (63× magnification) shows PV-ir staining in IRL of WT mice; bottom image shows lack of auto-fluorescence in the green channel. *N* = 5–7 per group, # signify overall effect for genotype, δ signify overall effect of age, **p* < 0.05, ***p* < 0.01, = signify trend (*p* < 0.1). Scale bar = 100 μm for 10× magnification and 60 μm for 63× magnification.

captured at ×10 zoom. Each brain region, PrL, Cg and IRL, was digitally captured in a single image in each hemisphere; Samples examined related to Bregma co-ordinates 2.68, 2.32 and 2.08. Images were uploaded and conjoined on ImageJ 1.48v (Nation Institute of Health, USA) where each region (cingulate, infralimbic and prelimbic) was then defined and clearly demarcated in ImageJ by being drawn onto the image using Bregma co-ordinates. Area measurements of each region were taken using the scale as set by microscope configuration where 1.080 μm = 1 pixel accordingly, to examine cell density (cell/mm²). Cell density analyses were performed manually by the experimenter, blind to the animal groups, using ImageJ counter. Specific criteria for labelled cell bodies were set in the Image J plugins software for detection thresholds.

2.6. Statistical analysis

All data are expressed as the mean ± the standard error of the mean (SEM). Two-way analysis of variance (ANOVA) with age and genotype as the two main factors was used to analyse the developmental curves. If *p* < 0.05, differences were considered to be statistically significant. If significant effects of age were found, this was followed by a Bonferroni-corrected post-hoc comparison of the main effect of age (10 different age groups). If a significant age × genotype interaction was found, this was followed by Bonferroni's corrected post-hoc multiple comparison of the effects of genotype at each individual week (GraphPad PRISM 7, version 7.01; GraphPad Software Inc., San Diego, CA, USA). For the total interneuron cell density in the mPFC, three-way ANOVAs were performed, with age, genotype and subregion as main factors (IBM SPSS Statistics, Armonk, NY, USA).

3. Results

3.1. The effects of age and BDNF^{+/-} genotype on parvalbumin expression in the mPFC

In female mice a significant effect of age was found for PV protein expression with levels increasing steadily throughout adolescence to adulthood (Fig. 1A, age, $F_{(9, 109)} = 8.898$, *p* < 0.0001). No significant effect of genotype and no genotype × age interaction was found.

Examining cell density across the entire mPFC, three-way ANOVA (region, age and genotype) revealed a significant main effect of region ($F_{(2, 72)} = 18.59$, *p* < 0.001) but no effect of age or genotype. There was, however, a genotype × region interaction ($F_{(2, 72)} = 13.11$, *p* < 0.001). Given the different projections and function modality of the subregions of the mPFC, we performed individual analyses for each subregion. Two-way ANOVAs showed that, in the PrL (Fig. 1C), there was no significant effect of age or genotype. However, a significant effect of genotype was found for PV-ir density in the IRL ($F_{(1, 24)} = 5.928$, *p* = 0.0227, Fig. 1D) and Cg ($F_{(1, 24)} = 19.42$, *p* = 0.0002, Fig. 1E) with BDNF^{+/-} showing less PV-ir in the IRL but more in the Cg compared to WT mice. No significant effect of age and no age × genotype interactions were found in the IRL or Cg. Representative images in Fig. 1B show the opposing genotype effects in the IRL and Cg.

In male mice a significant effect of age (Fig. 2A, $F_{(9, 75)} = 3.698$, *p* = 0.0007) and a significant effect of genotype was found for protein expression in the mPFC (genotype, $F_{(1, 75)} = 26.85$, *p* < 0.0001), however no age × genotype interaction was found. Here PV expression increased with age in both genotypes but expression levels were significantly lower in BDNF^{+/-} mice (Fig. 2A).

For PV-ir cell density in the entire mPFC of male mice, three-way ANOVA revealed a significant main effect of genotype ($F_{(1, 69)} = 4.17$,

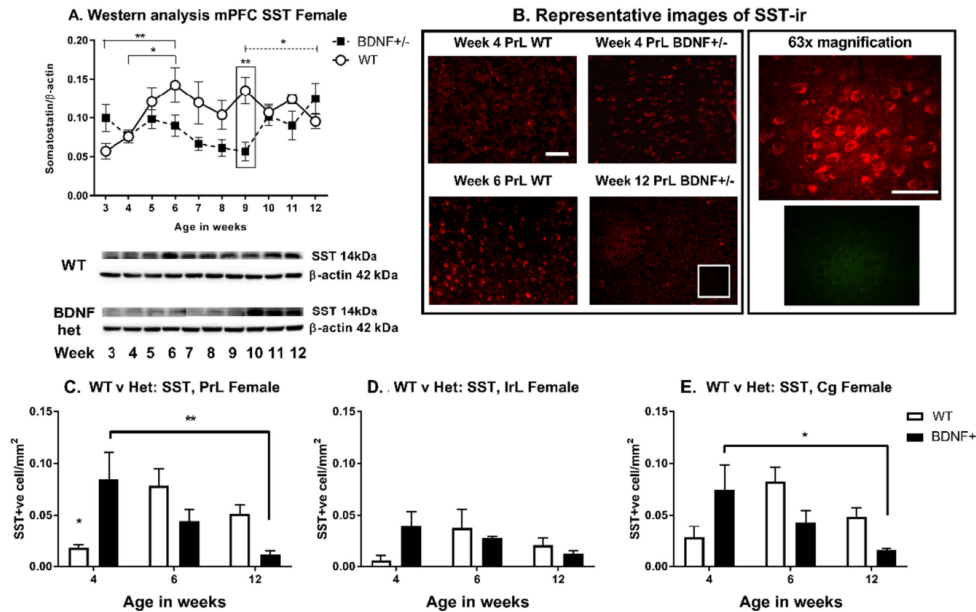


Fig. 3. Female SST protein expression and cell density in the mPFC. Female WT mice show a significant increase in SST protein expression from week 3 to week 6. BDNF mice, on the other hand, do not show a significant increase until after week 9, when it catches up to WT level (A). SST-ir density shows significant decrease across age in the BDNF +/- mice and trends for increased density in WT mice in the PrL (C) and Cg (E) but not in the IRL (D). Representative images (B) in the left box (10× magnification) show the increase in SST-ir density from week 4–6 in WT PrL and the decrease in density in BDNF +/- from week 4–12. Top image in the right box (63× magnification) shows SST-ir staining in Cg of WT mice; bottom image shows lack of auto-fluorescence in the green channel. $N = 5-7/\text{group}$. Negative control minus primary antibody is displayed in the small box situated in the right corner. * $p < 0.05$, ** $p < 0.01$. Solid lines of significance represent differences in WT. Dashed lines represent differences in BDNF +/- . Scale bar = 100 μm for 10× magnification and 60 μm for 63× magnification.

$p = 0.045$) and a significant main effect of region ($F_{(2, 69)} = 4.08$, $p = 0.021$). There was also a significant genotype \times age ($F_{(2, 69)} = 10.95$, $p < 0.001$) and a genotype \times region interaction ($F_{(2, 69)} = 4.81$, $p = 0.011$). This prompted us to look at individual sub-regions, whereby two-way ANOVA revealed a significant age \times genotype interaction in the discrete subregions of the PrL (Fig. 2C, $F_{(2, 23)} = 4.442$, $p = 0.0234$) and IRL (Fig. 2D, $F_{(2, 23)} = 6.866$, $p = 0.0046$). This interaction was not found in the Cg (Fig. 2E). Post-hoc analysis revealed an increase in PV-ir density in the WT mice, from weeks 4 to 6 in the PrL and IRL ($t_{(23)} = 3.041$, $p = 0.0174$ and $t_{(23)} = 3.700$, $p = 0.0035$ respectively). Furthermore, a significant genotype effect was found in the IRL (Fig. 2E: genotype, $F_{(1, 23)} = 7.799$, $p = 0.0103$), with the BDNF +/- mice showing lower cell density. These data indicate that PV cell density increases from weeks 4–6 in WT, but not BDNF +/- mice. Representative images (Fig. 2B) show the genotype differences in the WT and BDNF +/- at weeks 4 and 6.

3.2. The effects of age and BDNF +/- genotype on somatostatin expression in the mPFC

In female mice, a significant effect of genotype (Fig. 3A, $F_{(1, 83)} = 10.52$, $p = 0.0017$), a trend for an effect of age ($F_{(9, 83)} = 1.945$, $p = 0.056$) and a significant genotype \times age interaction ($F_{(9, 83)} = 3.256$, $p = 0.002$) was found for somatostatin (SST) protein expression level throughout adolescence. Here WT mice showed a significant rise in SST protein level, which peaked at week 6 and levelled out thereafter. In contrast, BDNF +/- mice showed a significant delay in this rise in SST, with levels starting to increase later at week 10. This was evidenced by post-hoc multiple comparisons which found

significant differences between week 3 and week 6 ($t_{(83)} = 5.72$, $p = 0.004$), and week 4 and week 6 ($t_{(83)} = 4.675$, $p = 0.04$) in WT mice, but a significant difference between week 9 and week 12 in BDNF +/- mice ($t_{(83)} = 4.788$, $p = 0.03$). In addition to the overall genotype effect, a significant difference between WT and BDNF +/- mice was found at week 9 ($t_{(83)} = 3.656$, $p = 0.0045$).

Examining SST-ir cell density in female mice, three-way ANOVA saw no significant main effects of genotype but revealed significant effects of age ($F_{(2, 56)} = 5.04$, $p = 0.01$) and region ($F_{(2, 56)} = 6.035$, $p = 0.004$). There was also a genotype \times age interaction ($F_{(2, 56)} = 14.1$, $p < 0.001$). In the discrete region of PrL, two-way ANOVA revealed a significant age \times genotype interaction (Fig. 3C, $F_{(2, 19)} = 6.933$, $p = 0.006$). SST-ir density was significantly higher in BDNF +/- mice at 4 weeks compared to WT ($t_{(19)} = 2.874$, $p = 0.029$), and SST-ir density is significantly lower in BDNF +/- at 12 weeks compared to 4 weeks ($t_{(19)} = 1.445$, $p = 0.009$). A trend for increased SST-ir density in WT from 4 to 6 weeks was also apparent in the PrL ($t_{(19)} = 2.488$, $p = 0.067$). No changes were detected in the IRL (Fig. 3D). In the Cg, there again was a significant age \times genotype interaction (Fig. 3E, $F_{(2, 18)} = 4.614$, $p = 0.0241$), with post-hoc analysis further showing significantly lower SST-ir density in BDNF +/- at week 12 compared to week 4 ($t_{(18)} = 2.867$, $p = 0.031$). There was also a trend for increased SST-ir density in WT at 6 weeks compared to 4 weeks ($t_{(18)} = 2.329$, $p = 0.095$).

In contrast to the females, SST protein expression level in male mice showed no significant effects of age ($F_{(9, 85)} = 1.269$, $p = 0.2654$) or genotype ($F_{(1, 85)} = 0.9503$, $p = 0.3324$) and no age \times genotype interaction on somatostatin expression in the mPFC (Fig. 4A).

Analysis of SST-ir cell density for the entire mPFC region, three-way

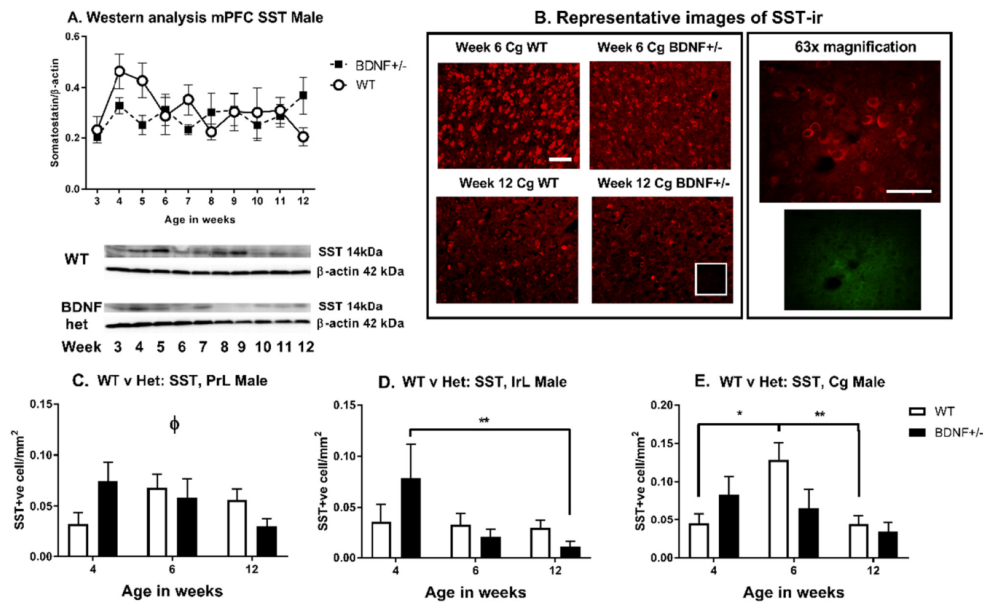


Fig. 4. Male SST protein expression and cell density in the mPFC. In Males, neither WT nor BDNF +/– show changes in SST protein expression in the mPFC. Protein level between genotypes was also comparable (A). Trend for decreasing cell counts in the BDNF +/– is apparent in the PrL (C) and the Irl (D). In the Cg, WT show a peak in cell density at 6 weeks (E). Representative images (B) in the left box (10× magnification) show the decrease in SST-ir density in WT Cg from week 6–12 and the relatively steady density in BDNF +/–. Negative control minus primary antibody is displayed in the small box situated in the right corner. Top image in the right box (63× magnification) shows SST-ir staining in Cg of WT mice; bottom image shows lack of auto-fluorescence in the green channel. $N = 5$ –7/group, Φ signify significant genotype \times age interaction, $*p < 0.05$, $**p < 0.01$. Solid lines of significance represent differences in WT. Dashed lines represent differences in BDNF +/–. Scale bar = 100 μ m for 10× magnification and 60 μ m for 63× magnification.

ANOVA revealed main effects of age ($F_{(2, 52)} = 6.44$, $p = 0.003$) and region ($F_{(2, 52)} = 6.40$, $p = 0.003$) but not of genotype. There was also a significant genotype \times age interaction ($F_{(2, 52)} = 8.26$, $p = 0.001$).

Examining each subregion, two-way ANOVA analysis of the PrL revealed a significant genotype \times age interaction (Fig. 4C, $F_{(2, 17)} = 3.778$, $p = 0.044$) but no significant post-hoc differences upon further analysis, although there was a trend for reduced SST-ir density in BDNF +/– at 12 weeks compared to 4 weeks ($t_{(17)} = 2.593$, $p = 0.057$). In the Irl, there was a significant main effect of age (Fig. 4D, $F_{(2, 16)} = 3.688$, $p = 0.048$), with post-hoc analysis showing a trend for reduced SST-ir density at 12 weeks compared to 4 weeks ($t_{(16)} = 2.655$, $p = 0.052$). In the Cg, there was a significant main effect of age (Fig. 4E, $F_{(2, 19)} = 5.329$, $p = 0.015$) and a significant age \times genotype interaction ($F_{(2, 19)} = 3.694$, $p = 0.044$). Post-hoc analysis show that in WT Cg, SST-ir density is significantly higher at 6 weeks of age compared to both 4 weeks ($t_{(19)} = 3.313$, $p = 0.011$) and 12 weeks ($t_{(19)} = 3.516$, $p = 0.007$). No differences were detected in BDNF +/– across ages.

3.3. The effects of age and BDNF +/– genotype on calretinin expression in the mPFC

In female mice, a significant effect of age was found for Cal protein levels ($F_{(9, 80)} = 4.398$, $p < 0.0001$) but no significant effect of genotype and no genotype \times age interaction was found (Fig. 5A). Here, post-hoc multiple comparisons for the main effect of age found significant differences between week 5 and weeks 7 and 9 ($t_{(80)} = 3.418$, $p = 0.045$; $t_{(80)} = 3.549$, $p = 0.029$), week 7 and weeks 10 and 12 ($t_{(80)} = 4.269$, $p = 0.002$; $t_{(80)} = 3.737$, $p = 0.016$) and week 9 and weeks 10 and 12 ($t_{(80)} = 4.383$, $p = 0.002$; $t_{(80)} = 3.862$, $p = 0.01$).

Examining Cal-ir cell density in the entire mPFC in female mice, three-way ANOVA revealed no significant main effects of genotype, age or region. Looking at individual subregions, two-way ANOVA saw a significant age \times genotype interaction in the Cg (Fig. 5E, $F_{(2, 24)} = 4.343$, $p = 0.0246$) that was not seen in the other brain regions (Fig. 5C, D). In the Cg, post-hoc analysis revealed that there was a significant increase in Cal-ir density from week 4 to week 12 ($t_{(24)} = 2.627$, $p = 0.0444$) in WT mice but with no significant effect of age in BDNF +/–. Representative images as shown in Fig. 5B show an increase in Cal-ir density in from week 4 to week 12 in WT but not BDNF +/– mice.

Males also showed significant effects of age ($F_{(9, 87)} = 2.807$, $p = 0.006$), but no significant effect of genotype ($F_{(1, 87)} = 0.6187$, $p = 0.4337$) and no genotype \times age interaction (Fig. 6A, $F_{(9, 87)} = 0.9418$, $p = 0.4937$). Here male mice showed a declining trajectory of Cal protein levels in the mPFC, with significant differences found between week 3 and weeks 7 ($t_{(87)} = 4.016$, $p = 0.006$), 8 ($t_{(87)} = 3.479$, $p = 0.036$), 10 ($t_{(87)} = 3.399$, $p = 0.046$), 11 ($t_{(87)} = 3.651$, $p = 0.02$) and 12 ($t_{(87)} = 3.914$, $p = 0.008$).

For Cal-ir cell density in the entire mPFC of male mice, three-way ANOVA revealed significant main effects of genotype ($F_{(1, 69)} = 5.36$, $p = 0.024$), age ($F_{(2, 69)} = 6.80$, $p = 0.002$) and region ($F_{(2, 69)} = 3.53$, $p = 0.035$). There was also a genotype \times age interaction ($F_{(2, 69)} = 7.35$, $p = 0.001$). Examining each subregion, two-way ANOVA in the PrL revealed a significant genotype \times age interaction (Fig. 6C, $F_{(2, 23)} = 3.77$, $p = 0.038$). Post-hoc analysis showed that WT PrL exhibited a significant increase in Cal-ir density from 4 to 6 weeks of age ($t_{(23)} = 3.28$, $p = 0.01$). There was a trend for WT to have increased Cal-ir density compared to BDNF +/– at week 6 ($t_{(23)} = 2.475$, $p = 0.063$). In the Irl, there was a significant effect of age (Fig. 6D, $F_{(2, 23)} = 3.77$, $p = 0.038$).

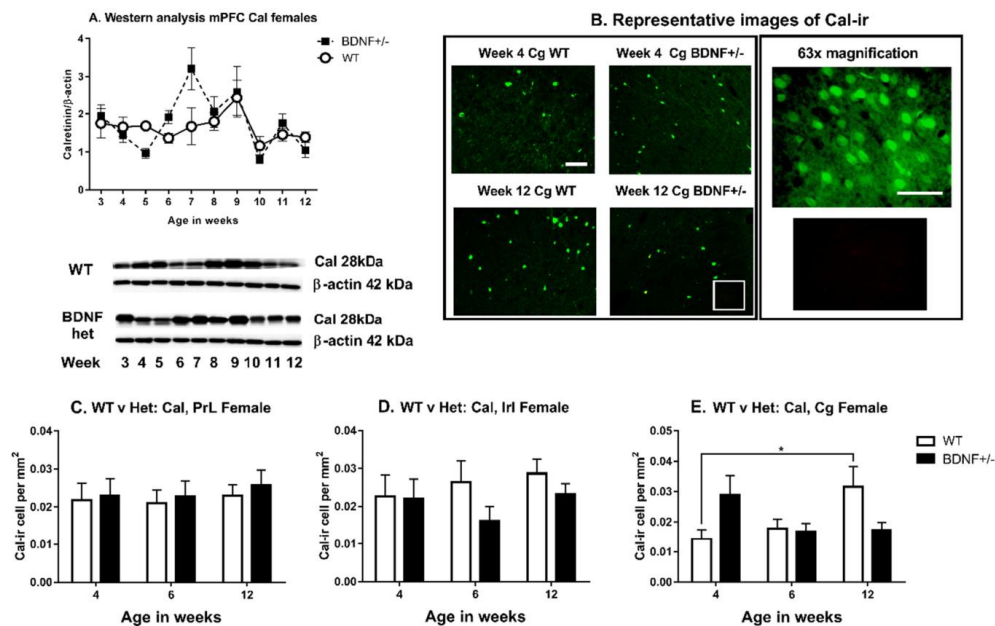


Fig. 5. Female Cal protein expression and cell density in the mPFC. Female Cal expression did not differ between genotypes. In WT mice, no changes were observed across age. However, female BDNF +/− exhibited changes in Cal expression with a significant increase in expression from week 5 followed by a decrease in expression from week 9 onwards (A). This was reflected in the PrL (C) and the Irl (D). In the Cg, however, the WTs show a significant increase in Cal-ir density at 12 weeks (E). Representative images (B) in the left box (10× magnification) show the increase in Cal-ir density in WT Cg from week 4–12 and the relative stable density in BDNF +/−. Negative control minus primary antibody is displayed in the small box situated in the right corner. Top image in the right box (63× magnification) shows Cal-ir staining in Irl of WT mice; bottom image shows lack of auto-fluorescence in the red channel. $N = 5-7/\text{group}$, * $p < 0.05$. Scale bar = 100 μm for 10× magnification and 60 μm for 63× magnification.

$_{23}) = 7.233$, $p = 0.0037$) but no significant effect of genotype or genotype × age interaction. Post-hoc analysis revealed that there is an overall increase in Cal-ir density from 4 to 6 weeks of age ($t_{(23)} = 3.757$, $p = 0.003$). In the Cg, there was a significant genotype × age interaction (Fig. 6E, $F_{(2, 23)} = 3.988$, $p = 0.033$). Post-hoc analysis did not reveal any significant differences albeit there were trends for lower Cal-ir density in the BDNF +/− group at 12 weeks of age compared to WT of the same age ($t_{(23)} = 2.417$, $p = 0.072$) and compared to BDNF +/− at week 4 ($t_{(23)} = 2.397$, $p = 0.075$). Representative images as shown in Fig. 6B show the significant increase in Cal-ir density in the PrL from week 4 to week 6 in WT but not BDNF +/− mice.

3.4. Mature BDNF expression in the medial prefrontal cortex

In females, the mBDNF expression across development exhibited significant main effects of genotype ($F_{(1, 77)} = 44.09$, $p < 0.001$) and age ($F_{(9, 77)} = 7.57$, $p < 0.001$). There was also a significant genotype × age interaction ($F_{(9, 77)} = 3.169$, $p = 0.003$). Post-hoc analysis show significantly higher mBDNF protein level in WT compared to BDNF +/− mice at ages 4, 6, 7 and 12 weeks and trending at 9 weeks (Fig. 7A). In male mice, analysis of mBDNF expression saw significant main effect of genotype ($F_{(1, 68)} = 5.04$, $p = 0.028$) and a trend for significant effect of age ($F_{(9, 68)} = 1.99$, $p = 0.054$), with WT showing overall higher levels of mBDNF compared to BDNF +/− mice. There was however no significant genotype × age interaction (Fig. 7B).

3.5. Correlations between mBDNF and interneuron expressions

Regression analysis of mBDNF protein expression with PV protein

expression found positive correlations between the two proteins in both female WT ($F_{(1, 39)} = 12.48$, $R^2 = 0.242$, $p = 0.001$) and BDNF +/− mice ($F_{(1, 44)} = 11.79$, $R^2 = 0.211$, $p = 0.001$) (Fig. 8A and B respectively). In males, a significant positive correlation was observed in WT ($F_{(1, 36)} = 15.86$, $R^2 = 0.306$, $p = 0.0003$) but not in BDNF +/− mice ($F_{(1, 37)} = 0.342$, $R^2 = 0.02$, $p = 0.342$) (Fig. 8C and D respectively).

Regression analysis of mBDNF protein expression with SST protein expression found positive correlations between the two proteins in both female WT ($F_{(1, 36)} = 5.66$, $R^2 = 0.136$, $p = 0.023$) and BDNF +/− mice ($F_{(1, 41)} = 20.56$, $R^2 = 0.334$, $p < 0.0001$) (Fig. 8E and F respectively). In males, there were no significant correlations between mBDNF and SST expression in either WT ($F_{(1, 39)} = 1.01$, $R^2 = 0.025$, $p = 0.321$) or BDNF +/− mice ($F_{(1, 40)} = 0.004$, $R^2 = 9.24 \times 10^{-5}$, $p = 0.952$) (Fig. 8G and H respectively).

Regression analysis of mBDNF protein expression with Cal protein expression found no correlation in WT female mice ($F_{(1, 40)} < 0.0001$, $R^2 = 1.76 \times 10^{-5}$, $p = 0.979$) but a significant negative correlation in BDNF +/− female mice ($F_{(1, 38)} = 4.75$, $R^2 = 0.111$, $p = 0.036$) (Fig. 8I and J respectively). In males, there were no significant correlations in either WT ($F_{(1, 39)} = 0.351$, $R^2 = 0.022$, $p = 0.351$) or BDNF +/− mice ($F_{(1, 42)} = 1.636$, $R^2 = 0.037$, $p = 0.207$) (Fig. 8K and L respectively).

3.6. Correlation between PV and Cal protein expressions

Regression analyses of PV and Cal protein expression levels showed a significant negative correlation in both WT ($F_{(1, 44)} = 18.94$, $R^2 = 0.301$, $p < 0.001$) and BDNF +/− males ($F_{(1, 45)} = 4.566$, $R^2 = 0.092$, $p = 0.038$) (Fig. 9A and B). In female mice, there was no

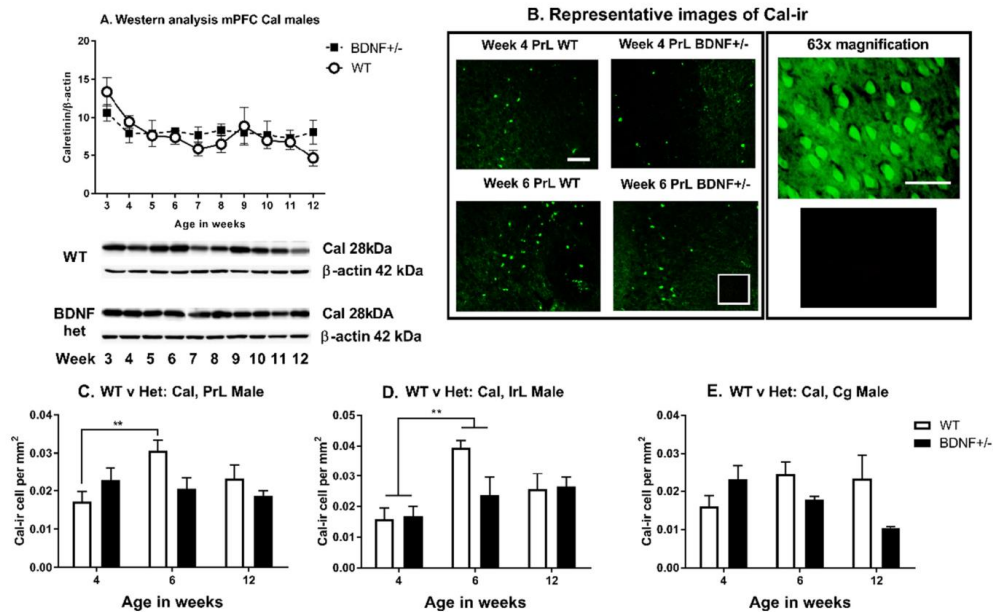


Fig. 6. Male Cal protein expression and cell density in the mPFC. Male mice did not exhibit genotype differences in CR expression. WT male show a significant and steady reduction in CR expression whereas expression in BDNF +/− mice remained flat (A). In the PrL (C) and IrL (D), WT Cal-ir density increased from week 4 to week 6 but was flat in BDNF +/−. No significant alterations were detected in the Cg for either genotype (E). Representative images (B) in the left box (10× magnification) show the increase in Cal-ir density in WT PrL from week 4–6 and the relative unaltered density in BDNF +/−. Negative control minus primary antibody is displayed in the small box situated in the right corner. Top image in the right box (63× magnification) shows Cal-ir staining in PrL of WT mice; bottom image shows lack of auto-fluorescence in the red channel. $N = 5-7/\text{group}$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar = 100 μm for 10× magnification and 60 μm for 63× magnification.

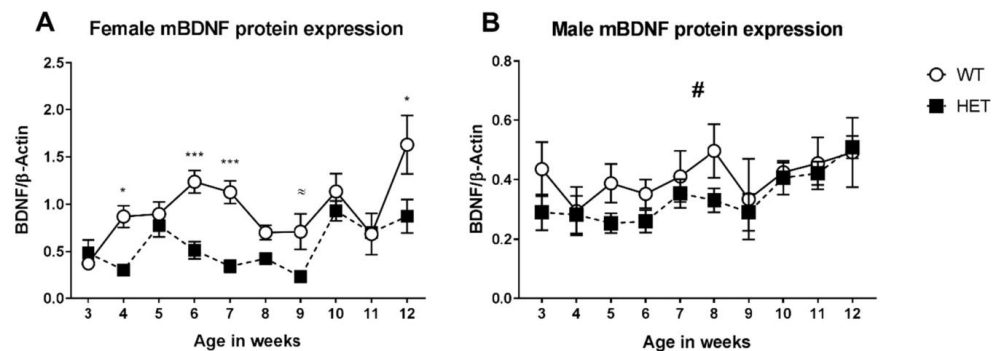


Fig. 7. Female and male mBDNF protein expression in the mPFC across development. Female mBDNF expression (A) was significantly higher in WT compared to BDNF +/− mice at weeks 4, 6, 7 and 12 and trending at week 9. Male WT (B) show an overall significantly higher mBDNF expression compared to BDNF +/− mice. $N = 2-6/\text{group}$, # signify significant overall effect of genotype, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $\approx p < 0.1$.

significant correlation between PV and Cal protein expression in WT mice ($F_{(1, 48)} = 2.381$, $R^2 = 0.047$, $p = 0.129$) but a trend for a negative correlation in BDNF +/− mice ($F_{(1, 44)} = 3.71$, $R^2 = 0.078$, $p = 0.061$) (Fig. 9C and D).

4. Discussion

The results in this study showcase both the dynamic nature of GABAergic interneuron expression during adolescence in the mPFC and

marked sex-differences in their cell density. It also highlights the variation between different interneurons in their response to reduced BDNF in males and females.

Throughout development, particularly during adolescence, PV mRNA expression increases in the dorsal lateral prefrontal cortex of humans (Fung et al., 2010) and in both the mPFC (Caballero et al., 2014) and ventral hippocampus (Caballero et al., 2013) of rats. Our finding of an ascending expression of PV protein level in female WT and BDNF +/− mice throughout the developmental period corroborates

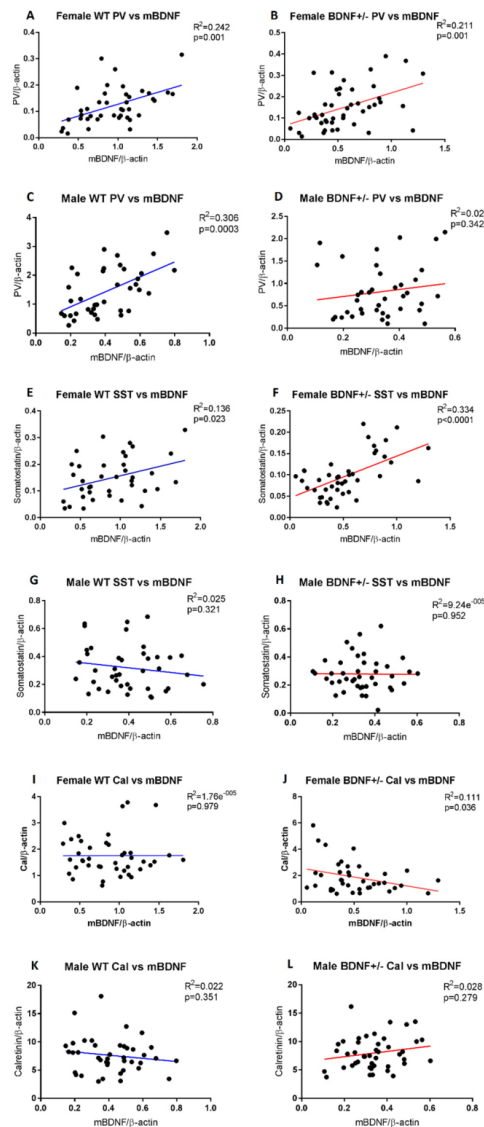


Fig. 8. Linear regression of mBDNF protein expression and GABAergic interneuron marker protein expressions in the mPFC. Significant positive correlation between mBDNF and PV protein expressions were observed in female WT and BDNF +/– mice (A and B) as well as in male WT but not BDNF +/– mice (C and D). There was a significant positive correlation between mBDNF and SST protein expression in female WT and BDNF +/– mice (E and F) but not in male WT or BDNF +/– mice (G and H). There was no significant correlation between mBDNF and Cal in WT female mice (I) but there was a significant negative correlation in female BDNF +/– mice (J). No correlation was observed in either male WT (K) or BDNF +/– mice for Cal and mBDNF protein expression (L). $N = 2$ –6/group.

these earlier findings. Immunohistochemical analysis however, when disentangling the subregions, shows that BDNF +/– mice exhibit overall higher PV-ir density in the Cg but reduced density in the Irl compared to WT mice. This may suggest that the Irl PV is more sensitive to BDNF deficiency. Alternatively, reduced PV in the Irl but increased PV expression in the Cg of BDNF +/– mice may be a result of an altered migration pathway of PV interneurons, as BDNF has an important role in regulating interneuron migration in the cortex during development (Polleux et al., 2002). This hypothesis would require further detailed analysis of early migration of PV interneurons in BDNF +/– mice. Future studies to assess neurogenesis, apoptosis and the presence of GABAergic populations within the white matter will help to decipher how BDNF heterozygosity causes reductions in cell density.

In males, whereas WT mice display a similar ascending expression profile of PV protein expression throughout adolescent maturation, the protein expression in BDNF +/– mice remains low throughout the ages examined. This was also evident from PV-ir cell density in the mPFC, which increased with age in WT mice but remained unchanged in BDNF +/– mice, matching the protein expression. BDNF plays a role in promoting differentiation and maturation of GABAergic neurons (Waterhouse et al., 2012). Here, the lack of change in female BDNF +/– mice compared to WT, in opposition to the male BDNF +/– specific alteration in PV expression, suggests that estradiol is protective for PV neurons against loss of trophic support from BDNF. The increase in PV cell density in WT mice was most notable in the Irl, where levels in BDNF +/– mice remained significantly lower. In contrast, PV cell density in the Cg was largely unchanged by age or genotype. Once more, our results show a demarcation between Irl and Cg in their sensitivity to BDNF influence. A recent paper indeed found that sensory deprivation for 28 days from birth resulted in a decrease in PV cell counts in the Irl but not in the PrL or the Cg cortices (Ueno et al., 2015), suggesting this subregion is particularly sensitive to deficits in activity-driven BDNF. The PrL and Irl have greater connectivity with limbic structures compared to the Cg, and as such are involved in the regulation of cognitive activities such as spatial planning (Hok et al., 2005). The more ventral Irl has particularly distinct roles in extinction learning that is heavily dependent on BDNF (Pattwell et al., 2012; Peters et al., 2010). The apparent developmental sensitivity to BDNF levels uncovered here may equate to vulnerability to stress as suggested by a recent study (Moreines et al., 2017) and our own two-hit study, where spatial deficits were detected in male but not female BDNF +/– mice after corticosterone treatment (Klug et al., 2012). However, given the constitutive nature of the BDNF +/– model, further work is required using more temporally and spatially precise models to confirm this relationship.

In light of previous findings of reduced PV neurons in the prefrontal cortex (Beasley and Reynolds, 1997; Beasley et al., 2002), and protracted PV maturation with reduced mRNA and protein expression in the dorsal lateral prefrontal cortex of people with schizophrenia (Fung et al., 2010; Glausier et al., 2014), our data suggest BDNF is a possible mechanism to explain this abnormal PV expression. Correlations have been reported between reduced BDNF and TrkB mRNA expression and reduced PV in the prefrontal cortex of schizophrenia patients as well as mice with genetically reduced TrkB expression (Hashimoto et al., 2005). The fact that male but not female BDNF +/– displayed significantly lower PV protein expression and cell density across the mPFC suggests estradiol may have a protective effect in female BDNF +/– mice, supporting our previous findings where estradiol modulated PV expression in the hippocampus of female WT mice during maturation (Wu et al., 2014) and is required for normal gamma oscillation and memory performance (Schroeder et al., 2017).

In the current study, the delayed SST developmental trajectory in terms of protein expression observed in the female BDNF +/– mice compared to WT mice suggests a role of BDNF in the maturation of SST interneurons in the mPFC during adolescence. In males on the other hand, the unchanging SST protein expression points to a sexually-

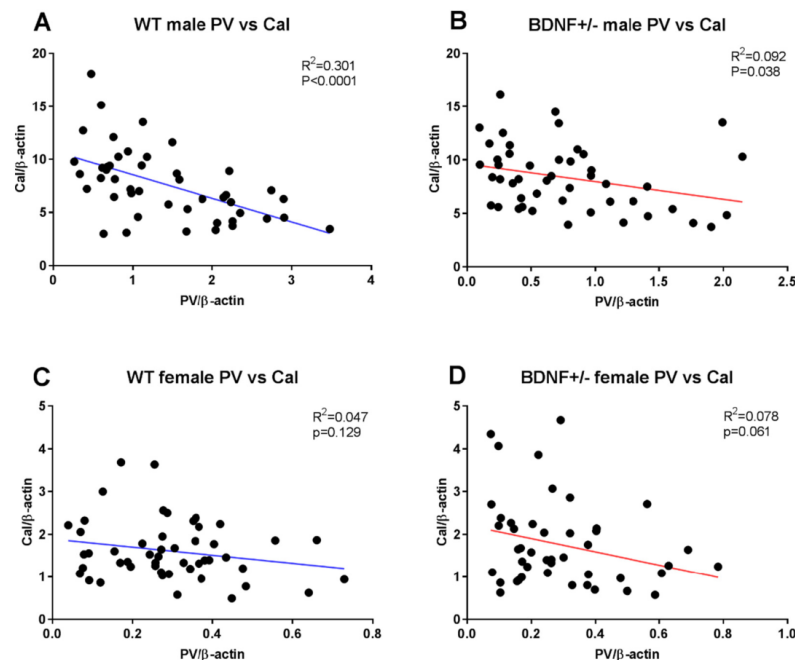


Fig. 9. Regression analyses of PV and Cal protein expression in mPFC. Significant negative correlation between PV and Cal protein expressions was observed in WT (A) and BDNF +/– mice (B). However, no significant correlations were seen in females for either WT (C) or BDNF +/– mice (D).

dimorphic developmental trajectory, which may be mediated by sex steroid hormones, as SST mRNA expression is upregulated by testosterone and not estradiol (Argente et al., 1990), hence testosterone may protect males from reduced BDNF.

SST-ir density in WT females follows a similar pattern to the protein expression with an increase in cell density from week 4–6. Interestingly, the SST-ir density in female BDNF +/– mPFC has the opposite trajectory to the SST protein expression. It has been reported that SST neurons of adult schizophrenia patients produce less SST mRNA per cell compared to controls (Morris et al., 2008). Here we suggest that the initial increase in SST neuron density in BDNF +/– could be a compensatory response for lack of cell functionality. The subsequent reduction of SST-ir density in conjunction with increased SST protein expression could signify recovery of cellular function. Further electrophysiological tests to assess functionality of these cells at different time points would be required to confirm this hypothesis. It is possible that in schizophrenia patients, a similar initial reduction in SST neuron functionality can represent a vulnerability to environmental factors – reduced SST is a risk factor to stress-induced anxiety/depressive-like behaviour in mice (Lin and Sibille, 2015). The cell density in male mice for total mPFC mirrors the protein expression data with no significant changes. A previous study reported sex-specific effects of forebrain BDNF loss on anxiety/depression phenotype with female mice being more affected by BDNF loss than males (Autry et al., 2009). Furthermore in a human post-mortem study reductions in SST mRNA expression were more pronounced in females with major depression compared to males (Seney et al., 2013). Thus, taken together the above studies and ours suggest that female SST positive interneurons may be more vulnerable to loss of BDNF and consequent anxiety/depression related behaviours which ensue following mPFC SST reduction.

Cal protein expression in WT male mice significantly decreases with

age. However, Cal levels in BDNF +/– male mice remained unaltered across the developmental periods. Studies of Cal expression in schizophrenia suggest it is relatively unaltered in cortical areas of patients compared to PV or SST (Hashimoto et al., 2008b; Hashimoto et al., 2003; Volk et al., 2012). In male rats, the developmental trajectory of Cal protein expression has been found to decrease across development from juvenile, through adolescence till adulthood, a trajectory opposite to that of PV and similar to our findings in male WT mice (Caballero et al., 2014). Evidence suggests Cal interneurons innervate PV interneurons and assert inhibition (Caputi et al., 2009) and that a switch occurs in favour of PV across development which may underlie advances in higher cognition maturation (Caballero et al., 2014). Indeed we observed significant negative correlations between PV and Cal protein expressions in both WT and BDNF +/– mPFC, albeit less significant in the BDNF +/– mice. What appears to be a failure of making this switch in BDNF +/– mice suggests irregular maturation in the male forebrain as a consequence of reduced BDNF. In female mice on the other hand, Cal protein and cell density levels were relatively unchanged. There was also no correlation between PV and Cal protein expressions. The relatively stable Cal expression in females may be due to the lower basal expression of Cal observed in female rodents (Brager et al., 2000; Lephart et al., 1997).

It is noticeable that the normalised signal intensity for protein expressions of interneuron markers are consistently higher in males compared to females in the current study. While the Western blot analyses for each sex were conducted separately to accommodate the multiple age groups as well as genotypes, and therefore direct comparisons may not be an accurate reflection of comparative levels of expression, future study should directly examine potential sex differences regarding interneuron marker expressions. To the best of our knowledge, direct comparison of interneuron marker protein expression

in the mPFC has not been done. This might be informative towards explaining sex differences in phenotypes pertaining to schizophrenia.

The mBDNF protein level in the mPFC is reduced in BDNF +/– mice of both sexes compared to their WT counterparts, albeit more pronounced in the females. This sex difference is similar to our previous results, whereby female BDNF +/– show a more robust reduction of BDNF than males (Hill and van den Buuse, 2011). However, as protein expression of both sexes was examined separately, some differences might be due to inter-experimental differences in Western sensitivity. Future studies should directly compare male and female BDNF expression to directly compare protein expressions at developmental stages. Both sexes show an ascending trajectory of mBDNF expression across development. As expected, levels of mBDNF protein were correlated to PV protein levels in both male and female WT mice. However, male BDNF +/– mice showed no correlation between mBDNF and PV, highlighting once again a reciprocal disturbance in their adolescent development. The ability of female BDNF +/– but not males to maintain a WT-like PV protein expression profile across development, as well as exhibiting much milder alterations in PV-neuron density in the mPFC, and preserving a positive correlation between mBDNF protein level and PV, suggest certain compensatory mechanisms are occurring in female but not male mice, possibly via estrogen signalling, onto converging intracellular targets such as the MAPK/ERK and the PI3K pathways (Hill, 2012). The evidence presented confirms the epidemiological data, which favours female schizophrenia patients, especially in the cognitive domain. We also suggest a potential mechanism – the ability of females, possibly through estrogen signalling, to preserve relatively normal development of GABAergic interneurons, particularly PV-interneurons, in the face of reduced BDNF, throughout the adolescent transition. These data lend further support to the notion that the BDNF and estrogen signalling pathways are promising therapeutic targets, especially in regards to the hitherto untreatable cognitive deficits in schizophrenia (Du and Hill, 2015; Owens et al., 2017).

5. Conclusion

The findings of this study are the first to describe idiosyncrasies during the maturation processes of PV, SST and Cal in the mPFC in sex-specific and reduced BDNF environments, during the vulnerable adolescent period. In particular, the female milieu appears protective against the negative effects that reduced BDNF have on PV expression as observed in male BDNF +/– mice. Conversely, SST in the mPFC seems to have especially important roles in females and hence its expression alters dynamically during adolescence.

Future studies should examine the mechanisms underlying the sexually dimorphic nature of interneuron development which may provide new avenues of targeted treatment particularly in regards to cognitive ability in male versus female patients.

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Conflicts of interest

None.

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The Importance of Distinguishing Allocentric and Egocentric Search Strategies in Rodent Hippocampal-Dependent Spatial Memory Paradigms: Getting More Out of Your Data

Adrienne M. Grech, Jay Patrick Nakamura and Rachel Anne Hill

Additional information is available at the end of the chapter

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Abstract

While the brain works as a dynamic network, with no brain region solely responsible for any particular function, it is generally accepted that the hippocampus plays a major role in memory. Spatial memory operates through the hippocampus with communication with the prefrontal and parietal cortices. This chapter will focus on two separate reference frames involved in spatial memory, egocentric and allocentric, and outline the differences of these reference frames and associated search strategies with relevance to behavioural neuroscience. The importance of dissociating these search strategies is put forward, and steps researchers can take to do so are suggested. Neurophysiological and clinical differences between these spatial reference frames are outlined to further support the view that distinguishing them would be beneficial.

Keywords: allocentric, egocentric, hippocampus, maze, navigation, networks, spatial memory

1. Introduction

Spatial memory is the cognitive process of noticing, encoding, and retrieving landmarks in the surrounding environment, to allow an organism to navigate and exist in the world. It is important for survival, by enabling searching and finding safety and food and being able to return to found places without issue. It is the domain of the hippocampus and medial temporal lobe,

with links to the retrosplenial cortex and parietal cortex [1]. Seminal studies in humans and animals have demonstrated the important role that the hippocampus plays in navigating the world around us [2, 3]. In humans, damage to the temporal lobe causes disturbances to spatial navigation [4], and similarly, humans employed in roles that require fantastic spatial navigation skills have enlargement of the hippocampus and its connections [5, 6]. In parallel, through multiple manipulations such as lesion, electrophysiological and optogenetic studies, the hippocampus has been shown to be equally important to animal spatial memory. Disruptions to hippocampal tissue or silencing of neurons in the hippocampus leads to spatial memory deficits [7, 8]. This parallel role of the hippocampus in both humans and animals allows research to be performed on these animals with the insights gained able to be extrapolated to humans.

2. Spatial memory in behavioural testing

Behaviourally characterising an animal model of disease often involves a battery of tests that investigate the animal's motivation, locomotor activity, startle reflex, anxiety, fear response, social behaviour, learning, memory and other emotional and cognitive traits. Dysfunctions in these behaviours are used to infer structural and functional changes in the brain, and the recovery of performance on these tests is used to evaluate the effectiveness of potential therapeutics. These inferences are only accurate with the use of appropriate tests with high specificity both for the behaviour in question and in terms of the specific brain regions recruited during test performance. Therefore, behavioural tests that are specific to one domain or behavioural tests that can correctly dissociate multiple domains should be used. Rodent spatial memory tests, often mazes, are commonly used in preclinical drug development and fundamental science experiments. The use of these behavioural tests dates back over a century, and a plethora of maze designs have been developed since then to probe different aspects of learning and memory [9]. Complex networks of brain regions and neuron populations are required to orientate and navigate using information such as environmental, vestibular and proprioceptive cues [10]. The current general consensus is that spatial memory encompasses two distinct but related reference frames, egocentric and allocentric. Here, we outline the differences between these reference frames and their relevance in behavioural neuroscience and discuss the merits of placing a stronger emphasis on distinguishing egocentric and allocentric search strategies in spatial memory tests.

3. What are allocentric and egocentric search strategies?

The egocentric reference frame is also referred to as a fixed, self-centred or first-person perspective. Egocentric navigation is based on direction (left-right) responses and actions independent of environmental cues. Directional decisions are made at single or sequential choice points; however, these locations are not used as cues and are therefore still egocentric in nature [11]. For example, memorising routes based on sequential turns would employ a mostly egocentric strategy (**Figure 1A**). Path integration, the summation of travelled vectors to deduce current position, is an example of an egocentric strategy that can navigate through

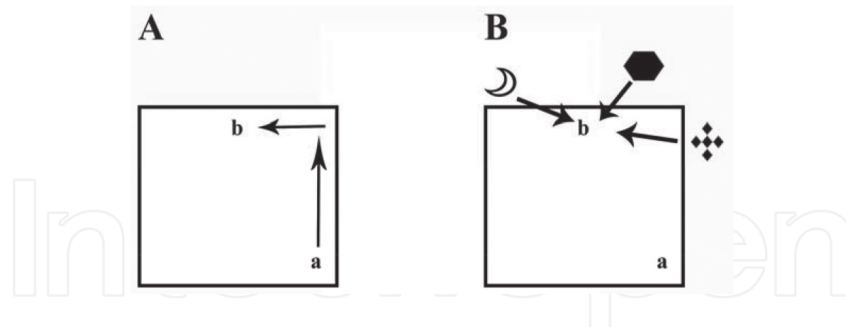


Figure 1. Schematic of egocentric (A) and allocentric (B) frames in a spatial memory task. Within each arena, (a) is the start position and (b) is the goal location. Egocentric strategies are referenced from self with set directions and distances to the goal (shown indirect here but may also be direct). Note that if the start position (Aa) is changed, the strategy would fail to reach the goal (Ab). Allocentric strategies relate the location of the goal to visual cues. Note that if the start position (Ba) is changed, the strategy would still successfully locate goal (Bb). If the visual cues are moved, the strategy would fail to reach the goal (Bb).

novel paths. The allocentric reference frame, on the other hand, can be thought of as a third-person perspective. Allocentric navigation utilises external cues or landmarks in relation to each other to navigate and is independent of self (**Figure 1B**). Utilising compass directions (north, south, east, west) is an example of allocentric reference frame use as these directions are relative to the Earth and do not change depending on the orientation of the navigator [12]. An advantage of allocentric navigation is the flexibility of being able to locate novel points from various start locations as long as the external cues remain the same. In situations where external cues are changing, minimal or absent, egocentric strategies become more salient [1].

Navigating environments outside of experimental settings requires the use of both allocentric and egocentric reference frames, with relative saliencies falling within a spectrum [1]. Experiments in controlled settings with specifically designed spatial memory tasks aim to dissociate these reference frames; however, it is argued that complete dissociation is not achieved [1]. Nevertheless, the employment of more precise tasks as well as the use of more rigorous analytical techniques allows greater dissociation and investigation into navigational strategy preference and specific dysfunctions in reference frames. Nonspatial strategies such as random or serial searches can often be successful in that they result in lower latencies to a goal. These, however, are not indicative of spatial memory, and measures should be put in place to detect such strategy use. The following section provides an overview of the various spatial memory tasks currently used in behavioural neuroscience and their ability to effectively probe egocentric and allocentric search strategies.

4. Spatial memory and navigation paradigms

There are a large variety of behavioural tests for both rodents and humans that provide a measure of spatial memory and navigation [9, 13, 14]. Generally, rodent spatial memory tests

utilise maze apparatus that have a goal area that the animals must find, learn and remember. These goals can be positive reinforcements such as food rewards, escapes from negative stimuli such as water or bright light or a result of instinctive behaviour such as exploratory drive. Human spatial memory testing, on the other hand, is mostly conducted on virtual reality setups that create controlled three-dimensional environments with goals usually being explained to the subject by the researcher. More recently, steps have been taken to combine aspects from both animal and human tests to increase the similarity and therefore translatability of these tests. Virtual reality versions of rodent tests have been developed for humans [15], and virtual reality and touchscreen setups for rodents that were developed from human equivalents have also become popular [16, 17]. Distinguishing allocentric and egocentric reference frames and search strategies used in spatial memory tasks for rodents differs depending on the type of test. Some tasks are designed to encourage employment of a single strategy, and so performance on that task is reflective of the saliency of that particular reference frame. Other tasks can be completed with a combination of allocentric and egocentric strategies, and subsequent analysis or probe tests are needed to infer deficits or preferences in these reference frames. Consideration of what types of spatial navigation are being tested, and extra steps to dissociate these strategies are often overlooked, despite the relative ease of implementing such measures. Below we discuss popular maze apparatus used to investigate spatial memory and various tests, controls and analyses that can help distinguish egocentric and allocentric navigation.

Spatial memory can be investigated through a variety of tests on mazes such as the Y-maze, cheeseboard maze, Morris water maze, Star maze, Barnes maze, radial arm maze and T-maze. These mazes encompass investigation of a range of spatial memory, including long-term, short-term and working memory, as well as cognitive flexibility. Tests that probe allocentric reference frames include the use of static visual cues which the rodent can use to develop a cognitive map. Efforts are made to minimise proximal cues and create open, unobstructed spaces to avoid non-allocentric strategies. The opposite is true for egocentric tasks where visual cues are minimised or made irrelevant (incorrect or random). The most accurate way of testing for egocentric strategies is to perform a test in the dark, which ensures removal of visual distal cues that could be used for allocentric strategies [18]. Many apparatus that are used to investigate egocentric navigation restrict movements to narrow channels or arms to create distinct choice points where egocentric strategies are encouraged [19].

Constructed in the shape of a capitalised 'T', the **T-maze (Figure 2A)** is a simple apparatus used to probe working and short-term spatial memory. Due to the shape of the maze, only two options, a 90-degree left or right turns, are available to the rodent when leaving the start arm. The T-maze can be unbaited, baited or use negative stimuli to drive exploration of the maze [20]. Generally, one of the arms is correct (unexplored, food/water rewarded, containing escape platform) and is learnt in the presence of intact memory. Internal and external visual cues can be used to probe navigational strategy [21]. Briefly, animals can be trained with the presence of extra-maze visual cues and an intra-maze visual cue. Reaching the goal arm can be achieved by either remembering to turn in the correct direction, move towards or avoid the intra-maze cue or move to the correct area in relation to the static external cues. Following successful acquisition of the task, animals can be tested on probe trials which involve systematically switching the cues or correct turn direction so that they are now incorrect. Indeed,

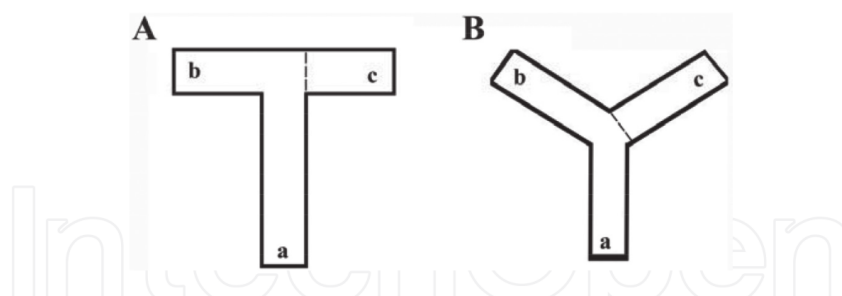


Figure 2. Schematic of a T-maze and Y-maze. (A) is the start location and the (B) and (C) arms are the choice arms. One choice arm (C shown here) may be physically blocked during the first phase of unbaited tests to create a novelty seeking drive to that arm when made accessible in the second phase.

rats were shown to have an overall preference for a direction-based strategy on the T-maze, suggesting that this apparatus encourages egocentric navigation [21]. Using similar visual cue manipulations on the T-maze, transgenic mice expressing an Alzheimer's disease-related mutation were shown to have specific allocentric place learning deficits in the absence of a general disruption in learning and memory, highlighting the importance of including these probe tests when possible [22].

The **Y-maze** (**Figure 2B**) works much in the same way as a T-maze; however, the apparatus is designed in a Y shape with three equal arms at 120 degrees from each other. Unbaited tests are popular on this apparatus, relying on the animal's innate preference to explore previously unexplored areas. Short-term memory can be tested by blocking access to one of the arms in the first phase of the test and observing the time spent in that arm in the second phase where all three arms can be accessed. There is a variable delay between phases to control short-term memory load of the task. This novel arm preference task is a test for allocentric spatial memory as rodents use both intra- and extra-maze cues to remember the location of the novel arm. Working memory can also be tested by allowing the animal to freely explore all three arms and observing if they chose to enter the arm most recently explored or they alternate and enter the more novel arm—this is called spontaneous alternation. Spontaneous alternation can also be investigated on the T-maze; however, because the arms of the Y-maze are equal (and can each become new start arms), alternation can be continuously measured without constant investigator interaction. Modifying the protocol to include baited arms and including or removing the use of proximal and distal cues allows for the investigation of allocentric and egocentric strategies [23–25].

The **Biel water maze** was developed by William Biel [26] and is constructed of multiple T-mazes that interconnect to create a labyrinth in which rodents must navigate from the 'Start' to 'Goal' to escape the maze. The maze is run in visible light, and no explicit distal cues are provided; in addition, the maze is covered by a large container to minimise access to both distal and proximal cues. Parameters that are used to measure egocentric navigation include errors across trials and escape latency. However, this maze had limitations in design and level of difficulty, most importantly that it was run in visible light which could provide distal or proximal cues from the box overhead [19]. The **Cincinnati water maze (CWM)** is an extension of the Biel water maze. It is a complex labyrinth water maze consisting of nine interconnecting

T-mazes (Figure 3). An experimental rodent must get from position A to position B and is motivated by its survival instinct to leave the water. It is designed to employ egocentric search strategies based on the physical dimensions and design of the maze that creates nine choice points (rather than six in the Biel water maze) at intersections where rodents are required to make a left or right turn. The CWM is constructed using an acrylic material so that the walls are smooth, and no proximal cues are available. The width of the channels ensures the rodent cannot climb the walls of the maze, and running the test in the dark under infrared light can act as a double insurance against the use of visual cues [19]. Generally, the number of errors, number of start return and latency to escape are the main parameters reported for this maze.

The radial arm maze (RAM) consists of a central circular area from which multiple arms radiate outwards. Rodent spatial memory is measured by the ability to remember the location of baited arms through the use of salient cues around the maze room (allocentric) [27] or an egocentric-focused paradigm that employs forced arm entry. An example of an egocentric paradigm using the RAM follows. In this instance the maze has automated doors that open and close to allow entry for the animal. The animal starts in one arm, and once the experiment starts, two adjacent arms to the start arm are opened to construct a Y shape. There will be a food reward at the end of one arm, determined for each mouse to be either left or right. The maze arm entered by the animal becomes the new start arm, which the animal is restricted to during an intertrial interval. Following an intertrial interval, two arms adjacent to the new start arm are opened, with the direction of arm (left or right) being correct with a food reward. The experiment continues in this fashion and requires the animal to navigate the maze in reference to its own position [28]. By limiting access to only three arms (in addition to the original start arm) at a time, this insures against a non-egocentric strategy to be used by the

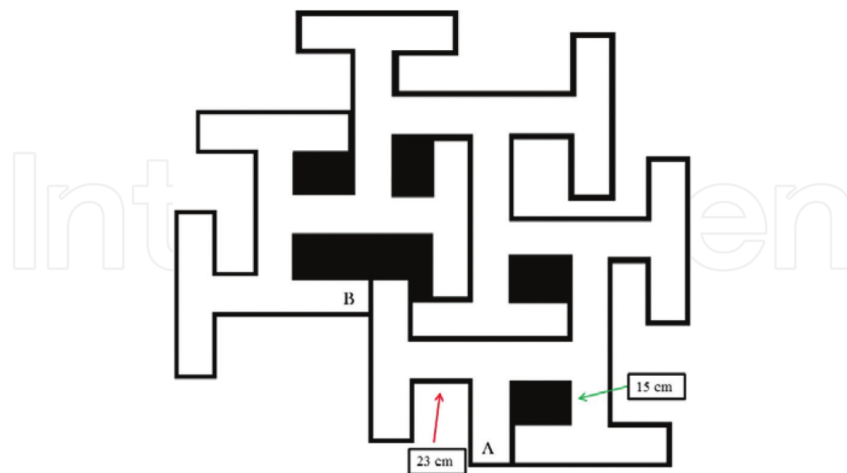


Figure 3. The Cincinnati water maze (CWM), original image from Vorhees and Williams [19], is a labyrinth-like maze that is performed in the dark. The forced left or right choice in addition to the lack of visual cues promotes egocentric strategies. (A) Is the start location and (B) is the escape platform.

animal. For example, if all arms of the RAM were available, the animal could use the serial strategy of entering each arm sequentially in order to find the food reward. For the RAM, measurements such as number of errors and rank of the first error [27] are reported to indicate memory performance. While the RAM can be used to investigate both egocentric and allocentric search strategies, the armless Morris water maze became the standard for allocentric testing [13], with the open opaque water acting as a mask for both choice points to learn a set sequence, and olfaction. In contrast to the armed designs of egocentric tests, mazes that target allocentric spatial strategies are designed to be open and free from intra-maze objects/edges that may act as choice points [13].

The Barnes maze is based upon the preference for dark, enclosed spaces by rodents. It is an open circular maze with holes around the perimeter (**Figure 4**). Underneath one of these holes is the 'target box' goal, which provides a small enclosed space for the rodent. During testing the maze is flooded with bright lights, sounds and/or air jets to provide motivation to find the goal. Distal cues are provided around the room to help the rodent navigate. Number of errors, escape latency and search strategies are commonly reported as a measure of spatial memory performance [20]. Visual cue manipulations on the Barnes maze show that distal cues are more salient than proximal cues, with animals trained without distal cues (with a marker at the goal location) showing decreased performance [29]. Thus this task tends to encourage allocentric strategies.

The Morris water maze (MWM) has been an integral part of neuroscience research as a gold standard when testing spatial memory in rodents since its introduction (Morris et al. [38]). The MWM utilises a large, circular pool with opaque water and a hidden escape platform (**Figure 5A**). Multiple distal cues are placed around the maze to aid the rodent to reach the

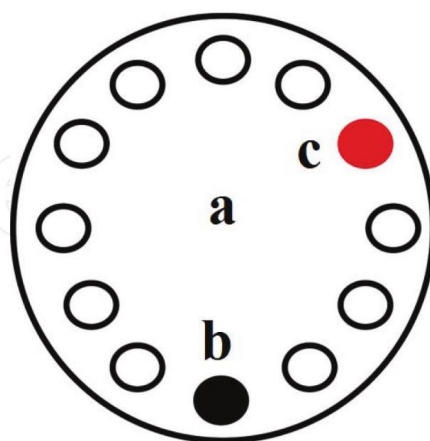


Figure 4. Schematic of the Barnes maze. Animals start in the Centre of the maze (A) and must find and remember the location of the hidden escape box (B). After acquisition, the correct location can be changed (C) to investigate cognitive flexibility.

hidden platform they use to escape. Most protocols are performed over multiple days, with multiple trials per day, and while the hidden platform position remains the same, starting position for the research animal is often changed to minimise egocentric strategies. However, if the start location is kept consistent, and the test is performed in the dark without external cues, rodents can complete the MWM using an egocentric strategy [30]. After training, the escape platform is removed, and reference memory is tested. Animals are expected to spend an increased amount of time in the quadrant where the goal previously was. The location of the goal can also be changed to investigate reversal learning and cognitive flexibility. The main motivation for the rodent to navigate the maze is to escape the water. The main advantage of the MWM when testing allocentric search strategies is the removal of intra-maze visual and olfactory cues with the use of opaque water. Indeed, the masking of any available olfactory cues is imperative due to the rodents' powerful sense of smell and the use of olfaction in their navigation [31]. However, the water in the MWM can also be a disadvantage, especially when working with mice because they are not natural swimmers in the wild and become stressed in the water [32].

The cheeseboard maze (CBM) (Figure 5B) is a dry version of the MWM and is similarly a long-term spatial memory test as well as a measure of cognitive flexibility. The CBM is a uniform circular arena with wells that can be baited. The wells radiate in lines evenly from the centre of the board. Spatial cues are placed around the CBM. Rodents are food deprived for the duration of the experiment to provide motivation to find the food reward. The location of the baited well is different for each animal and is kept constant across trials and days for each individual mouse. Animals should learn to use the spatial cues placed around the maze to find the baited well from the start area in the centre to receive the reward and are expected to use allocentric search strategies. Following acquisition of the goal location, the location of the food reward is changed, and the animal then must adopt a new learning strategy (reversal). This is a measure of cognitive flexibility and is testing the ability of the animal to ignore the initial position of the reward and learn the new location of the second reward. Compared to the MWM which relies on survival motivation, the CBM relies on hunger drive. Both tasks involve distal cues to guide the mouse to its goal, be it the platform of the MWM or the food reward of the CBM. These different motivations could influence the cognitive processing of

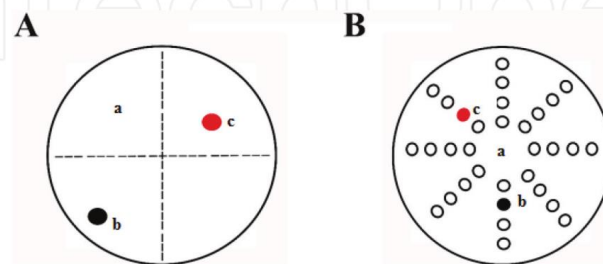


Figure 5. The Morris water maze (A) and the dry cheeseboard maze (B). (a) is the start location, (b) is the goal location, and (c) is a new goal location used to investigate cognitive flexibility. Both apparatus are circular, open-arena mazes that can contain goal locations in a range of xy coordinates.

the rodents. MWM has been criticised as unduly stressful [13], with the research animal having to employ avoidance learning. The CBM, while food deprivation may provide a similar stress [13], involves positive reinforcement through the food reward. There are some arguments that positive reinforcement may not be sufficient enough [13] to encourage the research animals to learn, in comparison to a test such as MWM where negative consequences must be avoided. It may be that each test provides a different angle to the study of cognition. Panicked stress may be detrimental to effective learning or a stronger drive compared to food deprivation. The main advantage of the MWM in terms of teasing out allocentric and egocentric strategies is that it is a cleaner allocentric maze. In the MWM, the use of opaque water that the rodents must swim through minimises the availability of choice points and olfactory cues. In comparison, the CBM is a maze that requires rodents to not only navigate using the distal cues but also around the wells. Hence, rodents may incorporate these wells into their navigation strategy—something that cannot be done in the MWM. This could provide an opportunity for the rodents to employ non-allocentric strategies, such as the serial strategy. This issue of detecting said egocentric versus allocentric search strategies is further discussed in the following section.

The Star maze (Figure 6), designed by Rondi-Reig et al. [33], is a purpose-built water maze that allows for the distinction of allocentric and egocentric search strategies. It is a circular water maze consisting of five water channels that form a central pentagon, and five water channels radiate out from this pentagon. The walls of the maze have a uniform colour, and the water is made opaque. The goal of the maze is to find the hidden platform in order to escape. Extra-maze cues on the walls are made available when analysing allocentric navigation. The setup of this maze allows for multiple protocols to test allocentric or egocentric navigation.

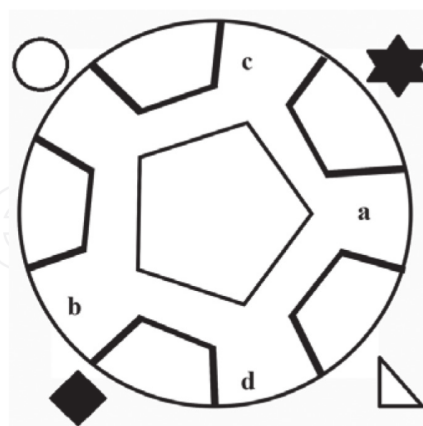


Figure 6. The Star maze, adapted from Rondi-Reig et al. [33], which is a water maze that allows for the investigation of spontaneous search strategy used by rodents. The design of the Star maze is such that either egocentric route learning or allocentric navigation can be analysed. For example, animals are trained from start position (a) to goal (b) until a threshold performance is reached. The start position is then moved to (c). An egocentric strategy would lead the animals to (d), whereas an allocentric strategy would continue to navigate to (b).

The first protocol, 'the multiple strategies version', is set up to investigate spontaneous navigation strategy that is employed by the rodent. The second protocol investigates egocentric navigation by setting up the maze so that a sequence of direction movements sends the rodent to the escape platform. The final protocol requires rodents to use the spatial cues provided in order to escape from randomly assigned start points [33]. This maze is a great setup as it allows the elucidation of individual search strategies, and given that it is a water maze, it controls for equal motivation and opportunity [13].

5. Analysing search strategies to compare the use of egocentric or allocentric search strategies

Spatial memory proficiency is commonly measured through a range of parameters in the above-mentioned mazes including latency, distance and time spent in target quadrants. However, evidence suggests that these analyses are not providing sophisticated enough insights into cognition and behaviour [34]. The Current trend is a deeper analysis of spatial navigation in order to produce more efficient research and more efficient use of research animals [34], moving beyond the well-known parameters of latency and distance. Research is now interested in the search strategy employed by research subjects and animals (**Figure 7**). Search strategy analysis can observe the complexity and dynamic nature of cognition employed in spatial memory mazes. For example, while different genotypes may have no significant differences in the typical parameters of latency, distance or target quadrant, a difference in approach to goal could exist and demonstrate changed cognition as a result of genotype. This may be more reflective of the innate differences that can exist in individual cognition despite similar anatomy. Of particular interest is the path trace analysis of allocentric tests in open field-type mazes, where movement is not restricted by walls (such as the MWM, CBM or Barnes maze). Although the absence of choice points aims to encourage allocentric strategies in these mazes, evidence suggests egocentric strategies can still be used; view-matching on distal cues can lead to egocentric cue guidance (e.g. face the star and then turn left) [35], which can successfully complete the task. Non-allocentric strategies such as serial strategies (visit all locations) and chaining (knowing distance from the edge of the maze) can also be successful strategies that also cannot be seen using traditional metrics (see **Figure 7**). These search strategies can be manually assigned through blinded categorisation or be analysed using automated algorithms. While historically latency and distance have been used as measures of cognitive disturbance in the MWM, time spent in the target quadrant on the probe day and search strategy are adjunct parameters that can provide a deeper analysis. Indeed, Rogers et al. [34] elegantly put forth how imperative investigating search strategy and setting up a high-powered experiment can be. Their study demonstrated not only the importance of high saliency cues but also the depth and breadth of information available through the analysis of search strategy.

The adoption of an allocentric search strategy is completely dependent on the quality of landmarks available [34]. This adds another consideration to the design of experiments for researchers; the setup of the maze must be carefully considered. Additionally, Rogers et al.

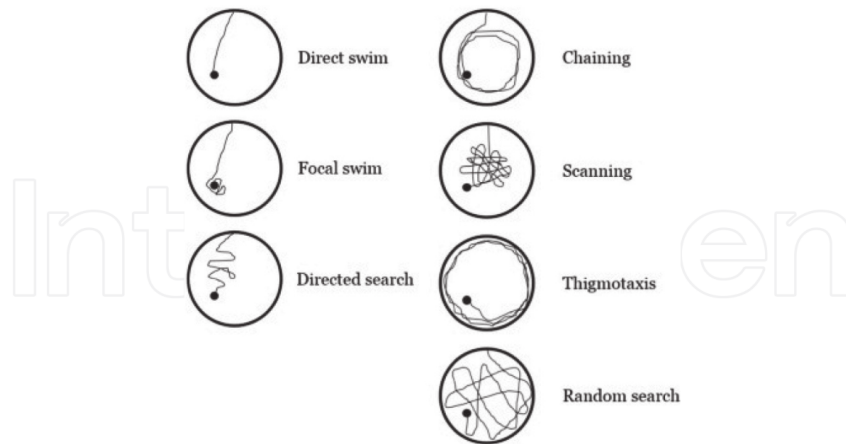


Figure 7. Selection of search strategies employed by rodents on the Morris water maze, adapted from Rogers et al. [34].

[34] demonstrated that the latency and path length parameters do not provide differentiation between the different search strategies and in fact do not provide a reliable analysis of spatial memory formation. From this arises the argument that not only does investigating search strategy allow for the elucidation of egocentric versus allocentric search strategies but that the saliency of distal cues allows the research animal to employ these strategies in the first place. It is important to note that more thorough methods for evaluating MWM performance have been suggested for a long time. The proximity measure, introduced in 1993, measures distance to the goal at a frequency of 10 Hz to get an average proximity throughout the trial. This measure was seen to be more sensitive than latency to the goal and was able to pick up subtle and otherwise masked effects [36]. Unfortunately, this measure is still currently under-reported and highlights the need to actively encourage extended analysis beyond latency, distance and time.

Building upon this, the study by Suzuki and Imayoshi [37] deftly investigated and presented a novel method of analysing navigation in the Barnes maze. The authors titled this 'network analysis method', which allowed for the visualisation of a rodent's exploratory patterns. The method involves several algorithms which initially determine the search strategy employed by a rodent (spatial, serial or random). Following this analysis, Suzuki and Imayoshi [37] were interested in determining if particular networks were associated with particular search strategies. A local network is the exploratory behaviour pattern of one mouse of one experimental group. Once local networks are established for all mice of an experimental group, a global network can be created from this data and demonstrates the exploratory behaviour of the whole experimental group. For this study, Suzuki and Imayoshi [37] focused on eight different exploratory behaviours that formed dynamic nodes. Following algorithmic analysis, links between the different nodes (i.e. exploratory behaviours) were established. The authors observed that as spatial learning is established across the experimental days, the global network is simplified,

and nodes surrounding the target area are stronger than indirect nodes with indirect links. Most importantly, as highlighted by Suzuki and Imayoshi [37], although significant differences in cognitions were subtle, these spatial navigation behaviours were able to be recognised and quantitatively analysed using the 'network analysis method'. The capacity to apply quantitative statistics to patterns of behaviour provides a fantastic opportunity to apply strong, scientific investigation into higher cognitive processing. This is a strong example of utilising search strategy analysis in order to identify the more dynamic substrates of the cognitive underpinnings of navigation. The successful identification of strengthened spatial memory by Suzuki and Imayoshi [37] using the 'network analysis method' demonstrates the brevity of utilising similar approaches when investigating spatial memory.

6. Neurophysiology of allocentric and egocentric strategies

Studies investigating the neurological correlates of egocentric and allocentric navigation have utilised lesion, electrophysiological and optogenetic techniques to better understand the distinct mechanisms underlying them. In many experimental and clinical settings, specific deficits in one reference frame but not the other are observed, further indicating separate mechanisms.

6.1. Lesion studies for identification of allocentric and egocentric brain networks

A number of studies have investigated the cognitive consequences of lesioning the hippocampus using spatial memory tests such as the MWM. The overwhelming consensus is that allocentric learning is impaired after hippocampal lesioning. One of the first studies to demonstrate this was by Morris et al. [38] in rats. They demonstrated that lesioning the hippocampus of rats resulted in an inability to navigate the MWM. This is supported by numerous other studies [7, 39, 40], which all found significant deficits in traditional spatial memory measurements such as time to platform, distance to platform and time spent in target quadrant (probe trial). Other lesion studies indicate the perirhinal cortex, entorhinal cortex and parietal cortices to be involved in allocentric search navigation [41–43]. Maze apparatus that can be utilised to test egocentric search strategies include RAM [44], Cincinnati water maze and Star maze [33]. While allocentric search strategies appear to be dependent majorly upon the temporal lobe components, egocentric navigation appears to have a broader network. A study using the RAM observed deficits in egocentric navigation after lesioning medial agranular cortices [44]. Comparatively, a fascinating study by Wolff et al. [45] demonstrated that region-specific lesions of the thalamus impaired egocentric and allocentric navigation independently. They postulated that lateral thalamic lesions interrupt communication between the striatum and frontal cortex, by destruction of the intralaminar nuclei. This interrupted pathway manifested as deficits in egocentric navigation. Indeed, studies have indicated that the dorsal striatum and head direction cells are involved in egocentric navigation [18]. The cerebellar-dentate nucleus has also been implicated in egocentric processes [46], demonstrating the complexity of the networks involved in these search strategies. While we have so far attempted to separate these two navigation strategies, they are not mutually exclusive. A fantastic review by

Ekstrom, Arnold and Iaria [1] goes into detail on theories that describe transitions between allocentric and egocentric strategies, as well as the overlap between them.

6.2. Electrophysiological studies for identification of allocentric and egocentric brain networks

There has been extensive research into the neural correlates of spatial memory and navigation. In the seminal book, *The Hippocampus as a Cognitive Map* [47, 48], O'Keefe and Nadel put forward evidence for a cognitive map of space in the hippocampus. A neural model for a spatial map was proposed, built by specialised populations of cells in the hippocampal formation that fire with direct relation to place (place cells). The flow of spatial information in this model begins with sensory and contextual stimuli from the neocortex moving through the entorhinal cortex where egocentric information is encoded. The signal then moves to the fascia dentata of the hippocampus where it is thought that this mix of information is organised and sent to the CA3 and CA1 field of the hippocampus. It is here that the construction of the spatial map is thought to be accommodated with place and misplace cell systems. This model paved the way for future research and identification of other specialised cell types such as head direction cells located between the entorhinal cortex and CA1 in the postsubiculum [49], boundary cells in the subiculum [50], grid cells in the entorhinal cortex [51] and speed cells in the medial entorhinal cortex [52]. Edvard and May-Britt Moser (grid cells), along with John O'Keefe (place cells), were awarded the Nobel prize in Physiology or Medicine in 2014 for their work in investigating these cells underlying the spatial representations of space in the brain. Grid cells, similar to place cells, fire in response to changing position in an environment [51]. These cells differ, however, in their response to a change in environment [53]. When exposed to a new environment, grid cells maintain their representation of space and can therefore represent universal metrics such as distance and direction. These properties suggest that grid cells are involved in path integration [54], a navigational method that integrates movement, direction and speed to compute location. Importantly, path integration primarily relies on an egocentric reference frame because the abovementioned movement, direction and speed are all relative to self [12]. On the other hand, place cells undergo remapping and adopt new, unrelated representations when exposed to novel environments. The resulting allocentric map includes locations predominantly independent of the path taken to get there [55].

Mechanistic differences between egocentric and allocentric reference frames are also observed in electrophysiological recordings. Theta oscillations, or the theta rhythm, are low-frequency (~7–9 Hz) local field potential oscillations that function as a temporal frame in which neurons fire action potentials [56]. Both place and grid cells demonstrate theta phase precession effects to differing levels during navigation. That is, as an animal travels closer to the peak firing field of a certain place or grid cell, that cell will fire earlier in the theta phase [57]. This adds an additional layer of encoded information that contributes to navigation. Furthermore, oscillatory activity has been shown to facilitate the coherency between brain regions involved in egocentric and allocentric navigation [58]. Specifically, low-gamma oscillations (25–50 Hz) between the CA1 and CA3 and high-gamma oscillations (65–140 Hz) between the CA1 and

entorhinal cortex. Indeed, these oscillatory frequency ranges in the CA1 are associated with changes in egocentric and allocentric behaviour [59].

6.3. Optogenetic studies for identification of allocentric and egocentric brain networks

Optogenetics is an outstanding technique to elucidate the functional relevance of particular neuron populations in specific brain regions and areas. A study by Andrews-Zwilling et al. [60] optogenetically inhibited hilar GABAergic neurons which led to a spatial memory retrieval impairment in the MWM. This study used the parameters escape latency and percentage time spent in target quadrant. However, there was no reported analysis of search strategy. As outlined by Rogers et al. [34], search strategy analysis is imperative to confirm spatial memory learning. For this study, it would be interesting to know the strategies employed by the mice and compare to controls, to see exactly how the optogenetic inhibition is affecting navigation. By knowing the effects upon search strategy, it provides further depth and breadth to understanding the cognitive processes occurring. Yamamoto et al. [8] further confirm a role for the hippocampus in spatial memory with their optogenetic inhibition of medial entorhinal cortex layer III (MEC) inputs to the CA1 of the hippocampus. This was demonstrated using the delayed nonmatch-to-place T-maze task, a working memory task that is based upon egocentric navigation, that is, it is based upon the successful alternation of turning left or right at a junction [61]. Building upon this, the study by Perusini et al. [62] demonstrated that optogenetically stimulating the dentate gyrus in aged mice improved memory retrieval in the contextual fear conditioning paradigm. This has great implications for the current problem of the world's extended life span and associated neurodegenerative diseases such as dementias. The hippocampus is a hub for memory and is linked to multiple networks, as demonstrated especially by Ito et al. [63]. Optogenetic inhibition of cells in the nucleus reuniens of the thalamus resulted in reduced trajectory-dependent firing of the CA1 region of the hippocampus. Projections from the medial prefrontal cortex to the nucleus reuniens which end in the CA1 hippocampus region are imperative to goal-directed map representation.

The studies examined above indicate that some regional differentiation exists between the individual networks involved in allocentric and egocentric navigation. Taken together, it would appear that the hippocampus and surrounding areas are strongly involved in spatial memory and in particular the allocentric search and egocentric navigation strategies. Understanding the effects upon spatial memory and navigation is enhanced by analysing the search strategies employed by research animals. Disruptions to normal functioning could result in compensatory mechanisms that disguise impairments to spatial memory, if the appropriate analyses are not performed. Future studies should use techniques such as optogenetics to specifically investigate cell populations in the hippocampus and associated areas and their role in spatial memory and allocentric and egocentric navigation strategies using specifically designed mazes such as the Star maze. It is widely accepted that the hippocampus has a role in spatial memory, but we are now starting to understand how disrupting spatial memory alters navigational pathways.

7. Search strategies and their relevance to ageing and disease

Further incentive to differentiate egocentric and allocentric navigation in spatial memory tests arises from evidence in studies of human ageing and disease showing that deficits are observed in specific search strategies. Studies in real-world environments such as supermarkets [64] and roads [65] confirm the anecdotally long-held belief that spatial memory performance worsens with normal ageing. Elderly humans also perform worse in virtual reality versions of mazes designed to investigate spatial memory [66] accompanied by changes in electrophysiological event-related potentials [67]. Allocentric navigation seems to be affected more so than egocentric navigation [25, 67], and specific deficits arising only when switching to an allocentric from an egocentric strategy have also been observed [68]. These behavioural changes may be a result of age-related changes in the hippocampus including decreased synapse function and long-term potentiation [69]. Declines in other domains such as working memory and sensory perception most likely also contribute to the decreased spatial memory performance seen in ageing; however, the vulnerability of allocentric over egocentric strategies prompts the need for further investigation into the mechanism behind this deficit. Interestingly, allocentric-specific deficits also seem to manifest in the young (6–7 years old) as well as the elderly [70], suggesting the deficit may be related to cognitive load.

Alongside ageing is an increase in risk for neurodegenerative disorders such as Alzheimer's disease (AD) and associated decline in memory. Topographical disorientation is an early symptom of AD that involves the inability to orientate in the environment and often leads to patients being prone to getting lost. A systematic review of egocentric and allocentric spatial ability in AD by Serino and colleagues [71] observed an allocentric deficit in both mild cognitive impairment and AD. Furthermore, a later study by Allison and colleagues showed allocentric-specific deficits can also be seen in asymptomatic preclinical AD, suggesting allocentric spatial memory tasks may be useful in the early diagnosis of AD [72]. Similar allocentric-specific deficits are also observed in neurodevelopmental disorders such as attention deficit hyperactivity disorder [73]. Although the ability to learn locations from allocentric representations has been shown to be decreased in patients with autism spectrum disorder (ASD) as well [74], there is sparse literature and agreement on this topic [75]. Cognitive symptoms are an untreated aspect of schizophrenia, and allocentric-specific deficits have been observed [76].

Many spatial memory deficits in cognitive decline and disease seem to preferentially affect the allocentric reference frame and navigational strategy. Constructing an allocentric cognitive map of an environment would allow navigation from any start point to a goal location compared to an egocentric sequence, which would only be viable from a single start point to reach a goal. Intuitively, allocentric search strategies are more complex than egocentric strategies and therefore may experience loss of function before the onset of more severe deficits that then go on to affect the egocentric reference frame. In a similar vein, there is also evidence to suggest that perhaps the allocentric reference frame is a culmination of many egocentric frames, meaning egocentric frames are likely to exist without allocentric frames but not vice versa [77]. This could explain the disproportionate dysfunction in allocentric abilities and the relative persistence of

egocentric ones. Another possibility is that specific navigational deficits are a reflection of inaccurate (unconscious) selection of the search strategy most suited for the task at hand [78].

8. Why is the distinction important?

Animal models allow the investigation of specific forms of memory and dysfunctional neuro-components, as a way to parallel human illness. Since humans and animals have analogous brain regions with similar functions, it is helpful to the expansion of biological knowledge to investigate possible disruptions in order to understand the fundamental neuroscience.

Distinguishing egocentric and allocentric search strategies in spatial memory tests is important because:

1. Accuracy and integrity of experimental results would be stronger. Due to the fact that one strategy may be preferentially affected over the other, not considering the distinction has a similar effect to not measuring the effect of an unknown variable. Results may become skewed, diluted or even completely masked.
2. There is a potential to discover novel therapeutic targets. Coupling behavioural data with known physiological and molecular pathways underlying these search strategies could elucidate specific deficits in disease.
3. They can function as more precise outcome variables that can potentially be utilised in early diagnosis of cognitive impairments. Detection of subtle deficits may also be improved.
4. Understanding the inner workings of our brains will be advanced.

9. Conclusions

Reviewed here is evidence supporting the distinction of egocentric and allocentric reference frames in spatial memory. These reference frames and their respective search strategies are closely related and are often used in combination when navigating. We argue that because these reference frames involve different mechanisms and they are differentially affected by experimental manipulations and disease, they should be appropriately dissociated when investigated. Rodent mazes such as the Star maze have been developed to tackle this issue by directly probing egocentric and allocentric strategies. Other, more widely used mazes such as the Y-maze and RAM are able to probe these strategies with slightly modified protocols. Open arena apparatus such as the MWM, CBM and Barnes maze can provide different insights on spatial memory performance, but an often overlooked and informative parameter is the qualitative measurement of path traces and investigation of search strategies. Not only has the investigation of search strategy been shown to be required to confirm the creation of an allocentric map, it provides a depth and breadth to understanding the cognitive processes occurring post-experimental intervention or modification. We strongly encourage and recommend the adoption of search strategy analysis and comparison between experimental groups, in order to gain the most from your data.

Author details

Adrienne M. Grech[†], Jay Patrick Nakamura[†] and Rachel Anne Hill^{*}

^{*}Address all correspondence to: rachel.hill@monash.edu

Department of Psychiatry, School of Clinical Sciences, Monash University, Clayton, Australia

[†]Denotes equal authors.

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