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Cannabidiol as a potential preventative treatment in a *Neuregulin 1* mouse model of schizophrenia

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Statement of authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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(Signature)

Declarations

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Abbreviations

2-AG	2- Arachidonoylglycerol
5HT1-A	5-hydroxytryptamine 1A
ACC	Anterior cingulate cortex
AEA	Anandamide
ANOVA	Analysis of variance
ASR	Average startle response
BSA	Bovine serum albumin
BDNF	Brain derived neurotrophic factor
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CB	Cheeseboard
CBD	Cannabidiol
COMT	Catechol-O-methyltransferase
CREB	cAMP-response element binding protein
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
CYP450	Cytochrome P450
D2	Dopamine receptor 2
D3	Dopamine receptor 3
dB	Decibel
DISC1	Disrupted in schizophrenia 1
ELISA	Enzyme linked immunosorbent assay
FAAH	Fatty acid amide hydrolase
FC	Fear conditioning
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GAD67	Glutamate decarboxylase 67
GPR55	G protein-coupled receptor 55
GWAS	Genome wide association study
HPC	Hippocampus
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
i.p.	Intraperitoneal
ISI	Interstimulus interval
ITI	Intertrial interval
LDT	Light-dark test
MAGL	Monoacylglycerol lipase
MIA	Maternal immune activation
MWM	Morris water maze
NAcc	Nucleus accumbens
NMDA	N-Methyl-D-aspartic acid
NORT	Novel object recognition test
NRG1	Neuregulin 1
Nrg1 TM HET	Heterozygous *neuregulin 1* transmembrane mutant mouse

OF	Open field
PBS	Phosphate buffered saline
PGC-1α	Peroxisome-proliferator-activated receptor-gamma co-activator 1 alpha
PND	Postnatal day
PFC	Prefrontal cortex
Poly I:C	Polyinosinic:polycytidylic acid
PPI	Prepulse inhibition
PV	Parvalbumin
RIPA	Radioimmunoprecipitation assay
RM	Repeated measures
SEM	Standard error of the mean
SI	Social interaction
SNPs	Single-nucleotide polymorphism
SPT	Social preference test
THC	Δ^9-tetrahydrocannabinol
TNF-α	Tumour necrosis factor alpha
TRKB	Tyrosine receptor kinase B
TRPV1	Transient receptor potential cation channel vanilloid 1
VEH	Vehicle
VTA	Ventral tegmental area
WT	Wild type

Abstract

Cannabidiol (CBD) is a non-psychoactive cannabinoid that has antipsychotic-like and anti-inflammatory properties, however its potential as a preventative drug in schizophrenia has not yet been investigated. Brain maturation during adolescent development creates a window where CBD could potentially limit the development of schizophrenia. The *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mouse shows face, predictive, and construct validity as a mouse model of schizophrenia. This project sought to determine if CBD given in adolescence could prevent the development of the schizophrenia-relevant phenotype in *Nrg1 TM* HET mice, as well as prevent susceptibility to the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC), in these mice. In Experiment 1, the baseline behavioural and neuroinflammatory phenotype of *Nrg1 TM* HET mice was first re-established at the novel Western Sydney University behavioural laboratory. *Nrg1* mutant mice exhibited hyperlocomotion, social interaction deficits, reduced startle response, and increased sensitivity to 3 mg/kg THC, with a trend for reduced sensorimotor gating. There were no changes in neuroinflammatory markers. In Experiment 2, *Nrg1* mutant and wild type-like (WT) animals were treated daily with 30 mg/kg CBD for 3 weeks during adolescence, and then tested for hyperlocomotion, social behaviour, sensorimotor gating and fear-associated learning and memory (tests informed by Experiment 1) during the subsequent 3 weeks while treatment continued. A week after these, mice completed a behavioural test battery under acute THC treatment. Chronic CBD increased locomotion in both genotypes, and after an extended period increased social behaviours in all mice, as well as reducing levels of glutamate decarboxylase (GAD_{67}) in the hippocampus. Combined, prior CBD and acute THC impaired startle habituation in *Nrg1* mutants, but not WT mice. THC alone increased social behaviours in *Nrg1* mice. In Experiment 3, *Nrg1 TM* HET and WT mice were administered CBD during adolescence and then left in the home cage until adulthood (5-6 mo) before undergoing

behavioural testing. CBD had converse effects, and reduced anxiety in mutants and overall sociability and hippocampal levels of cannabinoid 1 (CB_1) receptors in both genotypes, though these were found to be increased in the hippocampus of mutant animals at this age. Combined, a prior chronic course of CBD then paired with later acute THC in adulthood decreased startle in *Nrg1* mutants, but not WT mice. These data suggest chronic adolescent CBD has persistent effects on the brain and behaviour and may potentiate later effects of THC, particularly in *Nrg1 TM* HET mice. As such, it may not be suitable as a preventative drug in relation to schizophrenia.

Chapter 1: Introduction

1.1 Schizophrenia – symptoms, prevalence, and development

Schizophrenia is a debilitating neurological disorder suffered by millions of people worldwide, an estimated 0.5-1% of the population (1). It is classified by the presence of behaviours from three categories of symptoms: positive, negative and cognitive (2). Positive symptoms are symptoms which occur in addition to normal experience, such as auditory or visual hallucinations, delusions (e.g. grandeur or persecution), as well as disorganised thought/behaviour and catatonia (3, 4). Psychosis is a combination of positive symptoms such as hallucinations, delusions, and disorganised thought or speech, that results in the patient feeling disconnected from reality (5). Positive symptoms limit the ability of individuals to return to normal life because of the way they affect perception and impair daily function (6). Negative symptoms are classified as a loss of typical behaviour and include anhedonia, an inability to attain pleasure from any aspect of life; social withdrawal, where an individual avoids contact with others; as well as apathy, poverty of speech and loss of motivation (7). Cognitive symptoms include diminished executive function, such as poor memory and attention, as well as impaired recall (8, 9). Cognitive symptoms are considered core symptoms of schizophrenia and are a strong predictor of a patient's ability to return to the workforce (10).

For patients with schizophrenia, these symptoms can be relentless throughout their life. Over their lifetime, up to 80% of individuals with schizophrenia will be unemployed for significant periods of time (11). Individuals with schizophrenia are more likely to experience homelessness during their life (12), and to abuse addictive substances (13). Patients also are more likely to engage in risk-taking behaviour, and because of this have a higher chance of being infected with human immunodeficiency virus (HIV) (14), as well as being incarcerated

(15). Premature death due to suicide is also more prevalent in patients with schizophrenia, with around 4% of patients ending their life at some stage of the disease process, and over 20% of patients attempting suicide at some time (16-18). Total medical costs annually per individual with schizophrenia in Australia are around $77,000 (19). Adverse effects caused by medications used to treat schizophrenia can over time lead to severe health conditions such as diabetes and heart disease, which also contribute to a higher risk of premature death in patients (20, 21).

Schizophrenia generally develops between 12 and 35 years of age, and is a chronic life-long condition (2, 22, 23). The 0.5-1% prevalence of schizophrenia is fairly consistent worldwide, and as a condition it has been documented throughout history, first being recognised as a disorder in the 1800s (24, 25). Schizophrenia is hypothesised to develop over three stages (Fig. 1.1), and begins with a premorbid neurodevelopmental period during prenatal development and/or adolescence, where the patient is at risk of developing the disorder due to environmental factors such as drug/child abuse, maternal infection, significant stress and trauma, or genetic risk (2, 5, 26, 27). While symptoms are often absent in the premorbid period and this is mostly considered an at-risk stage, the next stage, the prodromal period, is when mild symptoms occur (27, 28). Prodromal symptoms such as delusional thinking (an attenuated positive symptom), anxiety and irritability (mood symptoms), distractibility (cognitive symptoms), social withdrawal and obsessive thinking (negative symptoms) occur during this period, but cannot be considered diagnostic as they overlap with other disorders (e.g. bipolar disorder, some personality disorders), and must be experienced for 12 months before a formal diagnosis is made (2, 27). The prodromal period is not characterised by any true psychotic symptoms e.g. delusions, hallucinations. The first occurrence of a psychotic episode is called first-episode psychosis (26). This is the beginning of the neuroprogressive, deteriorative stage.

Once patients have experienced positive symptoms for one month, in addition to six months of negative and cognitive symptoms, an individual is diagnosed with schizophrenia (2, 8, 29).

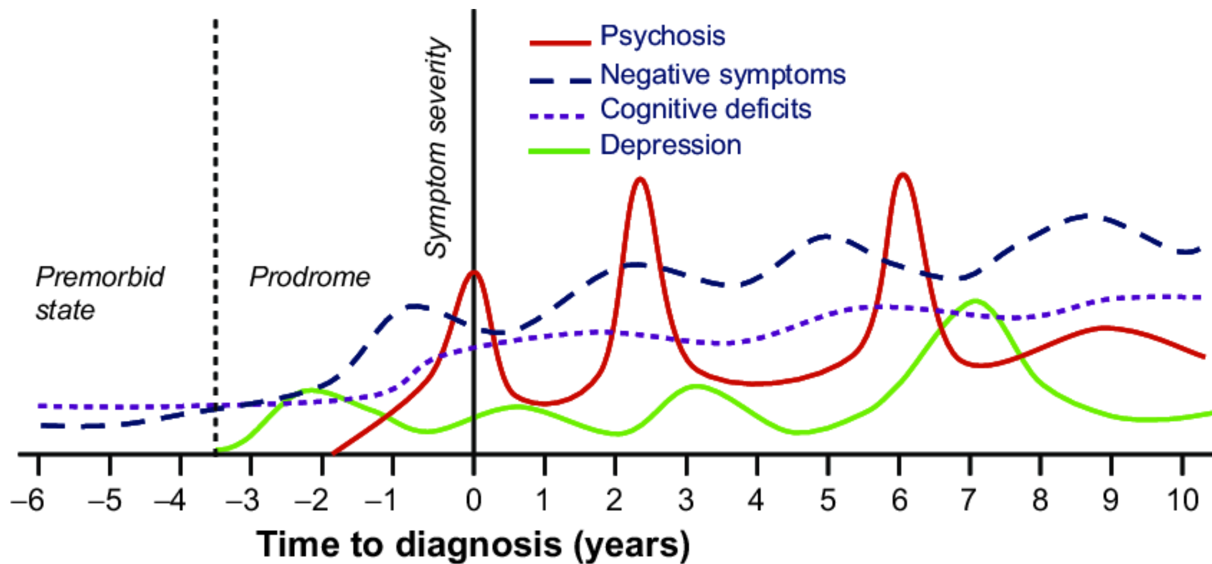

Fig. 1.1: The progression of symptoms in the development of schizophrenia (taken from Yasui-Furukori 2012 (30)), where the unbroken vertical line represents first-episode psychosis and notable presence of cognitive and negative symptoms.

Because of this long period of development, diagnosis can be a convoluted and time-consuming process, involving multiple possible pre-diagnoses and medications (29). This is also due to the number of symptoms (such as mood changes, poor focus, psychotic symptoms) that could also be indicative of other mental health disorders, such as bipolar disorder, schizoaffective disorder, or some personality disorders (e.g. schizotypal personality disorder, multiple personality disorder) (31, 32). Due to this extended time course, patients may not have a stable or present treatment course in place while the diagnosis remains pending.

1.2 Pathology associated with schizophrenia

Schizophrenia is a complex disorder and symptoms can be attributed to pathology in several different brain regions. Pathology associated with schizophrenia may also be different depending on the stage of the disorder. Despite this, some factors have been repeatedly

implicated in schizophrenia development and symptomology. These factors include cell loss, impaired white matter tract integrity, dysregulation of neurotransmitter systems (such as dopamine, glutamate, and γ-aminobutyric acid (GABA)) and an increase in a toxic cellular environment driven by inflammation.

1.2.1 Gross structural pathology

Structurally, the brain in schizophrenia patients can have a lower cerebral volume than that of healthy patients, with reduced gray matter volume in the frontal lobes (involved in executive functions such as decision making, cognitive fluidity, short term memory), temporal lobes (involved in auditory/speech and comprehension processes and memory processes), hippocampus and parahippocampus (both involved in learning and memory), and amygdala (part of the limbic system; responds to emotive stimuli and is critical for emotionally charged memory), as well as evidence of enlarged lateral ventricles (33, 34). Functional magnetic resonance imagining (fMRI) studies indicate these brain regions are linked to schizophrenia symptoms: abnormal activation in the frontal cortex and the temporal lobes contributes to positive symptoms, abnormal activation in the limbic system is related to negative symptoms, and abnormal activation of the hippocampus is involved in poor memory and cognitive symptoms (35, 36). White matter also appears to be diminished in patients with schizophrenia, suggesting that while deficits in cortical volume may account for some symptomology, abnormalities in connectivity may also be important in the disorder's pathophysiology (34, 37, 38). Implicating connectivity as a driver of symptoms also opens up which brain regions are investigated in relation, as it implies deficits in specific regions may not be solely at fault. For example, subcortical regions like the hippocampus and striatum connectivity to the prefrontal cortex is coming more into focus with regards to the origin of positive and cognitive symptoms (39). Deficits in connectivity within the prefrontal and associative areas, rather than a loss of

glial or neuronal bodies, has been suggested to play a large role in the generation of psychotic symptoms (40). This is due to the dysregulation of excitatory and inhibitory pathways that moderate normal cognitive function, creating an imbalance in both electrical and chemical properties that lead to hallucinations and delusions (41, 42).

1.2.2 Cellular morphology

While gray matter appears to be reduced in the frontal, temporal and parietal lobes in schizophrenia patients, cell density in these areas has been found to be greater in patients, (43, 44). White matter in the prefrontal cortex (PFC) also shows increased cell density, and neurons can be maldistributed within cortical layers (45). suggesting that cell morphology and migration are relevant to the development and presentation of the disorder. Neuronal morphology appears also affected, with overall reductions found in neuronal cell size, dendritic spine length, dendritic spine density, and synaptic proteins evident in areas related to the symptomology of schizophrenia, such as the PFC and hippocampus (40, 46-48).

Other cell types (e.g. glia) have also been shown to be altered in schizophrenia. Glial cells such as oligodendrocytes, astrocytes, and microglia support neuronal function, immune function, and connectivity within the brain. Abnormalities in the number, density, and morphology of these cells have been observed in post-mortem brain tissue from individuals with schizophrenia (49-51). Decreased oligodendrocyte density in the PFC has been found in patients with schizophrenia, suggesting poorer connectivity between PFC neurons (52, 53). Astrocyte density is also decreased in the hippocampus, while activated microglia in the hippocampus are highest in a subset of patients with acute paranoid schizophrenia, suggesting increased inflammation may be present in schizophrenia patients and may relate to symptomology (54, 55). Because of their key role in the regulation of the brain's immune response, microglia have also been important to consider morphologically. Increased

microgliosis (defined as the reaction of the brain to pathogenic threat and categorised partly by increased activation of immuno-reactive microglia; which is up to 57% in some layers) in the frontal and temporal cortices has been found in post-mortem examinations of institutionalised patients with chronic schizophrenia (56). Microgliosis has also been found in the PFC, anterior cingulate cortex (ACC), and thalamus of schizophrenia patients who committed suicide compared to non-suicidal schizophrenia controls (49), suggesting some connection between microglial activation and suicidal behaviour in schizophrenia. This is reflected in post-mortem schizophrenia studies, where significant microgliosis and microglial activation was found in the ACC and thalamus of the brains of schizophrenia patients that committed suicide (57).

Microglial cells also initiate neuroinflammatory cascades, and therefore play a key role in synaptic pruning as they actively engulf and degrade synapses during neural development (58). This is particularly important as over-pruning during neural development in adolescence is speculated to be involved in schizophrenia aetiology (59, 60). Morphology is also affected in microglia and in turn causes hyperactivity or dysfunction: oligodendrocytes in the PFC and hippocampus of schizophrenia patients have abnormal myelin structure and proliferation, while astrocytes are swollen and dystrophic in the hippocampus (49, 52, 54, 61). Immunoregulatory microglia have a higher density and activation in the brains of patients with schizophrenia, and a high density of microglia is correlated with increased risk for the disorder (57, 62-66). Patients with schizophrenia can exhibit an overall increase in microglial activation (67), as well as increased density of activated microglia in the frontal and temporal cortices (56). These results suggest that cellular morphology, particularly in supporting cells such as microglia, could contribute to the neuropathology of schizophrenia.

1.2.3 Neurotransmitter dysfunction

Cell morphology may not be the only factor affected in the brain in schizophrenia. Connectivity in the brain also relies on chemical transmission between cells. One of the major hypotheses explaining schizophrenia symptoms is the 'dopamine hypothesis', which implicates excessive dopaminergic activity in the mesolimbic pathway with positive symptoms and insufficient dopaminergic activity in the mesocortical pathway with negative symptoms (68-71). Psychotic symptoms are associated with elevated presynaptic dopamine synthesis and elevated dopamine D_2 receptors in the striatum in patients (72), specifically in the associative striatum as opposed to the ventral striatum as previously thought (73). Negative symptoms have been associated with hypodopaminergia in the mesocortical pathway between the VTA and the PFC (68) (Fig. 1.2). Many antipsychotics target D_2 receptors, either 'tightly' (i.e. first-generation, binding tighter and with a higher affinity than dopamine, thus causing extrapyramidal symptoms and parkinsonism) or 'loosely' (i.e. second-generation, binding less tight than dopamine, thus causing less extrapyramidal symptoms) (74, 75).

Glutamatergic receptor dysfunction also appears to play a role in schizophrenia symptoms for the development of positive and negative symptoms due to its role as an excitatory neurotransmitter (41, 76, 77). Reduced N-methyl-D-aspartate (NMDA) receptor activity in the hippocampus and frontal cortex produces negative and cognitive symptoms, and these symptoms can be mitigated by administering drugs that enhance NMDA receptor function (78). Administration of NMDA receptor antagonists (thus blocking NMDA receptors) such as ketamine induces a psychotic-like state by disinhibiting glutamatergic activity in the cortex in healthy controls, and also induces psychosis in stable chronic schizophrenia patients (79). This suggests that excess glutamate release in the cortex via NMDA receptor blockade may contribute to some psychotic symptoms. Recent electrophysiology research has also shown that clozapine, a second-generation antipsychotic drug, exhibits some efficacy at the

glycine binding site of the NMDA receptor (80). Glycine can also augment antipsychotic efficacy (81), which suggests an important role of antipsychotics regulating NMDA receptors for the improvement in schizophrenia symptoms (82). Collectively, this suggests NMDA receptor hypofunction contributes to the symptom expression in schizophrenia, perhaps separate to alterations to the dopaminergic system.

Malfunction in GABAergic parvalbumin-containing cortical neurons has been linked to cognitive impairments in schizophrenia (42, 83). In cortical pathways, inhibition from GABAergic interneurons is key for producing γ wave oscillations (30-80Hz), which are critical for cortical information transmission in the brain. Furthermore, excitotoxic loss of NMDA receptor-bearing GABAergic neurons has been shown to cause excessive dopaminergic input into corticolimbic regions, which can induce glutamatergic system hypofunction (84) (Fig. 1.2). Glutamic acid decarboxylase (GAD_{67}), a rate-limiting isoenzyme that controls the synthesis of cytoplasmic GABA from glutamate, is also downregulated in the prefrontal cortex of schizophrenia and bipolar patients with psychosis (85), as well as the hippocampus of patients with schizophrenia (86), suggesting an involvement of this enzyme in the reduction of free GABA in schizophrenia (87).

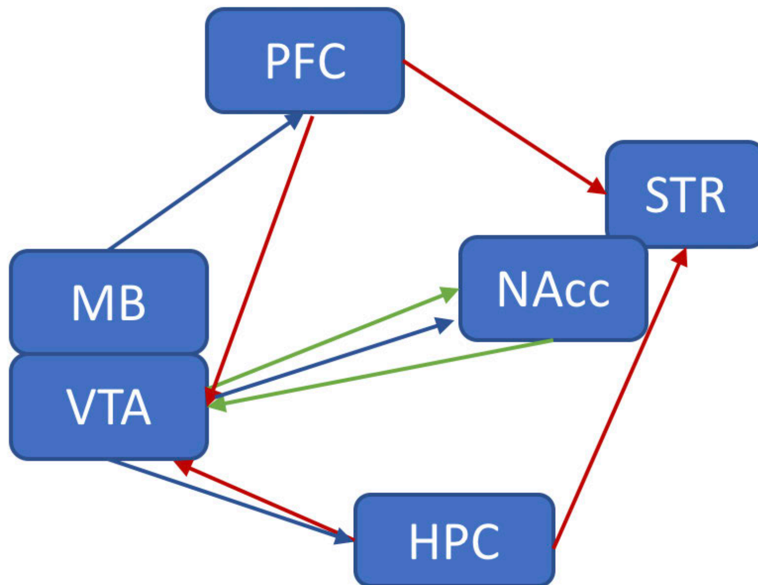

Fig. 1.2: Pathways associated with the development of psychosis in schizophrenia, adapted from (88): neurotransmitter systems in the hippocampus, midbrain, corpus striatum, and prefrontal cortex, are shown as dopamine (blue), glutamate (red), and γ-aminobutyric acid (GABA) (green).

1.2.4 Neuroinflammation

Cytokines affect cell differentiation, survival, and growth, and they have been of particular interest in relation to neuronal development in schizophrenia. Post-mortem tissue analyses reveal neuronal degeneration, abnormal white matter, and reduced neurogenesis, which can be caused by increased pro-inflammatory cytokines during key brain developmental periods (e.g. perinatal, adolescence) (62, 89). Indeed, there is evidence for an increased concentration of pro-inflammatory cytokines in some patients with schizophrenia compared to healthy controls (89-93), suggesting neuroinflammation may play a role in schizophrenia. Importantly, neuroinflammation in clinical schizophrenia populations varies depending on the stage and severity of the disorder, type of medication, and chronicity of treatment regime [see reviews e.g. (62, 93-98)]. In particular, elevated levels of interleukin-1 beta (IL-1β) in the cerebrospinal fluid (CSF) are associated with first-episode psychosis, suggesting circulating IL-1β may trigger positive symptoms of schizophrenia (89, 90). Interleukin-6 (IL-6) is also elevated in a subset of individuals with schizophrenia (89, 92, 99, 100). Tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine in the CSF, has been associated with acute psychosis in both first episode patients and schizophrenia patients in an acute psychotic state (89, 90), and is elevated in the serum of schizophrenia patients compared to controls (89, 92, 99). Antipsychotics such as flupentixol, trifluperidol, haloperidol, and risperidone have been shown to inhibit the release of TNF-α, IL-1β, and IL-6 by microglial cells (62, 101-103), a finding reflected in patient studies (see meta-analysis: (104)). Antipsychotic-mediated inhibition of TNF-α has also been related to an improvement in cognitive symptoms in patients (105), indicating a link between increased neuroinflammation and some symptomology of schizophrenia. These studies together suggest that immune activation may be present during the development and progression of schizophrenia, which may also contribute to the ongoing

expression of the disorder. Thus, pharmacological treatments which reduce inflammation may be able to reduce some schizophrenia symptoms.

1.3 Potential causes of schizophrenia

Schizophrenia is a highly complex disorder which appears to develop from contributions of several genetic and/or environmental insults, requiring a combination of either gene-gene, gene-environment, or environment-environment interactions (106-109). The multiple-hit hypothesis suggests that schizophrenia develops following exposure to a combination of genetic and/or environmental risk factors, with a heightened sensitivity to these factors at particular developmental periods (e.g. prenatal period, adolescence) (94, 110). Examining the aetiology of schizophrenia and modelling it as a disease can therefore be a challenge, as these factors are diverse and difficult to both track in patients and model comprehensively in animals (111, 112). Furthermore, how these various factors interact is currently not well understood (111, 113).

1.3.1 Genetic risk factors for schizophrenia

Twin studies have revealed that the development of schizophrenia involves a major genetic component, with between 73-91% (81% point estimate) heritability (114, 115). Studies examining gene-psychiatric associations have found several different chromosomal regions that may be related to schizophrenia development (116). Further studies have focused on these regions and used different methods to identify single nucleotide polymorphisms (SNPs) in the regions, and genes that contain these SNPs or haplotypes, which are sets of SNPs (114, 115). Genome wide association studies (GWAS) and linkage analyses have also identified many genes that are associated with schizophrenia and have been able to identify the degree of risk for developing schizophrenia associated with different genes (117-120). More recent studies

have shown that some major genes identified by earlier GWAS may confer no more risk to the disorder than chance, however, within these candidate genes some variants have been linked to schizophrenia in further GWAS, suggesting that the relationship between some genes and schizophrenia development may rely on either specific polymorphisms or combinations thereof not previously identified (121-123). This also suggests that while genes may be generally associated, variants of these may confer risk to different subtypes of the disorder and may also interact with environmental factors and other genes/variants (119, 124). The relevance of gene-gene and gene-environment interactions may also be obscured in GWAS studies, as some mutations that may interact with others to increase predisposition would not be highlighted as candidate genes, and environmental factors during development or later life could also affect genes in different manners, which would also not show up in analysis (125).

The following genes identified by GWAS have been of particular interest because of their involvement in important biological processes relevant to schizophrenia: *Catechol-O-methyltransferase (COMT), Disrupted in schizophrenia 1 (DISC1), Brain-derived neurotrophic factor* (*BDNF*), and *Neuregulin 1* (*NRG1*) (123, 126, 127). *COMT* plays a key role in regulating dopamine metabolism in the brain, and one SNP was shown to elevate dopamine metabolism and therefore decreasing dopamine levels in the PFC (128). Some meta-analyses published in the almost two decades since *COMT* was implicated have suggested though that this gene may confer less risk than previously thought (129). *DISC-1* regulates dendritic spine morphology and function in the brain (130, 131). Earlier studies found significant associations between some mutations in *DISC-1* and schizophrenia development; however, these were low frequency mutations, and more recent meta-analyses have also suggested that all the common mutations in the *DISC-1* gene confer low risk for schizophrenia (132, 133). The mutations in *DISC-1* that do confer high risk for schizophrenia are infrequent in the general population (132). *BDNF* has been implicated in the development of both

schizophrenia and bipolar disorder (134). *BDNF* has a significant influence on the overexpression of dopamine receptor 3 (D_3) in the brain in the striatum and nucleus accumbens, and also regulates postsynaptic NMDA receptor activity in the hippocampus, which may alter glutamatergic activity in this region and connected regions in patients with schizophrenia (135 , 136, 137).

NRG1 was first associated with increased relative risk for developing schizophrenia in 2002 by Stefansson and colleagues (127) and is involved in neuronal growth regulation, cell signalling, and neuron migration (138). *NRG1* is a widely studied gene that confers some risk of schizophrenia development (138-140). The risk haplotype identified by Stefansson and colleagues has been replicated in several cohorts around the world (123, 127, 141, 142). This is of particular interest as the *NRG1* haplotype identified by Stefansson and colleagues was found in up to 30% of Icelandic, Australian and Scottish schizophrenia patients (127, 141, 143), making *NRG1* a common risk variant for schizophrenia in these populations.

Schizophrenia has a complex aetiology that may not be able to be fully understood by simplistic categorisation. Identifying single genes may not allow for full understanding of genetic contribution to the disorder, and other non-genetic factors may have strong impacts on its developmental pathology and also later symptomology.

1.3.2 Environmental risk factors for schizophrenia

Environmental risk factors for schizophrenia include prenatal stress or infection (e.g. influenza, maternal stress), obstetric complications (e.g. premature birth, traumatic birth), adolescent drug abuse (e.g. cannabis or psychostimulant abuse), adolescent trauma (e.g. sexual abuse, emotional abuse), and high adolescent stress (e.g. low socioeconomic status) (26, 100, 106, 144-148). It is our current understanding that the risk of these environmental factors influencing schizophrenia development is heightened if an individual experiences them during

a period of neurodevelopmental susceptibility (110). Prenatal development and adolescence are key stages of neurodevelopment, where the brain is sensitive to environmental, and therefore chemical insults. It is hypothesised that insults during these stages increase risk for schizophrenia by causing excessive synaptic pruning, or changes in neuronal migration (59, 149, 150). Furthermore, environmental factors can also increase risk for elevated neuroinflammation in the brain during adolescence (93, 145, 151-153).

To give one particular example, drug abuse with psychoactive drugs (i.e. methamphetamine) during adolescence significantly increases the risk of developing schizophrenia (154). Furthermore, one of the most commonly abused and easily available illicit psychoactive drugs, cannabis, has been linked to earlier disease onset, earlier symptom development, and overall increased risk for developing schizophrenia (155-158) in particular when chronic abuse occurred during adolescence (159). Indeed, patients with schizophrenia are significantly more likely to have abused cannabis during adolescence (155, 160). The well-established link between cannabis use and risk for schizophrenia suggests cannabinoids and/or the endogenous cannabinoid system may be involved as a component / cumulative factor in the development of the disorder.

1.3.3 Gene x environment interactions

The interaction between specific genes and environmental stressors has been of much interest in psychiatric disease research in the last two decades (106). Factors such as adolescent stress/trauma and cannabis abuse occur more often in the overall adolescent population than schizophrenia (159, 161), and cannabis abuse alone does not lead to a psychotic disorder (162). Even combining environmental effects such as childhood trauma and later cannabis abuse does not outright cause psychosis (163). This supports the idea that while environmental factors may have some effect on the development of schizophrenia and psychosis, they do not cause the

disorder outright, and may interact with other factors (e.g. genetic predisposition) to bring about disease onset. Some genes have been linked with psychosis development and earlier adolescent cannabis use, such as a functional polymorphism in the *COMT* gene (164). Another variant in *COMT* has also been linked with high stress and later psychosis development (165). Furthermore, prenatal immune activation can interact with *DISC1* to produce psychopathology (166). These interactions show that some mutations can increase the risk of psychosis development when paired with certain environmental factors.

1.3.4 A *Neuregulin 1* mouse model of schizophrenia

A mutation in the transmembrane domain region of *NRG1* found in patients with schizophrenia (167) has been modelled in the heterozygous *Nrg1* transmembrane domain mutant (*Nrg1 TM* HET) mouse, which exhibits schizophrenia-relevant behaviours and brain pathology (168-181). These rodent behavioural and pathological changes must follow a specific set of validity conditions in order to be related back to clinical schizophrenia. These validity conditions include: face validity, whereby the model must show behaviours which mimic those seen in the clinical condition; construct validity; whereby the causative factor in the disease is modelled (i.e. a specific gene or environmental risk factor or interaction); and predictive validity, whereby the model must show a similar response to therapeutic agents relevant to the disease (or in the case of schizophrenia, e.g. a heightened response to disease-triggering drugs).

Nrg1 TM HET mice display face validity to clinical schizophrenia in changes to locomotor hyperactivity (which, although can be relevant to several mental health conditions e.g. attention deficit hyperactivity disorder, substance use problems, can be caused by dysregulations to some schizophrenia-relevant neurotransmitter systems relating to positive symptoms e.g. dopaminergic and glutamatergic system perturbations), reduced social interaction (representing negative symptoms), impaired sensorimotor gating (considered an

endophenotype for schizophrenia, and is impaired in patients), reduced interest in a novel object and reduced contextual fear-association (representing impaired cognition in patients) (173, 177, 182, 183). Furthermore, the behavioural phenotype of these mice is age-dependent and develops in adulthood (177).

The *Nrg1 TM* HET mouse model also has construct validity (i.e. heterozygous 'knockout' of transmembrane [*TM*]-domain NRG1), as the gene mutation is found in patients with schizophrenia (127, 141). *Nrg1 TM* HET mice display imbalanced glutamatergic and dopaminergic receptor expression compared to age-matched controls, with increased NMDA receptors in the NAcc (at 14 but not 20 weeks), decreased NMDA receptors in the thalamus (at 20 but not 14 weeks), and decreased D_2 receptor expression in the striatum (at 14 and 20 weeks) (179), which are relevant to changes found in patients as mentioned previously. They also show changes in cytokine profiles relevant to patient studies. Female *Nrg1* mutant mice display decreased serum cytokine levels of IL-6, IL-8 and IL-10 and increased TNF-α (in early adulthood), while males show decreased serum IL-1β and TNF-α, suggesting altered inflammation in these mice (173). These mice also show predictive validity (i.e. interactions with medications that reflect what occurs in clinical populations), as when administered clozapine, locomotor hyperactivity is reversed (127). In line with predictive validity, *Nrg1 TM* HET mice are also more susceptible to environmental risk factors such as cannabinoids (184-186), changes to their housing conditions (177, 187), and restraint stress (169, 174), demonstrating that genetic predisposition to schizophrenia can increase sensitivity to environmental factors relevant for the disorder (i.e. drugs of abuse, stress). Male *Nrg1 TM* HET mice show a stronger behavioural and inflammatory phenotype than females (173, 183, 184, 186, 188), which is similar to clinical findings where males experience more severe symptoms than females (189).

Preclinical research with this model indicates that the *Nrg1* gene interacts with environmental factors such as drug use, maternal immune activation, and prenatal/adolescent stress, and this interaction leads to an increase in the severity of schizophrenia-relevant behaviours in mice (99, 170-174, 180, 184, 186, 190, 191). These behaviours in *Nrg1 TM* HET mice are worsened by exposure to stress during adolescence and adulthood, such as restraint and social stress (169-174). Maternal immune activation using Polyinosinic:polycytidylic acid (Poly I:C; an immunostimulant) also exacerbates the behavioural phenotype of these mice in adulthood (180). This demonstrates that schizophrenia-relevant environmental factors in the prenatal stage can exacerbate later behavioural impairment, similar to what is seen in patients (175, 192). *Nrg1 TM* HET mice display increased sensitivity to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound in cannabis, at both a behavioural and molecular level in adulthood and adolescence (168, 184-186), as well as increased sensitivity to the behavioural effects of the psychostimulant methamphetamine in adolescence (191).

Research with *Nrg1 TM* HET mice shows clear gene-environment interactions and supports these as an important component in both symptomology and development. This mouse model is therefore an ideal candidate model for understanding the complex relationship between these two factors.

1.4 Current treatment options for schizophrenia

Once diagnosed, there is no uniformly successful treatment plan for schizophrenia, and pharmacological interventions can be difficult to tailor to individual patients (23). This may be due to differences in action of antipsychotic medications, as well as general differences in symptom prevalence and severity that cause schizophrenia to resemble more of a spectrum disorder (193). There is a narrow range of available medications, and compliance to treatment strategies is extremely low in patients with schizophrenia, with factors influencing the high non-compliance rate (up to 27%) ranging from poor disease insight to adverse medication reactions such as weight gain and sexual dysfunction (20, 194).

Pharmacological treatments for schizophrenia treat primarily positive symptoms and psychosis, with negative and cognitive symptoms less improved by medications (195, 196). Antipsychotic medication is the main type of medication prescribed to patients with schizophrenia, and these can be broadly classified as first- and second-generation antipsychotics. First-generation antipsychotic medications, such as haloperidol and chlorpromazine, are primarily dopamine D_2 receptors antagonists, thereby providing relief from positive symptoms (197, 198). These first-generation antipsychotics are accompanied by significant extrapyramidal side effects including hyperprolactinemia, tremor, slurred speech, and dystonia, the latter of which is due to excess dopamine D_2 receptor blockade in nigrostriatal pathways. This has led to the development of second-generation antipsychotics that have fewer of these extrapyramidal symptoms (199). Some examples of second-generation antipsychotics are amilsulpride, clozapine, olanzapine, risperidone, and aripiprazole. In addition to acting on dopamine D_2 receptors, second-generation antipsychotics bind to multiple different receptor domains, including serotoninergic, adrenergic, histamine, and muscarinic receptors (200-202). Second-generation antipsychotics have lower risk of extra-pyramidal symptoms due to their lower affinity to dopamine D_2 receptors and their blockade of serotonin receptors, and some

are also mildly more successful in treating negative symptoms and cognitive impairment than first generation antipsychotics (199, 203). Despite this, the more commonly prescribed second-generation antipsychotics still have a range of adverse side effects including significant weight gain, insulin resistance, metabolic syndrome, diabetes and heart disease, and sexual dysfunction particularly leads to a low rate of adherence in younger people (16, 21, 204). Clozapine, a potent second-generation antipsychotic, has a 2% mortality rate due to conditions such as clozapine-induced agranulocytosis, a condition where white blood cells are targeted within the body and the immune system fails, and is therefore often prescribed as a last resort when other treatments fail (205). Second-generation antipsychotics, though more nuanced than first-generation antipsychotics in their modes of action, also have limited efficacy for negative and cognitive symptoms, as they primarily relieve psychotic symptoms, which is problematic as cognitive symptoms often determine the level of an individual's ability to function in daily life (206, 207). Third-generation antipsychotics have also been described since the discovery of aripiprazole, a partial or selective D_2 agonist (208, 209). Aripiprazole effects include partial agonism of dopamine D2 receptors, partial agonism of serotonin 5-HT1A receptors, and antagonism of 5-HT2 receptors, while remaining more effective as an antipsychotic and being accompanied by less extra-pyramidal and orthostatic side-effects (208). It can however cause akathisia (a movement disorder) and tremor in some patients (210).

Overall, side effects of antipsychotic medications lead not only to a concerning lack of medication compliance, but also a higher mortality rate in patients with schizophrenia due to health issues such as metabolic syndrome and heart disease, which can be caused by antipsychotic treatment (16). In addition, 30% of patients are treatment resistant and do not respond to any antipsychotic medication (1). Because of the adverse effects of antipsychotic medication, novel pharmacotherapies for treating schizophrenia are needed to lessen the financial and personal burden of schizophrenia (1, 12).

1.5 Cannabidiol (CBD) as a novel treatment for schizophrenia

1.5.1 The endocannabinoid system

The human body has an endogenous cannabinoid (endocannabinoid) system which comprises cannabinoid-specific receptors, ligands and enzymes, and was of interest first when an endogenous membrane receptor for THC was discovered in the mid 1990's (211). This system is complex and continues to be studied in the context of aberrant pathology in many different neurological diseases, such as depression, anxiety, Parkinson's disease, Alzheimer's disease, stroke, multiple sclerosis, and schizophrenia (211, 212). Its metabolites and receptors are involved either directly or indirectly in multiple key neural processes, including cell signalling, memory consolidation, and neuronal transmission (213).

High concentrations of cannabinoid 1 and 2 (CB_1/CB_2) receptors are found in schizophrenia-relevant brain regions. CB_1 receptors are located primarily presynaptically in the limbic system (hypothalamus, amygdala, thalamus, and hippocampus), cerebellum and PFC, while CB_2 receptors are found mostly on microglia cells in these regions (214, 215). CB_1 also increases significantly in these regions between prenatal development and adolescence as the endocannabinoid system develops (216). As well as this, endocannabinoids regulate synaptic plasticity by CB_1-mediated activity, resulting in changes in the release of neurotransmitters at excitatory and inhibitory synapses in the hippocampus, cerebral cortex, cerebellum, striatum, and amygdala (217). *N*-arachidonoylethanolamine, or anandamide (AEA), and 2-arachidonoylglycerol (2-AG) are two endocannabinoids widely expressed both during and after brain development and have a high affinity for CB_1 receptors (218). AEA is involved in sleep regulation, memory consolidation, and the reward system (219), while 2-AG has a major role in suppressing GABA release from CB_1R-containing inhibitory axon terminals (220). Higher 2-AG metabolism and lower CB_1R protein levels have been reported in the PFC

of a cohort of schizophrenia patients (220). Finally, enzymes that are involved in cannabinoid synthesis and hydrolysis and play an important role in degrading endocannabinoids are fatty acid amide hydrolase (FAAH), the hydrolytic enzyme for AEA, and monoacylglycerol lipase (MAGL), the key hydrolytic enzyme of 2-AG (221).

1.5.2 Changes to the endocannabinoid system in schizophrenia

Alterations to the endocannabinoid system in people predisposed to developing schizophrenia may in part account for the high levels of cannabis use in patients, as well as why cannabis abuse is such a major risk for development of the disorder itself (155, 157). Perhaps most importantly in terms of schizophrenia-related pathology, CB_1 receptors are localised in glutamatergic and dopaminergic primary projecting neurons and GABAergic interneurons; CB_1 receptor expression is particularly high in GABAergic interneurons (222). This suggests a greater sensitivity to cannabinoids in the GABAergic system. Post-mortem tissue analyses have also found CB_1 receptors are less proliferous in the PFC of patients with schizophrenia, and CB_2 receptors have impaired functional binding in the PFC (220, 223). Cannabis abuse has been linked to an increase in CB_1 receptors in the caudate putamen in patients with schizophrenia (224), implying that dysregulation of endocannabinoid signalling may occur in patients with schizophrenia after cannabis exposure. Furthermore, cannabis use during adolescence greatly increases the risk for prodromal psychotic symptoms and first episode psychosis (218) but not every person who uses cannabis during adolescence will develop schizophrenia (159, 225). Considering the use of cannabis is exponentially more widespread than the diagnosis of schizophrenia, this suggests that some changes to the endogenous cannabinoid system may already be present before cannabis use precipitates the disorder. Furthermore, these changes could hold implications for cannabinoid-based therapeutics used

in schizophrenia treatment and prevention, especially where these concern sensitivity to later use of other cannabinoids.

Cerebrospinal fluid (CSF) AEA levels are elevated in both prodromal and psychosis-experiencing schizophrenia patients (226, 227), and prodromal patients with levels of AEA on the lower end of the spectrum have increased risk for developing psychosis earlier, suggesting that upregulation of AEA in the prodromal phase may be neuroprotective (227). Some cannabinoids such as CBD have also been shown to moderately inhibit the degradation of AEA and decrease psychotic symptoms in patients (228). Furthermore, frequent cannabis use in schizophrenia patients has been shown to downregulate AEA signalling, where AEA levels inversely correlate with psychotic symptoms, suggesting cannabis use can cause dysregulation of the endocannabinoid system which can impact on schizophrenia severity (229). Some enzymes that are involved in cannabinoid synthesis and hydrolysis have also been implicated in schizophrenia pathophysiology. For example, genetic deletion of FAAH enhances levels of AEA, which shows that this enzyme may have a role in the dysregulation of AEA metabolism in schizophrenia (230). Furthermore, both FAAH and MAGL are significantly increased in first-episode psychosis patients, and levels of FAAH are higher in male patients than females (231). This implicates the endocannabinoid system as involved in some aspects of schizophrenia pathology, and also suggests dysregulation of this system as a potential component of some symptoms in schizophrenia. Importantly, this highlights potential avenues for pharmacological treatment and intervention.

1.5.3 Effects of cannabinoids in schizophrenia

While the *cannabis sativa* plant contains at least 100 different cannabinoid compounds, two of the most widely researched compounds are THC and CBD. THC is the main psychoactive compound in cannabis and is of particular interest to schizophrenia as it has been consistently

linked to elevated risk for developing the disorder (157, 160, 232). THC use worsens symptoms of schizophrenia, and adolescent THC abuse is hypothesised to disrupt maturing circuitry both cortically and subcortically, and contribute to the development of schizophrenia, resulting in dysregulation of glutamatergic and GABAergic transmission in prefrontal cortical areas (155, 157). THC is a partial agonist of CB_1 and CB_2 receptors, but has higher affinity for CB_1 receptors (214). Chronic THC also impacts on the dopaminergic system, likely by acting on CB_1 receptors in the ventral tegmental area and increasing dopaminergic projections to the striatum, and decreasing dopamine levels in prefrontal regions (233).

CBD has generated significant research interest, as it has demonstrated anti-psychotic-like and non-intoxicating properties in both rodents and patients, and is used to medicate or partially treat disorders including Dravet syndrome, multiple sclerosis (MS), and stroke (228, 234-245). It has also been investigated as a potential antipsychotic in schizophrenia patients [(228, 246) see review: (247)]. When cannabis contains a high percentage of CBD, the psychoactive effects of THC are blunted (248). In the brain, CBD interacts modestly with CB_1 and CB_2 receptors as a negative allosteric modulator and antagonist, however, many of its downstream effects are hypothesised to be either initiated or continued by different receptors as it is also a serotonin 5-HT1A partial agonist and a D_2 partial agonist (235, 249, 250). CBD also acts as an agonist on transient receptor potential cation channel subfamily V member 1 (TRPV1, also known as the capsaicin receptor or vanilloid receptor 1) (251), which are expressed widely in both the central and peripheral nervous system. CBD can also modulate astrocyte activity and decrease neuroinflammation in the brain – one possible explanation for its antipsychotic-like effects (252). This mechanism may be triggered by the desensitisation of TRPV1 receptors to inflammatory stimuli, causing a decrease in microglial activation (249, 253-255). CBD also activates and desensitises perception-modulating TRPV2, TRPV3, and TRPV4 receptors, which act as ionotropic cannabinoid receptors as opposed to metabotropic

CB1 and CB2 (256). This is important, as THC acts potently at TRPV2, and moderately modulates TRPV3 and TRPV4 channels (257). CBD action on these could therefore desensitise these channels to the modulation of THC.

The relationship between THC and CBD is still poorly understood, but research suggests CBD can both modulate and potentiate the behavioural and neural effects of THC. Effects of THC alone in mice include locomotor suppression, hypothermia, antinociception and deficits in prepulse inhibition – the ability for mice to inhibit a startle response to a tone if predicted by a softer tone (258, 259). Interestingly, when THC is administered alongside CBD, CBD can inhibit some of the behavioural effects of THC (248, 258), suggesting that perhaps CBD may be acting on shared cannabinoid pathways and that less THC may be binding when CBD is present (260). However, the ratio of CBD and THC administered likely has a significant effect on how the cannabinoids interact, and timing the treatments so that CBD precedes THC may also alter behavioural pharmacokinetics. In a 1:1 ratio, pre-treatment with CBD prior to daily chronic THC (1 mg/kg, then 3 mg/kg after 7 days, and 10 mg/kg for a further 7 days) mildly potentiates THC-induced anxiogenic behaviour, locomotor suppression and social withdrawal, possibly by delaying the metabolism of THC by interrupting cytochrome P450 (CYP_{450}) enzymes used to metabolize both compounds (261). Interestingly, it does not alter the THC-induced decreases in CB_1 receptor binding, and neither compound has an effect on 5-HT1A receptor binding (261). Rats pre-treated with 20 mg/kg CBD are protected against deficits in social interaction caused by 1 mg/kg of THC (262), demonstrating this 20:1 ratio is effective in preventing the suppressing effect of THC on social behaviours. Other studies have also suggested that at higher CBD/THC ratios, a more pharmacodynamic relationship is favoured between the compounds, rather than pharmacokinetic (263). If CBD is working to partially mediate some of the psychoactive effects of THC by blocking the same receptor

profile or THC metabolism, timing of administration would be an important factor in how CBD acts this way, and dose ratio may need to favour CBD.

1.5.4 CBD in animal models of schizophrenia

CBD has shown promise in some animal models of schizophrenia as a potential antipsychotic drug when administered acutely. In adult mice, acute 30 and 60 mg/kg of intraperitoneal (i.p.) CBD reduced hyperlocomotion induced by a combination of ketamine- and D-amphetamine (drugs that produce psychosis-like states and are used to model psychosis in rodents) (264). This reduction in locomotion was not linked to any cataleptic response (264). 3, 10, or 30 mg/kg of CBD i.p. prior to MK-801 (an NMDA receptor antagonist that produces social deficits, hyperactivity, and sensorimotor deficits in rodents, used as a rodent model of schizophrenia) also improved social behaviour compared to rats treated with MK-801 alone (265). Mice treated with 5 mg/kg of CBD prior to 1 mg/kg MK-801 also showed a reversal in MK-801 disrupted prepulse inhibition (PPI) (i.e. CBD reversed sensorimotor gating deficits) (266).

While the above studies suggest CBD as an efficacious acute remedial treatment for schizophrenia-relevant behavioural impairments, it is important to determine if chronic CBD is equally effective in reducing schizophrenia-relevant behaviours. A limited number of studies have investigated the effects of chronic CBD on schizophrenia-relevant behaviours in rodents. Chronic (28 day) MK-801 treatment coupled with CBD treatment (15, 30, or 60 mg/kg) from the sixth day onwards attenuated PPI impairment in mice (267). CBD also reversed molecular changes induced by chronic MK-801 administration, e.g. increased FosB/ΔFosB expression and decreased parvalbumin expression in the mPFC, and decreased NMDAR GluN1 subunit gene in hippocampus (267). When treated with 1, 50, or 100 mg/kg of CBD for 21 days, adult male *Nrg1 TM* HET animals showed enhanced social interaction with 50 and 100 mg/kg, but

deficits in PPI and hyperlocomotion remained unchanged by CBD in this model (190). 50 and 100 mg/kg CBD also selectively increased $GABA_A$ receptor binding in the granular retrosplenial cortex of *Nrg1 TM* HET mice (190). Interestingly, $GABA_A$ blockade in the PFC has been linked to schizophrenia-relevant deficits in attention (268), suggesting a role for CBD in increasing inhibitory neurotransmission in these animals, which may help ameliorate cognitive impairment in schizophrenia. CBD may also be competing with metabolic pathways of MK-801 in this study, or blunting them due to co-activation – this provides a limitation of the above pharmacological studies of CBD, and so it would be important to measure alterations based on these compounds being administered at different time points in future studies.

Other studies have investigated the role of CBD in protecting against the effects of chronic stress and have found that a higher dose of 30 mg/kg of CBD given daily for 14 days promotes cell proliferation in the hippocampus via CB_1 and CB_2 activation, and improves behavioural deficits resulting from chronic unpredictable stress, suggesting that activation of these receptors plays an important role in the effects of chronic CBD (269, 270). This also suggests that a higher dose of CBD during adulthood may have a more beneficial effect. Furthermore, these studies collectively show that CBD shows some potential in treating schizophrenia-relevant symptoms and suggest that alteration to the hippocampus via the endocannabinoid pathway may be how CBD improves some aspects of schizophrenia.

1.5.5 CBD in clinical research

Clinical research has assessed CBD as a remedial treatment for some symptoms of schizophrenia. Patients treated daily with 1000 mg/kg of CBD after 6 weeks displayed lower levels of positive psychotic symptoms, as well as a significant improvement in cognitive performance (271), suggesting CBD may have antipsychotic-like properties. The authors also noted that CBD was well-tolerated by patients and that rates of adverse events were on par with

those of the placebo group (271). The effectiveness of 800 mg/kg CBD was compared with the commonly prescribed antipsychotic, amisulpride, in a 4-week trial (228). Both drugs led to significant clinical improvement of positive and negative symptoms, however, the side effect profile of CBD was significantly superior to amisulpride, and CBD only was found to increase serum anandamide levels (228). Another recent study investigated a single oral dose of 600 mg CBD in patients with psychosis, and positive and negative symptoms were assessed 60 mins before administration, and 270 mins after (272). The study also assessed glutamate levels in the left hippocampus using proton magnetic resonance spectroscopy (272). Compared to a placebo group, CBD-treated psychosis patients showed a significant increase in hippocampal glutamate, as well as a reduction in symptom severity (272). One study has failed to show improvement of schizophrenia symptoms by CBD: antipsychotic-treated outpatients were given 600 mg/kg of CBD for a 6-week trial alongside their stable medication regimen and their cognitive and psychotic symptoms were rated at the conclusion of the trial, however, their symptoms were not improved (273). While this may be because of the lower dose, it is also possible the effects of CBD are lost when co-treated with antipsychotic medication (273). This is possibly due to the fact that both antipsychotics and CBD share very closely several important hepatic enzyme metabolic pathways. CBD is metabolised by hepatic enzymes CYP2C19, CYP2D6, CYP34A, CYP1A1, CYP1A2, and CYP2C9 (256). Clozapine, olanzapine, and aripiprazole are all metabolised by CYP34A and CYP2D6, the former two also use CYP1A2, and clozapine is also metabolised by CYP2C19 (274). This shows a crossover between the two drugs metabolically that may cause drug-drug effects where one drug is preferentially metabolised over the other, delaying metabolism of one and leading to more diffuse or slower metabolism. Timing of administration of the two separate compounds could also alter the way either drug affected symptoms of individual patients. Importantly in the aforementioned study, for any clinical relevance of CBD therapy, the phytocannabinoid was

well tolerated in patients where antipsychotics are often not (273). The studies discussed here indicate some potential for CBD to treat schizophrenia symptoms.

1.5.6 Chronic CBD during adolescence

Most research has examined antipsychotic-like effects of CBD during adulthood. Effects of CBD treatment during adolescence, a period of important neurodevelopment and risk for schizophrenia, are relatively unexplored. A few studies have begun to address how adolescent CBD affects schizophrenia-relevant behaviours, and these are outlined below.

Adolescent CBD treatment can improve schizophrenia-relevant behaviours in animal models for the disease. Poly I:C is an established model of schizophrenia and is used during prenatal development to stimulate an immune response in rodents which in turn results in schizophrenia-relevant behavioural changes to later adult animals (275). Male Poly I:C rats treated for three weeks during adolescence with 10 mg/kg of CBD showed improvement in social interaction deficits caused by Poly I:C treatment as compared to Poly I:C controls, as well as improvements in spatial learning and object recognition memory caused by Poly I:C (276). In the brains of adolescent (PND81) Poly I:C male rats treated with CBD, Poly I:C-induced deficits in CB_1 receptor binding in the PFC reduced levels of GAD_{67}, the enzyme that converts glutamate to GABA, in the hippocampus (HPC) were reversed by adolescent CBD treatment (277). CBD treatment also increased parvalbumin (PV) levels in the HPC in both Poly I:C animals and controls whereas NMDA and GABA-A receptor binding and protein levels of FAAH were unchanged by CBD treatment (277). In female Poly I:C rats, CBD ameliorated adolescent Poly I:C-induced deficits in social interaction and object recognition (278). CBD corrected Poly I:C induced deficits in NMDAR binding in the PFC (278). Chronic CBD during adolescence also increased levels of GAD_{67} and PV protein levels in the HPC of Poly I:C treated offspring (278). However, in an interesting contrast to Poly I:C animals,

control rats treated with chronic CBD displayed deficits in social interaction and CB_1 and NMDAR binding in the PFC (278). This suggests that while chronic adolescent CBD may improve some schizophrenia-relevant domains in Poly I:C challenged mice, this may not be the case for healthy controls. Other studies investigating chronic adult CBD (20 mg/kg for 6 weeks) on healthy C57BL/6J mice have not found negative effects on behavioural paradigms, however (motor, long-term memory, spatial memory, anxiety) (279). In the animals from the above mentioned Poly I:C behavioural studies (241, 276), CBD treatment also normalised a Poly I:C-induced deficit in cholinergic markers in the PFC and HPC (280). Levels of cholinergic markers also positively correlated with working memory performance in the T-maze task but only in male animals (280). Importantly, the studies discussed in this section did not examine long term effects of chronic adolescent CBD. It is therefore unknown whether chronic adolescent CBD would have long-term beneficial effects on behaviour and schizophrenia-related pathology.

It is important to note that long-lasting effects of CBD on behavioural deficits and neurochemical changes relevant to schizophrenia post treatment cessation have not been explored. Furthermore, the chronic CBD effects specific to *Nrg1 TM* HET animals highlight the importance of understanding how CBD may interact with a genetic predisposition for the disease, as to the knowledge of the author chronic adolescent CBD has not been explored as a preventative treatment in any genetic rodent models of schizophrenia.

1.6 Gaps in the literature and the current study

Schizophrenia develops during adolescence and early adulthood, but few studies have looked at the impact of possible developmental interventions for the disorder. Due to the developmental nature of schizophrenia, pharmacological intervention during the premorbid or prodromal period could potentially lessen the impact on a patient's life by diminishing later

symptoms. There is evidence to support this approach: a preliminary study which administered risperidone during the prodromal stage and in first episode psychosis found that risperidone treatment over an 8-12-week period with doses of 1.0 and 1.8 mg/day, respectively, decreased the severity of thought and behaviour disturbance ratings by approximately 30%, and improved verbal learning by ~100% (281). This suggests that drugs that are effective in the treatment of psychosis in patients may also provide some preventative protection from the development of these disease symptoms. Interestingly, risperidone in low doses (0.3–3.0 mg/kg i.p.) given to young adult rats 30 min prior to a lipopolysaccharide (LPS, another model of inflammation that can result in schizophrenia-relevant behavioural changes) challenge has also been shown to prevent increased levels of IL-1β and TNF-α induced by LPS treatment in the brain cortex (282). This suggests that pharmacological intervention can inhibit some aspects of pathology, such as increased neuroinflammation. As discussed, CBD has anti-inflammatory properties and may also be able to decrease levels of neuroinflammation (283, 284).

There is an overall lack of research that investigates using pharmacological interventions during important early developmental periods to protect at-risk patients / model systems against the development of schizophrenia-like symptoms. Because schizophrenia often develops in late adolescence or early adulthood, it may be possible to treat at-risk populations with neuroprotective agents during adolescence that could potentially delay or ameliorate later symptom development. As such, the use of CBD to treat some mild dysfunctions in populations which are at-risk for psychosis has been trialled in a clinical setting, with some tentative results showing CBD can normalise motivational salience and moderate motor response, and tentatively alter cortisol response (285, 286). To understand whether CBD has a place as a preventative drug in schizophrenia, however, we must understand its neuro-behavioural effects both short and long term and in particular when given during adolescence. We must also understand whether CBD affects schizophrenia-relevant

systems when administered chronically, as this could have implications for later THC exposure.

The *Nrg1 TM* HET mouse provides a clinically relevant model in which to trial CBD as a preventative drug for schizophrenia therapy / prevention in at-risk individuals. Importantly, due to the delayed onset of the behavioural phenotype in these mice (i.e. 5-6 months, (177), it is possible to trial preventative treatments in this genetic mouse model. It is also possible to assess interactions between CBD and THC in this model, due to the susceptibility of *Nrg1* mutant mice to cannabinoids. I hypothesised that CBD may be acting therapeutically via mechanisms related to schizophrenia-relevant pathology in these mice and will thereby reduce behaviours relevant to schizophrenia, as well as interact with THC exposure.

Thus, the current study aimed to explore CBD as a potential preventative treatment in the *Nrg1 TM* HET mouse model for schizophrenia. The experiments are designed to investigate both short- and long-term effects of chronic adolescent CBD on schizophrenia-relevant behaviours and brain processes, and to also determine if chronic CBD modulates the neuro-behavioural susceptibility to THC in a genetic mouse model for *Nrg1*.

1.7 Aims

Aim One:

The behavioural phenotype previously recorded in *Nrg1 TM* HET animals has been shown to be sensitive to changes in environment during development and testing, such as cage system and housing enrichment. The neuroinflammatory phenotype is also sensitive to environmental factors such as stress. We therefore aim to investigate the baseline behavioural and pro-inflammatory phenotype of male *Nrg1 TM* HET mice and its sensitivity to acute treatment of THC in a new neuro-behavioural mouse test facility.

Aim Two:

CBD has the potential to protect against pathological neural changes to the *Nrg1 TM* HET mouse during adolescent development that cause later behavioural deficits, possibly by anti-inflammatory or endocannabinoid modulatory effects. We therefore aim to investigate the neuro-behavioural effects of chronic adolescent CBD in schizophrenia-relevant domains immediately post-treatment period at baseline and after acute THC challenge in *Nrg1 TM* HET mutant mice.

Aim Three:

CBD may alter neural systems long-term that cause persisting effects on animal behaviour relevant to schizophrenia. We therefore aim to investigate the neuro-behavioural effects of chronic adolescent CBD in schizophrenia-relevant domains in later adulthood in *Nrg1 TM* HET mice, and investigate whether a history of CBD treatment may modulate the response to later THC exposure.

1.8 Hypotheses

It is hypothesised that baseline behavioural schizophrenia-relevant deficits in *Nrg1 TM* HET mice will reflect those detected previously in this mouse model in our laboratory, including a behavioural phenotype of increased locomotion, reduced social interaction, altered fear context memory, reduced PPI, and reduced anxiety, as well as alterations in pro-inflammatory cytokine levels. It is also hypothesised that chronic treatment of CBD during adolescence will improve schizophrenia-relevant behavioural deficits in *Nrg1 TM* HET mice both during treatment and after an extended washout i.e. in later adulthood, and have both an immediate and long-lasting effect on underlying schizophrenia-related molecular markers and inflammation markers.

Lastly, it is hypothesised that chronic adolescent CBD will modulate the behavioural response of *Nrg1 TM* HET mice to an acute THC challenge.

Chapter 2: General Methods

For these studies, the following general methods were followed for established behavioural and molecular experimental procedures. Further detail on each experiment is provided in individual results chapters e.g. drug treatment, behavioural test order, tissue collection, and molecular markers examined in each cohort.

2.1 Animals

Male *Nrg1 TM* HET mice (previously described by (127), then in further detail by (177)) bred on a C57B6 background and non-mutant wild type-like (WT) littermates were bred and group housed in individually ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at the Animal BioResources (Moss Vale, Australia). Heterozygous males and WT females were bred to produce mixed litters of both *Nrg1 TM* HET animals and WT animals, and control WT mice were therefore littermates of mutant animals. At approximately 21-30 days old, mice were transported to the mouse holding and test facilities at Western Sydney University (WSU) and were transferred to group-housing in filter top cages with 2-3 animals being housed per cage (1144B: Techniplast, Rydalmere Australia) with corn cob bedding (Tecniplast Australia, Rydalmere, Australia), red domes (Able Scientific, Canning Vale, Western Australia) and tissues for nesting. Mice were kept in a 12:12 h light:dark schedule [light phase: white light (illumination: 124 lx), 0900-2100, dark phase: red light (illumination: < 2 lx), 2100-0900] and were tested between 0930 and 1400 in behavioural paradigms. Mice were fed *ad libitum* with mouse feed pellets (Gordon's Specialty Stockfeeds Pty Ltd., Yanderra, Australia) and water was available at all times. Adult or young adult (>3months), male A/J mice from Animal Resources Centre (Canning Vale, Australia) were used as conspecifics in all social-based tests. All experimental procedures and drug treatments etc. were approved by Western Sydney

University Animal Care and Ethics Committee (#A11746 and #A13298) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animal numbers are detailed separately per chapter.

2.2 Drug preparation and treatment regime

Powdered CBD (CBD: THC Pharm GmbH, Frankfurt/Main, Germany) was dissolved in equal parts of 100% ethanol and Tween 80 (Sigma-Aldrich Co, St Louis, USA). It was then diluted with 0.9% sodium chloride to the final concentration (5% ethanol, 5% Tween 80, 90% saline). A vehicle control (VEH) was prepared by mixing all components minus CBD. VEH and CBD were injected daily intraperitoneally (i.p.) at 30 mg/kg body weight using an injection volume of 10 ml/kg body weight. Mice were weighed every four days and dosage was adjusted accordingly. Male *Nrg1 TM* HET and WT controls were treated with either VEH or CBD i.p. daily, starting at postnatal day (PND) ~35 in adolescence (the development period defined previously in our laboratory in (183)), for three weeks. CBD or VEH were always injected in the afternoon (between 1200 – 1500) to limit effects of CBD on behavioural testing, which was conducted in the morning (0930 – 1400). For *Chapter 4* treatment continued throughout behavioural testing (total treatment duration: 6 weeks) until 24 h prior to tissue collection. In *Chapter 5* mice were injected for 3 weeks during adolescence (as per *Chapter 4*), after which injections ceased and animals were left in group housing until reaching 5-6 months of age, when behavioural testing began.

For the THC challenge, THC (100 mg/ml; THC Pharm GmbH) and VEH were prepared similarly to CBD (5% ethanol, 5% Tween 80, 90% saline). An acute dose of 3 mg/kg (a dose to which *Nrg1 TM* HET animals are sensitive to (287), outlined in Chapter 3) was injected i.p. 30 minutes before behavioural testing commenced, at approximately 75 days (±5 days) of age for adolescent animals, and 180 days (±5 days) of age for adult animals.

2.3 Behavioural Tests

Behavioural tests were conducted in the first half of the light phase between 0930 and 1400 in the Behavioural Neuroscience Facility at WSU. All tests were separated by an inter-test interval of at least 48 h and equipment and apparatus were cleaned with 80% ethanol between test runs unless specified otherwise.

Open Field (OF)

This test was used to assess locomotion and exploration behaviours relevant to positive symptom domains in schizophrenia (177, 288) and is an established test in this laboratory (289). Test mice were placed individually into infrared photobeam controlled test chambers (MED Associates Inc., St Albans, USA) for 30 minutes. The test arena (43.2 cm x 43.2 cm) was divided into a central and peripheral zone (MED software coordinates for central zone: 3/3, 3/13, 13/3, 13/13) and time and distance in these zones was measured (locomotion defined as two infrared beam breaks within 100 ms). The distance ratio (% distance/time in centre) and overall centre time were analysed to assess anxiety-related behaviours. Data are presented across 5-min blocks. Additional test parameters included *rearing* frequency (vertical activity), and small motor movement frequency.

Social Interaction (SI)

This test was used to measure social interaction behaviours relevant to negative symptom domains in schizophrenia (290) and has been measured in this laboratory previously in other models (289, 291). The apparatus consisted of a grey Perspex arena (35 x 35 x 30 cm). The paradigm was run in 10 min trials over two consecutive days. On the first day (habituation) test mice were placed in the arena alone and allowed to explore the apparatus freely for 10 min,

then were returned to the home cage. On the following day (test), each test animal was placed into the arena with an age-matched male A/J opponent in the opposite corner, and mice were allowed to interact freely for 10 min. A/J mice were used as they engage in passive social behaviours and do not typically instigate contact, allowing test subject mice social behaviour to be scored more clearly (292). Frequency of and time spent exerting socio-positive behaviours *sniffing, anogenital sniffing, climbing over/under, and following* were recorded manually using ANY-maze tracking software (Stoelting, Wood Dale, USA). These behaviours were combined to produce a total social interaction frequency/duration score.

Prepulse Inhibition (PPI)

This test assessed the acoustic startle response (ASR) and schizophrenia-relevant sensorimotor gating of animals and has been previously used in this laboratory in order to measure relevant deficits in this model (266). While there are a multitude of slightly varied protocols for this paradigm, deficits do not appear to be protocol-reliant (293). The apparatus consisted of Plexiglas mouse enclosures in startle chambers (SR-Lab, San Diego Instruments, San Diego, USA). The test was conducted over four days, beginning with three days of habituation to the apparatus and enclosure for 5 min each day with a constant background noise (70 dB). On the fourth day, the 35-min test trial was run and included a 5 min acclimatisation period with a 70 dB background noise, followed by 97 trials in a pseudorandomised order (5 x 70 dB trials (background); 5 x 100 dB trials; 15 x 120 dB trials (startle) and six sets of prepulse trials using either 74, 82 or 86 dB prepulses presented either 32, 64, 128 or 256 ms [variable interstimulus (prepulse-pulse) interval; ISI] prior to a startle pulse of 120 dB. The intertrial interval (ITI) between individual PPI trials varied randomly from 10 - 20 s. The startle response to each trial was calculated as the mean amplitude detected by the accelerometer. Percentage PPI (% PPI) was calculated as [(mean startle response (120 dB) - PPI response)/mean startle response (120

dB)] x 100. % PPI was averaged across ISI's to produce a mean % PPI for each prepulse intensity.

Fear Conditioning (FC)

This test was used to assess fear-associated learning and memory related to cognitive symptom domains in schizophrenia (188). The apparatus consisted of a fear conditioning chamber with a grid floor (MED-VFC-USB-M, Med Associates Inc., St Albans, VT, USA) (29.5 cm x 24.5 cm x 21 cm). Four animals were excluded in Chapter 3 due to equipment malfunction.

The test was run across three days: conditioning, context test, and cue test. During conditioning, mice were placed into the apparatus chamber for 7 min, and after 2 min an 80 dB conditioned stimulus (CS) cue was presented for 30 s, co-terminating with a 2 s 0.4 mA foot shock [unconditioned stimulus (US)]. The tone-shock pairing was repeated 2 min later and the test ended after another 2 min. During conditioning, a vanilla scent cue (Queen™ imitation vanilla essence) was present in the chamber. For the context test (24 hr later), mice were returned to the apparatus for 7 min with the vanilla scent cue present. For the cue test 24 h later, mice were returned to the apparatus for 9 min; however, the context of the apparatus was altered with a tent-shape covering around the base grid and no vanilla scent present. After 2 min in the cue test, the tone was played for 5 min, concluding 2 min before the end of the test.

Time spent *freezing* (immobile behaviour) was recorded across 1-min bins on all days using Video Freeze® (Med Associates Inc. - software setting: freezing threshold = 15; detection method = linear; minimum freezing duration = 30 frames) and presented as *freezing* over time. Responses to the cue presentation during the cue test were also analysed by comparing percentage of time spent *freezing* in the 2 min prior (i.e. no cue presentation) and the 5 min post cue onset (i.e. during cue presentation).

Cheeseboard (CB)

This test was used to assess spatial reference memory acquisition and retention (294). The test apparatus consisted of a circular board (1.1 m in diameter, elevated 60 cm from the floor, Fig. 2.1) with one flat side and one side with 32 wells (3 cm diameter, 1 cm depth) evenly distributed into 8 radial zones (4 wells in each zone, 5 cm apart – the last well is 10 cm from the edge). The test room walls were marked with clear visual cues (black symbols on a white background: square, circle, plus and minus).

Fig. 2.1: The cheeseboard maze apparatus.

During the 14 days of CB training and testing, mice were food deprived and kept at 85-90% of their initial body weight (body weight was monitored daily) during testing. They were fed approximately one pellet (approximately 2 g) per mouse upon completion of daily testing to keep them within this weight range.

All trials lasted 2 min with a 10 min ITI, and the food reward consisted of several drops of condensed milk in a 1:4 ratio with water, the location of which was different for each mouse but counterbalanced around the board between subjects. The test was run over 14 consecutive days and consisted of a 3-day-habituation period, where each test mouse was placed on the side of the board without wells for 3 trials each day. The next 5 days were part of a training phase where the mouse was placed on the side of the board with wells, with one well being baited with a food reward, where the mouse had 3 trials to learn the location of the food reward each day. The remaining wells on the board were brushed with a fine layer of condensed milk and water so that mice were unable to find the baited well using scent alone. If the mouse did not locate the food reward during the trial, then the mouse was picked up and placed at the edge of the food reward well. Latency to find the baited well was measured for this period both within trials and days. The criteria for task acquisition was taking < 20 s (average of 3 trials per day) to reach the baited well. Target wells were counterbalanced throughout the cohort, however the starting position was always at the centre of the board. Mice were placed facing in a different direction to their respective target well. Successful location of the target well was achieved when the mouse reached the well and began to consume the food reward. The target zone was defined as approximately a 1/8 wedge of the overall cheeseboard surface.

The probe test was conducted the day after all mice had reached the acquisition criteria (after 5 days of training, i.e. experimental test day 9), where no food reward was present, but

all wells were brushed with diluted sweetened condensed milk. The probe test lasted 2 min irrespective of whether animals found the target well. Time spent in the target zone (where food reward was normally located) was recorded using ANY-maze tracking software (Stoelting, Wood Dale, Illinois USA), and time in the target zone as a percentage of overall test time was calculated. The percentage of time spent in the target zone in the first 30 s was analysed against chance levels of exploration (12.5%) as other research has shown that once the animal has found the empty target well, it may start to engage in other search strategies to find the food reward in a new location (295, 296).

The day after the probe trial, the food reward location was changed and mice underwent 3 days of reversal training where they learned the new location of the reward. For each animal, the new reward well was at the opposite position on the board to the original reward. The criteria for reversal learning was < 20 s on average to find the reversal target well. A reversal probe test was conducted the day after the reversal acquisition criteria was met.

Novel Object Recognition Task (NORT)

This test assessed short-term object recognition memory and object exploration (183, 188). The NORT apparatus consisted of a single Perspex chamber (35 x 35 x 30 cm) and two distinct objects (toy giraffe and toy elephant [LEGO®DUPLO®, Billund, Denmark]; Fig. 2.2). This test was repeated with novel objects in a second cohort test battery as in the first test battery animals did not distinguish between the novel and familiar objects. For the second NORT test, a spiky and lumpy ball of similar size were used (Kmart, Australia). This test was conducted over a period of two consecutive days. On the first day, mice were habituated to the arena for 10 min. On the second day, there were 2 x 10 min trials. In the first trial, mice were placed in the arena with two identical objects located an equal distance from the centre and the walls of the apparatus and allowed to explore freely. After a 15 min ITI, animals were returned to the arena

with one familiar object and one novel object. Positions of the novel and familiar objects were counterbalanced within subjects to account for any spatial biases. Time spent *nosing* and *rearing* on these objects were recorded using ANY-Maze tracking software manually. These behaviours combined were used to measure 'total exploration' (time and frequency) of each object, as well as the percentage of *nosing* and *rearing on* and total exploration behaviours on the novel versus familiar object.

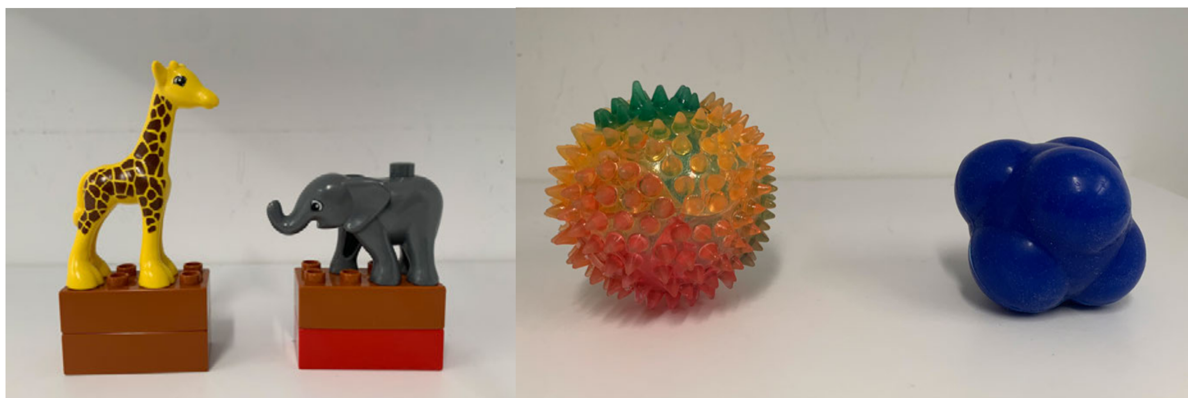

Fig. 2.2 Objects used in the novel object recognition task (NORT): Giraffe (left), elephant (middle left), spiky ball (middle right), and lumpy ball (right).

Light-Dark Test (LDT)

The LDT was used to measure anxiety in animals (297). For this test, the OF apparatus (MED Associates Inc., St Albans, USA) was equipped with an opaque dark box insert covering half the chamber (297). This separated a light zone (20 Lux) from a dark zone (< 2 Lux) by an opening in the centre of the insert (9 cm wide). Mice were placed into the opening facing toward the dark zone. The time spent and distance travelled in the two chambers (calculated to percentage in light or dark 'zone') were recorded for 10 min. Time spent in the aversive light chamber is presented as a percentage of overall test time and was used to measure anxiety.

Social Preference Test (SPT)

SPT was used to assess sociability and social recognition memory, the latter of which has found to be mildly impaired in *Nrg1 TM* HET mice (174, 181). The test apparatus consisted of a three-chamber box, where two outer chambers (16 cm x 18 cm x 20 cm) were united by a central chamber (16 cm x 18 cm x 20 cm). In the two outer chambers a circular mouse enclosure (diameter: 7 cm; height: 15 cm; bars spaced: 0.5 cm) was located against the far wall on each side of the apparatus (Fig. 2.3). The apparatus was filled with the same corn bedding used in mouse housing, to increase familiarity with the apparatus and control for glare from the plastic base (1 cm deep layer of bedding).

The test consisted of 3 x trials, beginning with a 5 min habituation trial in the apparatus. Mice were isolated in a cage identical to their home cage for 1 hour prior to habituation, and were returned to this cage between trials. During habituation, mice were allowed to freely explore the apparatus. The second trial was the sociability trial, where an age and sex-matched opponent (male A/J mouse) was placed in one mouse enclosure, and the other enclosure was empty. Test mice could explore the apparatus and the A/J mouse for 10 min. Time spent *nosing* the mouse enclosure and time spent in each chamber was measured. *Nosing* is defined as close social exploration (i.e. sniffing within 1-2 cm) of the control mouse. In the third trial (social novelty, 10 min), a novel opponent mouse was placed in the opposite enclosure while the mouse from the sociability trial was in the same enclosure. Thus, both enclosures contained mice, one familiar and one novel. Time spent *nosing* enclosures and time in chambers was measured. The ITI between trials was 3 min and the apparatus was unaltered between trials to limit any novel apparatus anxiety. The entire apparatus was cleaned with water and 80% ethanol between each test mouse, and fresh cob bedding was added. Data was recorded using ANY-Maze tracking software.

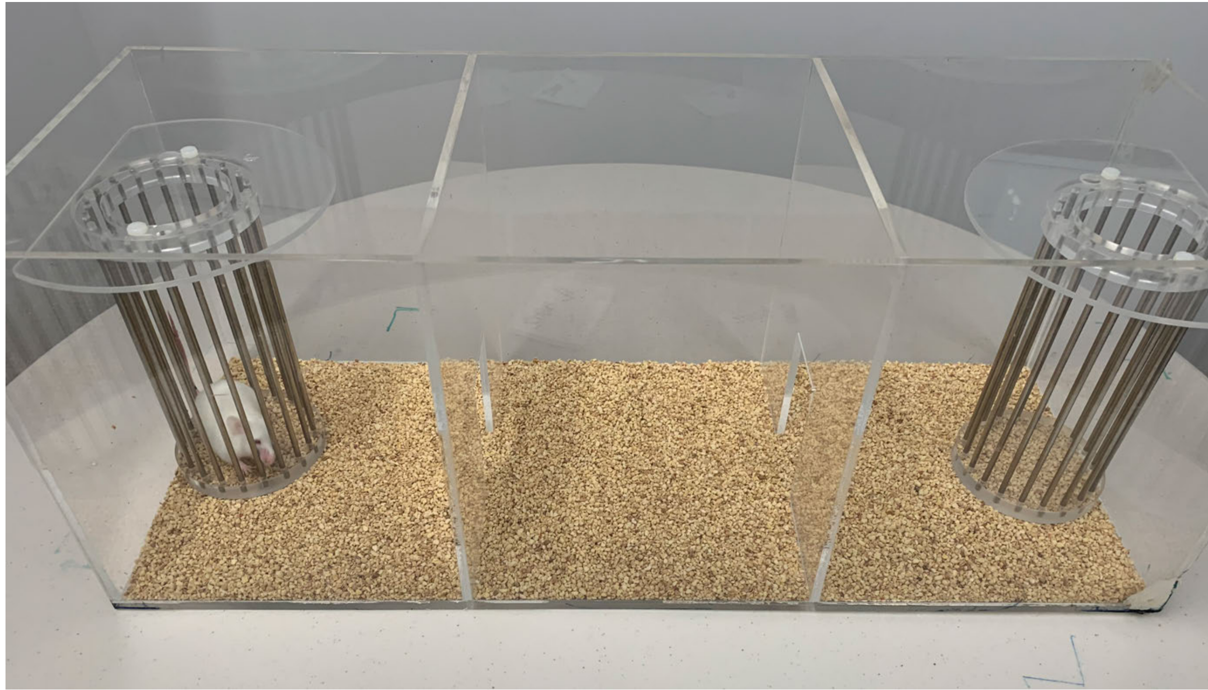

Fig. 2.3: Social preference test (SPT) and social novelty apparatus: The mouse is placed in the centre chamber and allowed to explore either empty chambers or chambers containing control mice inside enclosures.

2.4 Tissue Collection and preparation

One week after completion of behavioural testing, mice were anaesthetized with isoflurane gas and perfused with phosphate buffered saline (PBS) transcardially (298). Brains were removed post-mortem and divided sagitally. The left hemisphere was fixed in 4% paraformaldehyde for 24 h and then blocked in 30% sucrose for approximately 48 h, before being snap frozen in liquid nitrogen and stored in -80˚C. The right hemisphere was dissected for the hippocampus, prefrontal area, and striatum, which were snap frozen on dry ice upon removal and stored in -80˚C.

Frozen brain regions (5-20 mg) were homogenized manually with syringes of decreasing needle diameter (21, 25, 27 gauge) in 12 volumes of radioimmunoprecipitation assay buffer [RIPA; sodium chloride (5 M), Tris-HCl (1 M, pH 8.0), nonidet P-40, sodium deoxycholate (10 %), SDS (10%), Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (100X) and 10 uM PMSF]. Homogenates were centrifuged at 3,750 g for 20 min at

4°C and the soluble supernatant was collected. Supernatant was stored at -80˚C until used in experiments. Protein content of samples was quantified using Qubit protein assay kit (Life Technologies, Thermofisher Scientific, Carlsbad, USA).

2.5 Western Blot

Samples were diluted to a protein concentration of 2 μg/μl with NuPAGE™ reducing agent [10X] (Thermofisher Scientific) in a 1:7 dilution with NuPAGE™ LDS sample buffer [4X] (Thermofisher Scientific). 10 μl of sample was added to each well in Bolt™ 4-12% Bis-Tris 15-well minigels (Thermofisher Scientific) alongside a protein ladder. Gels were run in Bolt™ MES SDS Running Buffer [20X] in Thermofisher Mini Gel Tanks for approximately 1.5 hours at 100V. Gels were removed from housing cassettes and layered into a transfer sandwich with Bolt™ Transfer Buffer (20X) (combined with Bolt™ Antioxidant and methanol 1:10) (Thermofisher Scientific) and a nitrocellulose membrane and transferred in the tank for 1 hour at 10V. Once removed from the sandwich, membranes were stained with Ponceau S, Acid Red 112 reversible protein stain (Sigma-Aldrich) and membranes were cut to relevant sizes. Membranes were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline-Tween®20 (TBST) [1X] for 1 hour. Primary antibodies were incubated overnight at 4ºC in the following concentrations: CB_1 polyclonal serine 316 antibody (1:500, Thermofisher Scientific [BS-1683R]), GAD_{67} polyclonal antibody (1:2000, Thermofisher Scientific [PA5-21397]), Iba1 (1:1000, Novachem [019-19741]), actin housekeeper 1:1000 (Sigma-Aldrich [A2066]). Goat anti-rabbit IgG HRP-conjugated secondary antibody (1:10,000, Millipore [AP132P]) and enhanced chemiluminescence was used to detect signals. Signals were quantified using image J software. Data were normalised to actin levels and expressed as relative values of the highest signalling sample in the gel.

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

The concentration of TNF-α, IL-1β, and IL-10 protein in soluble homogenised hippocampal and prefrontal cortical tissue of *Nrg1 TM* HET mice and WT controls were quantified using Invitrogen TNF-α Mouse ELISA Kit (ThermoFisher Scientific), Invitrogen IL-1β ELISA Kit (ThermoFisher Scientific), and Invitrogen IL-10 ELISA kit (Thermofisher Scientific). Instructions as per kit were followed for each procedure.

2.7 Statistical analysis

Sample group sizes were between 15-25 mice and were based off previous publications using this model in our laboratory and all mouse data was included (i.e. no outliers detected or removed) (188). Four-way repeated measures (RM) analysis of variance (ANOVA) were used if distribution conditions were met to analyse main effects and interactions between factors 'genotype', 'CBD', and 'THC', and within factors '1-min block' (FC), 'block' (OF, PPI, & FC), 'startle block' (PPI), 'prepulse intensity' (PPI), and 'dB' (PPI). Three-way RM ANOVA were also used to analyse the main effects and interactions of 'genotype' and 'CBD' and these within factors if conditions were met. Three-way ANOVA were used to analyse main effects and interactions between 'genotype', 'CBD', and 'THC', and two-way ANOVA were used to analyse main effects and interactions between 'genotype' and 'CBD'. Two-way RM ANOVA were also used to investigate differences between 'genotype' within factors 'day', 'startle block', and 'prepulse intensity'. In case of significant interactions between factors, further ANOVA after splitting data by the corresponding factor were used to in order to investigate specific group differences. One-way ANOVA were used to analyse data where 'genotype' was the only between group factor, and single sample t-tests were used to investigate levels of activity (*nosing* [s], time [s], etc.) against possible chance. Sidak's multiple comparisons were used to compare dosage differences within groups in the THC dose test. Group differences

were regarded as significant if $p < 0.05$. F-values and degrees of freedom are presented for all ANOVAs. Data are shown as means ± standard error of means (SEM). Significant 'CBD' effects are reported by '#' (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). Significant 'genotype' effects are shown by '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Significant 'THC' effects are shown by '^' (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$). RM effects are shown by '+' (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$), and any trend effects are reported with a *T* and the corresponding *p* value. Statistical analyses were conducted using SPSS 27 for Mac and GraphPad Prism 8 for Mac.

Chapter 3: Baseline behavioural and neuroinflammatory phenotype of adult male *Nrg1 TM* HET mice

3.1 Introduction

Relating a preclinical rodent model to a disease phenotype requires that the model fit a specific set of validity conditions. This includes: face validity, whereby the model must show behaviours which mimic those seen in the clinical condition; construct validity; whereby the causative factor in the disease is modelled (e.g. a specific gene or environmental risk factor or interaction); and predictive validity, whereby the model must show a similar response to therapeutic agents relevant to the disease (or in the case of schizophrenia, e.g. a heightened response to symptom-triggering drugs).

The *Nrg1 TM* HET mouse model of genetic risk for schizophrenia has repeatedly demonstrated all three of these validity conditions across multiple laboratories, authors, and paradigms. The behavioural phenotype includes locomotor hyperactivity (relevant to positive symptoms/psychosis, see (177, 182, 299)), reduced social interaction (relevant to negative symptoms of schizophrenia, see (181, 300)), and impaired sensorimotor gating (also seen in patients with schizophrenia, see (301)) and appears age-dependent (i.e. develops at around 5-6 months of age (177)). Males show a more severe phenotype than females of this genotype (183, 188), which is in line with gender-specific differences found in schizophrenia patients (302).

Importantly, some of the key pathology seen in patients with schizophrenia has also been observed in *Nrg1* mutant animals. For example, NMDAR levels are increased in the cortex and nucleus accumbens of *Nrg1 TM* HET mice, but are reduced in the thalamus, while striatal D_2 receptors are reduced in *Nrg1* mutants compared to WT controls (179). Hippocampal NMDA receptor NR2B subunit phosphorylation is also lower in *Nrg1 TM* HET mice, and

elevated ongoing and reduced sensory-evoked gamma power is evident in this model, which is relevant to the hyperlocomotive phenotype in *Nrg1 TM* HET mice (178, 303). Reduced NMDA receptor activity in the hippocampus and frontal cortex of patients produces negative and cognitive symptoms (78), and excess glutamate release in the cortex via NMDA receptor blockade is theorised to contribute to some psychotic symptoms (79), suggesting these changes in *Nrg1* mutants are patient-relevant. Significant reductions in hippocampal expression of GAD_{67} and parvalbumin are also found in *Nrg1 TM* HET mice (304). Malfunction in GABAergic parvalbumin-containing cortical neurons has been linked to cognitive impairments in schizophrenia (42, 83). In addition, *Nrg1 TM* HET mice also have an altered cytokine profile from WT mice, with female *Nrg1* mutant mice displaying decreased serum cytokine levels of IL-6, IL-8 and IL-10 and increased TNF-α, while males show decreased serum IL-1β and TNF-α (173). Alterations in cytokines and microglial activation are hypothesised to be key in both the development and possibly symptomology of schizophrenia (64, 152, 305).

Importantly, *Nrg1 TM* HET mice are sensitive to environmental risk factors for schizophrenia during early life, adolescence and adulthood, drawing parallels with schizophrenia development and pathology. These factors include: maternal immune activation (180), which by itself can induce a robust preclinical model of schizophrenia (275, 306); adolescent and adult stress (169, 173, 174); limited environmental enrichment during development (177); and cannabinoids (such as THC) ((168, 184-186); review: (176)). Sensitivity to cannabinoids, and in particular THC, is also observed in patients with schizophrenia (157). Furthermore, *Nrg1 TM* HET mice also show sensitivity to different laboratory environments, with PPI deficits in this model being particularly vulnerable to particular conditions of the test laboratory as well as protocol differences (301) – not an unusual phenomenon when comparing genetic mouse models or different laboratory strains across different laboratories (307). The behavioural phenotype is also affected by housing type and

the environmental enrichment provided to animals during testing, with the phenotype being weaker with individually-ventilated housing as opposed to filter top housing, and the phenotype being enhanced by the inclusion of limited environmental enrichment (177, 187).

Considering the behavioural phenotype of *Nrg1 TM* HET mice is sensitive to changes in laboratory setting and housing type, it is necessary to confirm the behavioural phenotype and sensitivity to THC in *Nrg1 TM* HET mice when working in a newly established testing facility. This was the case for this current PhD research project as the research team led by Prof. Karl moved to the School of Medicine, Western Sydney University in early 2016, and established a new purpose-built mouse phenotyping facility. It was also necessary to confirm the dominant and key behavioural deficits in this mouse in order to determine testing parameters in further experiments. Housing conditions and test apparatus were kept as consistent as possible with previous publications using this model (177, 187). It was also necessary to confirm the neuroinflammatory profile of *Nrg1 TM* HET mice, as cytokine and microglia levels in these mice had not been examined in our laboratory. Importantly, elevated levels of cytokines or microglia could be a mechanism underlying schizophrenia-relevant behaviours in these mice and would provide a potential mechanism by which CBD could ameliorate the disease phenotype in this model. Thus, in Chapter 3, we thoroughly assessed the behavioural and neuroinflammatory phenotype of adult male *Nrg1 TM* HET mice, as well as their sensitivity to an acute THC challenge.

Aim One:

The behavioural phenotype previously recorded in *Nrg1 TM* HET animals has been shown to be sensitive to changes in environment during development and testing, such as cage system and housing enrichment. The neuroinflammatory phenotype is also sensitive to environmental factors such as stress. We therefore aim to investigate the baseline behavioural and pro-

inflammatory phenotype of male *Nrg1 TM* HET mice and its sensitivity to acute treatment of THC in a new neuro-behavioural mouse test facility.

3.2 Methods

Main behavioural tests, animal details, and drug preparation are outlined in Chapter 2. *Nrg1 TM* HET and WT control mice were group housed (2-3 animals per cage) throughout experiments in Chapter 3. Bodyweight was monitored every 48 h and significant changes across time and between genotype or treatment condition were not detected throughout the experiment (data not shown). Mice were tested at 5-7 months (± 4 weeks) of age, in three cohorts of 15-25 mice (see Table 3.2.1 for the number of animals per cohort). Each of the three cohorts completed 4-5 behavioural tests across a two-three-week period with minimum 48 h between different tests, using behavioural test methods described in Methods 2.3 (test order: Table 3.1). Cohort 3 was used to top up numbers for some tests (i.e. OF, SI, PPI, FC) where low *n* might obscure group effects, and then underwent additional testing with an acute THC challenge – mice were injected with vehicle, 3 or 5 mg/kg THC i.p. 30 min prior to OF testing in a quasi-randomised order, where each mouse was given each treatment on a different day with 24 h washout between (Methods 2.2). This was used to determine doses of THC which *Nrg1 TM* HET mice were more sensitive to, informing experiments in Chapter 4 and Chapter 5, similar to what has been reported previously (168, 186).

After behavioural testing concluded, mice were euthanised according to General Methods (Methods 2.4). For cohorts 1 and 2, brain tissue was collected according to General Methods (Methods 2.4). For cohort 3 no tissue was taken due to the additional THC testing. Mouse brain tissue was homogenised according to the General Methods (Methods 2.4). Western blot and ELISA protocols were performed according to General Methods (Methods 2.5 and 2.6 respectively), to measure cytokine levels of IL-10, IL-1β and TNF-α in the PFC and hippocampus, and levels of microglial activation marker Iba1 in the hippocampus, PFC, and striatum. Cytokine concentrations in the striatum were not analysed, as protein concentrations in this region were too low for implementing this technique.

Two-way RM ANOVA were used to investigate differences between the main between factor 'genotype' and to determine interactions with the within factors 'day', 'startle block', 'dB', 'cue', 'prepulse intensity', and 'THC dose'. In case of significant interactions, further ANOVA split by corresponding factor were used to investigate specific group differences. One-way ANOVA were used to analyse data where 'genotype' was the only between group factor, and single sample t-tests against chance levels were used to determine preferences (*nosing* time [s], time in zone [s], etc.) for NORT, SPT and CB tests. Sidak's multiple comparisons were used to compare individual doses in the THC test. Group differences were regarded as significant if $p < 0.05$. Trend effects were acknowledged if p was between 0.05 - 0.06. F-values and degrees of freedom are presented for all ANOVAs. Data are shown as means ± standard error of means (SEM). Significant 'genotype' effects are shown by '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Significant 'THC' effects are shown by '^' (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$). RM effects are shown by '+' (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$), and any trend effects are reported with a *T* and the corresponding p value. Statistical analyses were conducted using SPSS 27 for Mac and GraphPad Prism 8 for Mac.

Cohort	**Age at start of testing**	**WT – sample size**	***Nrg1 TM* HET – sample size**	**Tests Biography**
Cohort 1	24 ± 4 weeks	14	7	OF, NORT, SPT, CB
Cohort 2	24 ± 4 weeks	8	13	LDT, SI, PPI, NORT, FC
Cohort 3	24 ± 4 weeks	8	10	OF, SI, PPI, FC, OF (acute THC challenge)

Table 3.1: Animal numbers, age, and cohort testing schedules: Animals used and test order used to investigate the behavioural phenotype of wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Abbreviations: CB: cheeseboard, FC: fear conditioning, LDT: light-dark test, NORT: novel object recognition test, OF: open field, PPI: prepulse inhibition, SI: social interaction, SPT: social preference test.

3.3 Results

3.3.1 Locomotion and exploration

At 5-7 months of age, male *Nrg1 TM* HET mice displayed increased locomotion in the OF (i.e. distance travelled) compared to WT mice ['genotype': $F(1,36) = 5.8$; $p = 0.02$; Fig. 3.1A]. This was similar in the LDT, where *Nrg1 TM* HET mice travelled further in both zones ['genotype': $F(1,38) = 5.814$; $p = 0.02$; Table 3.2].

Exploratory behaviour (i.e. *rearing* frequency) in the OF was not different between the genotypes [one-way ANOVA: $F(1,36) = 1.9$; $p = 0.2$; Fig. 3.1B], nor were small motor movements affected by 'genotype' [$F(1,36) = 1.9$; $p = 0.2$; Fig. 3.1C]. This was similar in the LDT test, where total *rearing* was unaffected by 'genotype' [$F(1,20) = 0.3$; $p = 0.6$; Table 3.2], as were small motor movements [$F(1,20) = 2.9$; $p = 0.1$; Table 3.2].

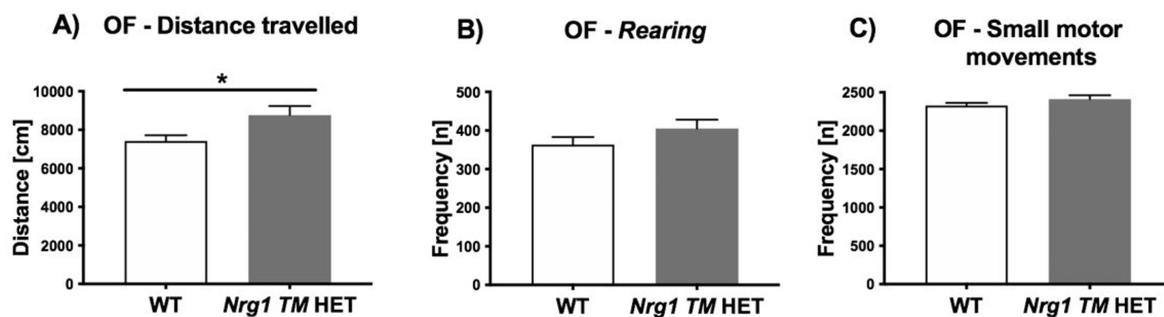

Fig. 3.1A-C: Open field (OF) locomotion and exploration: A) Total distance travelled [cm], B) total *rearing* frequency [n], and C) total small motor movement frequency [n] in the OF. Data expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Main effects of 'genotype' against WT mice are indicated by $*p < 0.05$.

3.3.2 Anxiety

In the OF test, no 'genotype' effects were found for time spent in the centre of the arena [$F(1,36) = 0.05$; $p = 0.8$; Table 3.2], or distance ratio in the centre [$F(1,36) = 3.2$; $p = 0.08$; Table 3.2], demonstrating no changes in anxiety-related OF behaviours in *Nrg1 TM* HET males

compared to WT controls. Likewise, in the LDT, the percent time in the light zone [$F(1,20) = 0.07$; $p = 0.8$; Table 3.2] and the percent distance in the light zone [$F(1,20) = 0.1$; $p = 0.7$; Table 3.2] did not differ between genotypes, suggesting no alterations in anxiety-related behaviour in this test in *Nrg1 TM* HET animals. Both genotypes travelled less in the light zone compared to the dark chamber, indicating this zone was aversive [two-way RM ANOVA for 'zone': $F(1,38) = 165.6$; $p < 0.0001$; no significant 'zone' by 'genotype' interaction] (Table 3.2).

Open field and light-dark test	**Genotype**	
Test / Measure	WT	*Nrg1 TM* HET
OF: centre time [s]	497 ± 34.3	508.3 ± 40.4
OF: distance ratio [%]	39.8 ± 1.5	43.8 ± 1.6
LDT: *rearing* [n]	81.9 ± 16.7	94.6 ± 15.7
LDT: small motor movements [n]	817.8 ± 22	752.7 ± 28.4
LDT: time in light zone [%]	37.7 ± 3	36.8 ± 1.7
LDT: distance in light zone [cm]	1471.5 ± 114.4	1774 ± 153 *
LDT: distance in dark zone [cm]	2311.8 ± 76.3	2667.3 ± 146.2 *
LDT: distance in light zone [%]	61.4 ± 2	60.5 ± 1.4
LDT: distance in dark zone [%]	38.6 ± 2	39.5 ± 1.4

Table 3.2: Locomotion and exploration in the open field (OF) and light-dark test (LDT): OF centre time [s], OF distance ratio [%], LDT *rearing* frequency [n], LDT small motor movement frequency [n], LDT time in the light zone [%], and LDT distance in the light and dark zones [cm/%] expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Main effects of 'genotype' against WT males are indicated with asterisks (*$p < 0.05$).

3.3.3 Social behaviours

Social interaction test: Social interaction (SI) was significantly reduced in *Nrg1 TM* HET animals, with decreased *nosing* time [$F(1,39) = 6$; $p = 0.02$], *climbing on/over* time [$F(1,39) = 8.3$; $p = 0.007$], and *climbing on/over* frequency [$F(1,39) = 5.5$; $p = 0.02$] in *Nrg1 TM* HET mice compared to WT controls (Table 3.3). Importantly, overall social interaction time was also significantly lower in *Nrg1 TM* HET animals [$F(1,39) = 4.9$; $p = 0.03$; Fig. 3.2]. For overview of all social behaviours recorded see Table 3.3.

Social interaction	**Genotype**	
Behaviour - Duration	WT	*Nrg1 TM* HET
Nosing [s]	84.7 ± 7.4	64.9 ± 4.3 *
Anogenital Sniffing [s]	44.5 ± 3.7	41.4 ± 3.4
Climbing On/Over [s]	25.5 ± 4.1	13.9 ± 1.6 **
Following [s]	5.7 ± 1.7	7.3 ± 1.4
Behaviour - Frequency	WT	*Nrg1 TM* HET
Nosing [n]	73.2 ± 3.8	71.4 ± 2.6
Anogenital Sniffing [n]	36.1 ± 2.2	39.1 ± 3.02
Climbing On/Over [n]	24.5 ± 3.2	16.8 ± 1.4 *
Following [n]	5.8 ± 1.5	8.8 ± 1.5

Table 3.3: Socio-positive behaviours in the social interaction (SI) test: Duration [s] and frequency [n] of *nosing, anogenital sniffing, climbing on/over*, and *following* the A/J mouse in the social interaction (SI) test. Data expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Main effects of 'genotype' against WT males are indicated with asterisks (*$p < 0.05$, **$p < 0.01$).

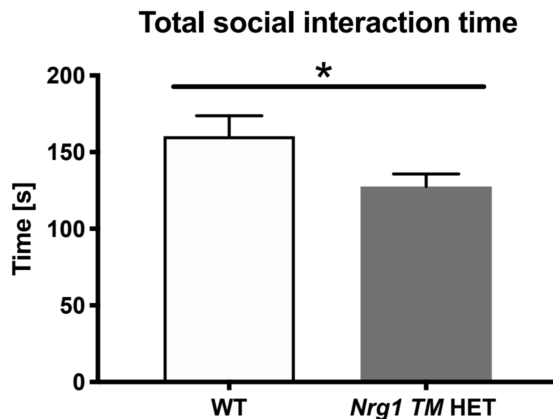

Fig. 3.2: Total interaction time [s] in the social interaction (SI) test: Data expressed as mean ± SEM for either wild type-like (WT) or *neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. *Nrg1 TM* HET animals displayed decreased total SI time (main effect of 'genotype': *$p < 0.05$).

Social preference and social novelty: Social preference was unaffected by 'genotype', with single sample t-tests for time in the chamber containing a mouse against chance levels (50%) indicating that male *Nrg1 TM* HET animals preferred the mouse to an empty chamber [$t = 3.4$, $df = 6$; $p = 0.01$], as did male WT animals [$t = 6.6$, $df = 13$; $p < 0.0001$; Fig. 3.3A].

In social novelty, a single sample t-test for the percentage time spent in the chamber of the novel of the mouse compared to chance showed neither *Nrg1 TM* HET [$t = 1.5$, $df = 6$; $p = 0.18$] nor WT [$t = 0.9$, $df = 10$; $p = 0.4$] animals preferred the chamber containing the novel mouse (Fig. 3.3B).

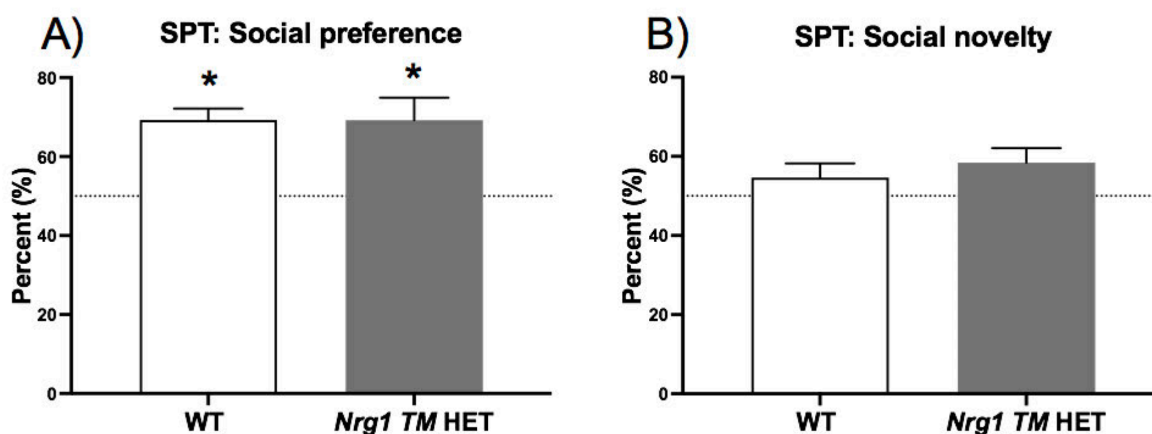

Fig. 3.3A-B: Social preference and social novelty: overall time as a percentage [%] of A) total time spent interacting with a mouse over no mouse, and B) time interacting with a novel mouse over a familiar mouse. All data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Social preference above chance levels (i.e. 50 %) is indicated with an asterisk (**p* < 0.05).

3.3.4 Sensorimotor gating

Prepulse inhibition: Startle responses increased overall as startle intensities increased [RM effect of 'dB', $F(2,68) = 85.06$; $p < 0.0001$]. *Nrg1 TM* HET animals exhibited a reduced acoustic startle response (ASR) across startle pulse intensities [two-way RM ANOVA main effect of 'genotype': $F(1,34) = 6.1$; $p = 0.02$; Fig. 3.4A]. An interaction between 'dB' and 'genotype' [$F(2,68) = 5.7$; $p = 0.005$] revealed that this genotype difference was dependent on startle pulse intensities. Indeed, one-way ANOVA at each startle intensity revealed reduced startle in *Nrg1* mutants only at 100 dB [$F(1,34) = 7.9$; $p = 0.008$] and 120 dB [$F(1,34) = 4.4$; $p = 0.04$] (Fig. 3.4A).

Two-way RM ANOVA for startle habituation comparing the first, middle, and last five startle responses demonstrated an overall reduction in startle across startle pulse presentations [RM ANOVA for 'startle block': $F(2,68) = 3.9$; $p = 0.02$; Fig. 3.4B]. *Nrg1 TM* HET mice startled less across the 120dB startle blocks in line with the above analysis ['genotype' $F(1,34) = 4.4$; $p = 0.04$; Fig. 3.4B], but no significant interaction was present between 'genotype' and

‘startle block’ [$F(2, 68) = 2.5$; $p = 0.09$], indicating that while ASR was lower in *Nrg1* mutant mice than WTs, startle habituation occurred in a similar manner between genotypes.

For %PPI, there was a significant RM effect of ‘prepulse intensity’ indicating animals exhibited greater prepulse inhibition with higher prepulse intensities [$F(2,68) = 151.4$; $p < 0.0001$]. Furthermore, a trend for overall reduced sensorimotor gating in *Nrg1 TM* HET mice compared to control mice was detected [$F(1,34) = 3.7$; $p = 0.056$; Fig. 3.4C], however, there was no interaction between ‘genotype’ and ‘prepulse intensity’ [$F(2, 68) = 0.6$; $p = 0.6$]. When analysing each prepulse individually (74, 82, 86 dB), overall reduced %PPI was detected in *Nrg1* mutant mice at the 86 dB prepulse [main effect of ‘genotype’: $F(1,34) = 5$; $p = 0.03$; Fig. 3.4C]. No effects of ‘genotype’ were found in other prepulse intensities.

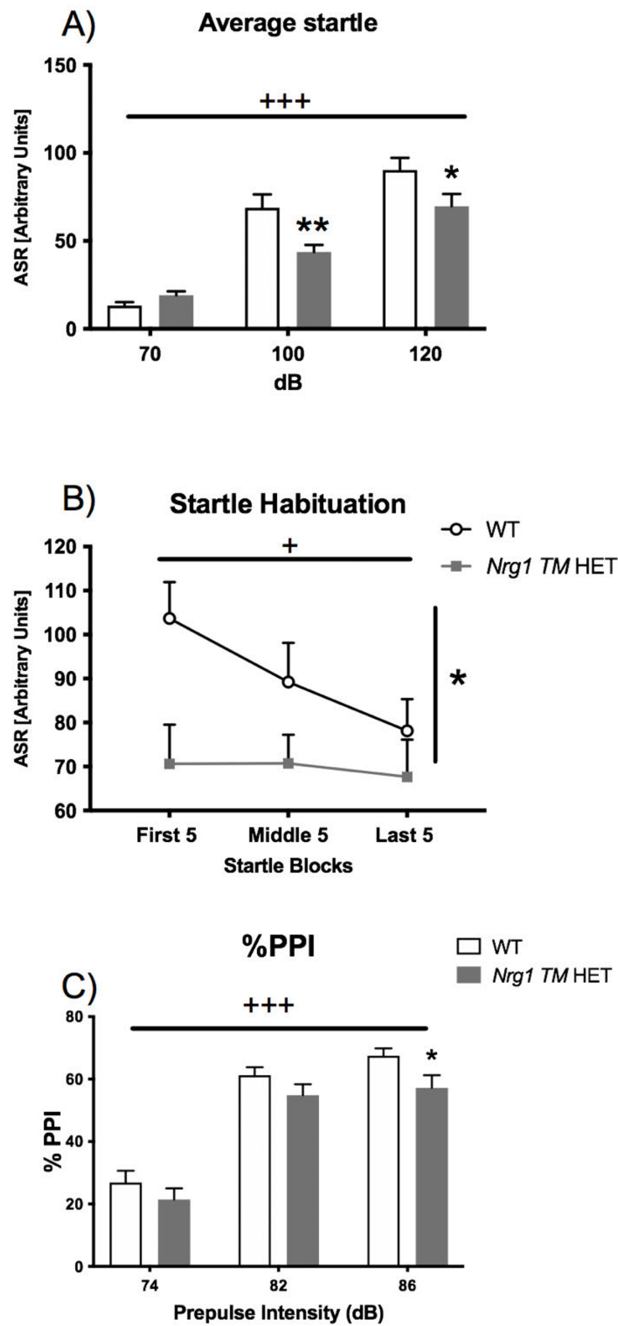

Fig. 3.4A-C: Acoustic startle response (ASR), startle habituation, and average % prepulse inhibition (PPI): A) average startle [arbitrary units] across prepulse intensities in PPI; B) startle habituation [arbitrary units] across the testing blocks; and C) average PPI [%] averaged across prepulse intensities, data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Effects of 'genotype' indicated with $^{*}p < 0.05$ and $^{**}p < 0.01$. RM effects indicated with $^{+}p < 0.05$ and $^{+++}p < 0.001$.

3.3.5 Learning and memory

Fear-associated memory in fear conditioning: There were no 'genotype' differences in baseline *freezing* [F(1,34) = 1.06; $p = 0.3$] (i.e. first 2 min of *freezing* during conditioning prior to the presentation of CS / US), indicating that no baseline differences in *freezing* confounded experimental test outcomes. Furthermore, during conditioning, all animals *froze* more after receiving foot shocks [two-way RM ANOVA for '1-min block': F(6,192) = 25.5; $p < 0.0001$], and there were no differences in *freezing* between *Nrg1 TM* HET and WT mice across time [no 'genotype' by '1-min block' interaction: F(6,192) = 1.2; $p = 0.3$; Fig. 3.5A].

Contextual fear-associated memory was unchanged in *Nrg1 TM* HET animals at 5-7 months of age as both genotypes displayed similar levels of *freezing* to the shock-associated context across 1-min blocks [no 'genotype' by '1-min block' interaction: F(6,216) = 0.7; $p = 0.7$; Fig. 3.5B]. A main effect of 'time' showed *freezing* behaviour changed across the test [F(6,216) = 4.6; $p = 0.0002$].

In the Cue test, an effect of 'time' showed that the *freezing* response changed across the test period and in response to cue on / off [F(8,288) = 57.1; $p < 0.0001$; Fig. 3.5C]. There was no interaction between 'genotype' and '1-min block' [F(8,288) = 0.9; $p = 0.5$], indicating *Nrg1* mutants *froze* in a similar manner to WTs during the Cue test. Comparing *freezing* prior and during cue presentation, average *freezing* was greater during cue presentation (averaged across minutes 3-7) compared to prior to cue presentation (averaged across first 2 minutes) ['cue': F(1,36) = 189.6; $p < 0.0001$]; this was not different between the genotypes [no 'cue' by 'genotype' interaction: F(1,36) = 0.4; $p = 0.5$; Fig. 3.5D].

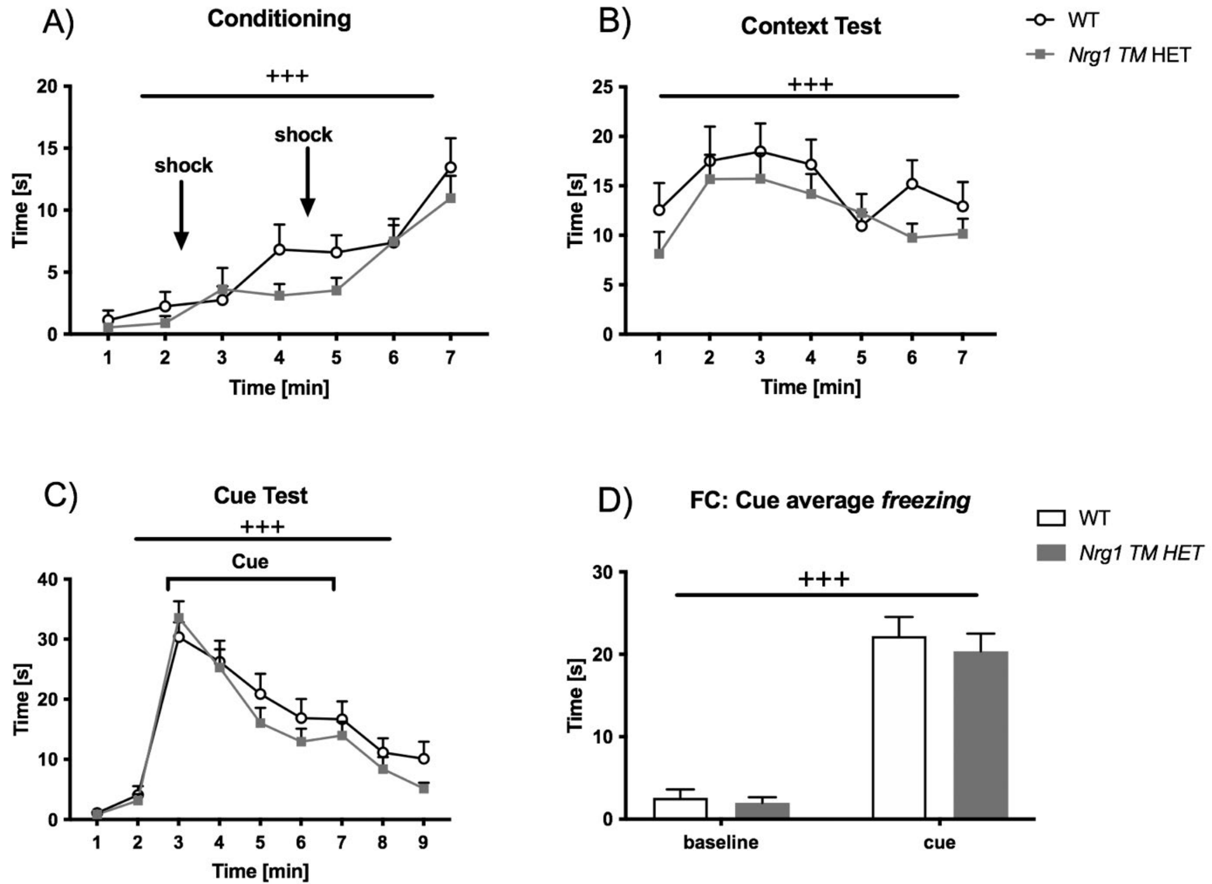

Fig. 3.5A-D: *Freezing* time [s] in fear conditioning: A) *freezing* [s] in the conditioning trial, B) *freezing* [s] in the context test, C) *freezing* [s] during the cue test, and D) *freezing* [s] in the cue test during the cue playing and at baseline expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. There were no genotype differences or interactions on any test day. RM effects indicated with $^{+++}p <$ 0.001.

Spatial learning and memory in the cheeseboard: All animals decreased their average latency to reach the target well (averaged across three daily trials) across training days, indicating successful learning [two-way RM ANOVA for 'day': F(4,64) = 21.3; p <0.0001; Fig. 3.6A]. *Nrg1 TM* HET animals showed no deficits in spatial learning compared to WT animals, as average latency did not differ across training days between genotypes [no 'genotype' by 'day' interaction: F(4,64) = 1.2; p = 0.3; Fig. 3.6A]. Long-term memory, investigated by analysing trial 1 across days showed that latency in the first trial decreased across days [F(4,64) = 6.4; p = 0.0002] regardless of genotype [no 'genotype' by 'day'

interaction: $F(4,64) = 2.2$; $p = 0.08$], suggesting intact long-term memory in all animals (data not shown). To investigate intermediate-term memory, trials 2 and 3 were also averaged per day. A main effect of ‘day’ showed that all animals spent less time finding the baited well in these trials as testing progressed [$F(4,64) = 22.1$; $p < 0.0001$] and a lack of ‘genotype’ x ‘day’ interaction suggested there were no differences in learning between genotypes for intermediate-term memory [$F(4,64) = 0.7$; $p = 0.6$; data not shown].

In the first 30 s of the probe test, single sample t-tests against chance levels (12.5 %) demonstrated that both genotypes spent more time in the target zone than chance, indicating spatial memory recall in the probe test [WT: t = 3.3, df = 10; $p = 0.008$; *Nrg1 TM* HET: t = 3.3, df = 6; $p = 0.01$; Fig. 3.7A]. The first 30 s is relevant as once the animal has found the empty target well, it may start to engage in other search strategies to find the food reward in a new location (295, 296). When analysing target zone across full test duration, neither *Nrg1* mutants [t = 2.2, df = 6; $p = 0.07$] nor WT animals [t = 1.9, df = 10; $p = 0.09$] spent more time in the target zone.

During reversal learning, daily average latency decreased across days in both genotypes as they learned the new location of the food reward [two-way RM ANOVA for ‘days’: $F(2,32) = 7.66$; $p = 0.002$; Fig. 3.6B]. *Nrg1 TM* HET animals displayed similar reversal learning performance compared to WT animals [no ‘day’ by ‘genotype’ interaction: $F(2,32) = 0.4$; $p = 0.7$]. Long-term memory in reversal did not improve over days [$F(2,32) = 1.7$; $p = 0.2$] and was unaffected by genotype [no ‘genotype’ by ‘day’ interaction: $F(2,32) = 1$; $p = 0.4$] (data not shown). Intermediate-term memory improved across days [$F(2,32) = 12.2$; $p = 0.0001$] and this was not different between the genotypes [no ‘day’ by ‘genotype’ interaction: $F(2,32) = 0.8$; $p = 0.4$] (data not shown).

Nrg1 TM HET animals showed preference for the target zone above chance in a single sample t-test (12.5 %) in the first 30 s of the reversal probe test [t = 2.8, df = 6; $p = 0.03$; Fig.

3.7B], but WT animals did not [t = 0.2, df = 10; $p = 0.9$; Fig. 3.7B]. *Nrg1* mutants also spent more time in the target zone in the test overall [t = 2.7, df = 6; $p = 0.04$] while WT animals did not [t = 1.06, df = 10; $p = 0.3$], suggesting that *Nrg1* animals may have improved memory for reversed spatial challenges.

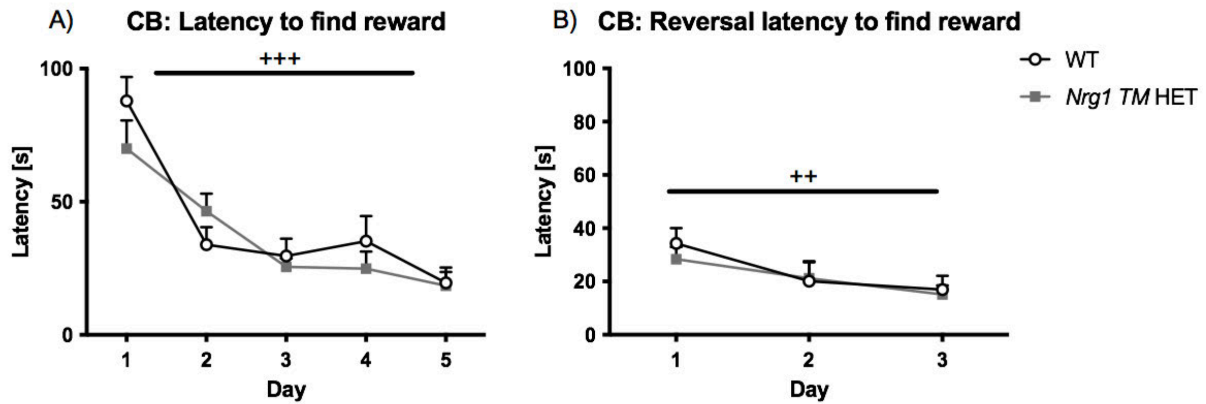

Fig. 3.6A-B: Daily average latency [s] to find the food reward during learning and reversal learning in the cheeseboard (CB) test: A) initial learning, and B) reversal learning the location of the baited food well. Data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. RM ANOVA showed successful learning i.e. a decrease of latency to reach the reward (s) of all animals during the 5 days of training (RM effects of 'day' $^{++}p < 0.01$, $^{+++}p < 0.001$).

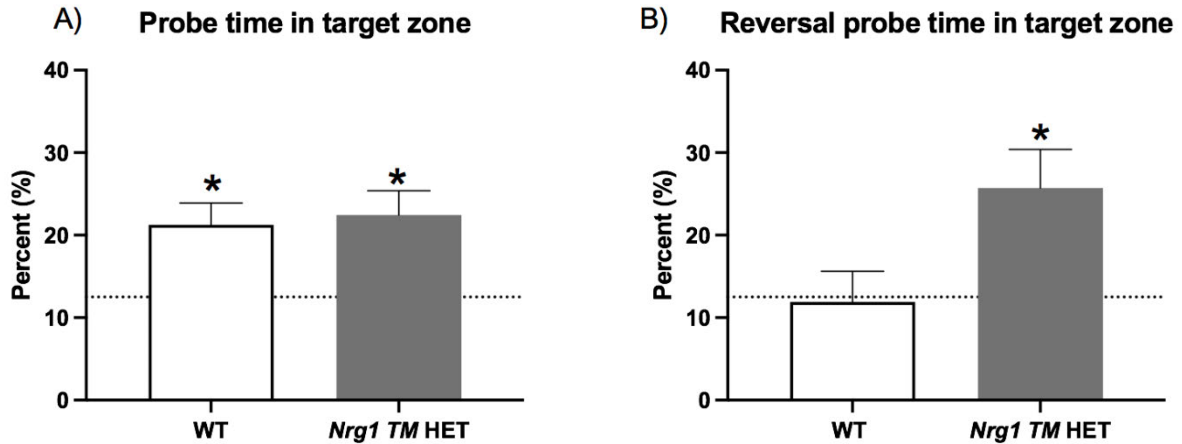

Fig. 3.7A-B: Spatial memory performance (i.e. percentage [%] of time spent) in the target zone during the probe trial and the reversal probe trial in the first 30 seconds of the cheeseboard (CB) probe tests: A) probe test; B) reversal probe test. All data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Time spent in the target zone higher than chance indicated with * $p < 0.05$.

Object recognition memory in the novel object recognition test: This test was carried out in cohorts 1 and 2 using different types of objects for each cohort to clarify if object selection was critical for experimental test outcomes.

For the NORT test for cohort 1, using the giraffe and elephant object combination, a single sample t-test for percentage of *nosing* the novel object against chance levels (50%) showed that neither *Nrg1* mutants [t = 1.04, df = 6; $p = 0.3$] nor WT [t = 0.5, df = 10; $p = 0.6$] animals preferred the novel object over the familiar object. When analysing the percentage of overall exploration (*nosing* + *rearing*) time against chance, both *Nrg1* mutants [t = 0.3, df = 10; $p = 0.7$] and WT animals [t = 1.02, df = 6; $p = 0.3$] did not prefer exploring the novel object above chance (Table 3.4).

Genotype	**WT**		***Nrg1 TM* HET**	
Object	Novel	Familiar	Novel	Familiar
Nosing %	48.7 ± 2.3	51.3 ± 2.3	54.3 ± 4.1	45.7 ± 4.1
Overall exploration %	50.8 ± 2.6	49.1 ± 2.6	53.3 ± 3.3	46.7 ± 3.3

Table 3.4: Total percentage [%] interacting with objects in the first novel object recognition test (NORT): Data expressed as mean ± SEM % for time *nosing* and overall exploration time for the novel and familiar objects for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice.

The second NORT used a spiky plastic ball and a lumpy rubber ball (Kmart, Australia). Analysing *nosing* in WT animals showed they did not prefer *nosing* the novel object above chance [t = 0.15, df = 9; p = 0.9], however *Nrg1* mutants did tend to spend more time *nosing* the novel object than chance levels [t = 2.6, df = 9; p = 0.059]. Overall percentage novel exploration time was also not above chance levels for *Nrg1* mutants [t = 1.6, df = 10; p = 0.14] or WT mice [t = 0.4, df = 8; p = 0.7] (Table 3.5).

Genotype	**WT**		***Nrg1 TM* HET**	
Object	Novel	Familiar	Novel	Familiar
Nosing %	50.7 ± 4.9	49.3 ± 4.9	55.8 ± 2.7	44.2 ± 2.7
Overall exploration %	48.3 ± 4.7	51.7 ± 4.7	55.9 ± 3.7	44.07 ± 3.7

Table 3.5: Total percentage [%] interacting with objects in the second novel object recognition test (NORT): Data expressed as mean ± SEM % for time *nosing*, frequency *rearing on*, and overall exploration time for the novel and familiar objects for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice.

3.3.6 Behavioural sensitivity to acute THC challenge in the open field test

Animals were treated acutely with either vehicle, 3 or 5 mg/kg of THC, to examine dose-dependent sensitivity to THC in *Nrg1 TM* HET animals (similar to (186, 308) but with no washout period). THC decreased both total ambulatory time [main effect of 'THC dose': $F(2,48) = 4.7$; $p = 0.01$] and distance travelled [main effect of 'THC dose': $F(2,48) = 6.4$; $p = 0.003$; Fig. 3.8A]. There were no effects of 'genotype' on total ambulatory time or distance travelled, and there were no interactions between 'genotype' and 'THC dose' (all p's > 0.05). Exploration was also reduced by THC treatment, with *rearing* frequency [main effect of 'THC dose': $F(2,48) = 17.9$; $p < 0.0001$; Fig. 3.8B] and small motor movements frequency ['THC dose' $F(2,48) = 10.1$; $p < 0.0001$; Fig. 3.8C] reduced by THC. No 'genotype' effects were present, and no interactions between 'genotype' and 'THC dose' were present for *rearing* and small motor movements (all p's > 0.05). Distance ratio was decreased by acute THC treatment [$F(2,48) = 3.3$; $p = 0.04$; Fig. 3.8D], suggesting an anxiogenic effect of THC but no effects of 'genotype' or 'THC dose' were found on OF centre time [all p's > 0.05; data not shown]. Finally, there were no interactions between 'THC dose' and 'genotype' for either anxiety parameter (all p's > .05).

To investigate THC dose response effects in each genotype and to assist the determination of which THC dose to be used for the following experiments, exploratory posthoc tests (i.e. Sidak's multiple comparisons) were performed to analyse within-group differences at each dose. Based on this analysis, 3 mg/kg and 5 mg/kg of THC suppressed locomotion distance in *Nrg1 TM* HET mice but the same sedative-like effect in WT mice was only observed after acute treatment with a dose of 5 mg/kg THC. Similarly, *rearing* frequency was decreased at both doses in *Nrg1 TM* HET animals and was decreased in WT animals at 5 mg/kg while only tending to be decreased at 3 mg/kg. Finally, small motor movement frequency was also decreased at both doses in *Nrg1* mutant animals but only at 5 mg/kg in WT animals.

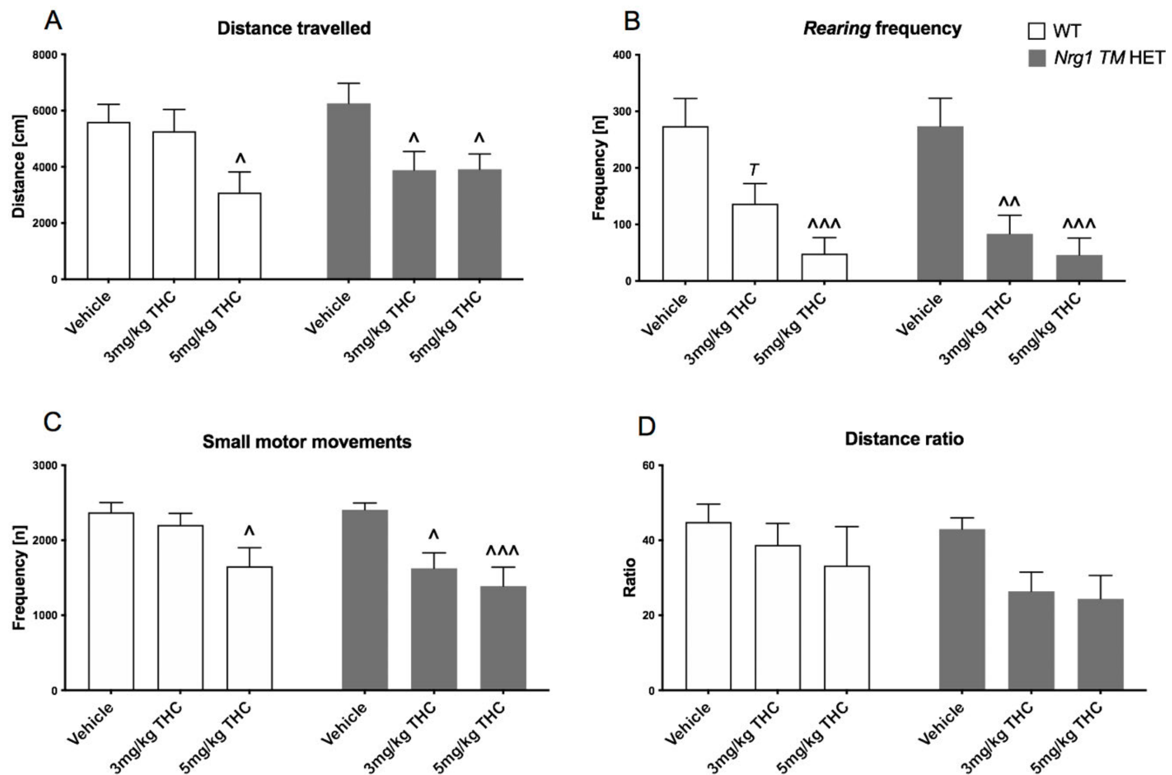

Fig. 3.8A-D: Behavioural measures following vehicle, 3 or 5 mg/kg THC i.p. in the open field (OF): Between wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Data presented as mean ± SEM for A) total distance travelled [cm]; B) total *rearing* frequency [n]; C) total small motor movement frequency [n]; D) distance ratio. Effects of 'THC' against corresponding vehicle group in multiple comparisons are indicated with ^$p < 0.05$, ^^$p < 0.01$, and ^^^$p < 0.001$.

3.3.7 Neuroinflammatory profile

Cytokines IL-10, IL-1β, and TNF-α were analysed in hippocampal and PFC tissue from male WT and *Nrg1 TM* HET animals using cytokine-specific ELISA kits. Levels of IL-10 in the hippocampus ['genotype': $F(1,21) = 1.8$; $p = 0.2$] and PFC ['genotype': $F(1,21) = 0.9$; $p = 0.3$] were unaffected by 'genotype'. This was also the case for levels of IL-1β in the hippocampus ['genotype': $F(1,21) = 1.6$; $p = 0.2$] and PFC ['genotype': $F(1,21) = 0.5$; $p = 0.5$]. Finally, levels of TNF-α in the hippocampus ['genotype': $F(1,21) = 1.2$; $p = 0.3$] and PFC ['genotype': $F(1,21) = 0.9$; $p = 0.3$] did not differ between genotypes at the age 5-7 months (Fig. 3.9).

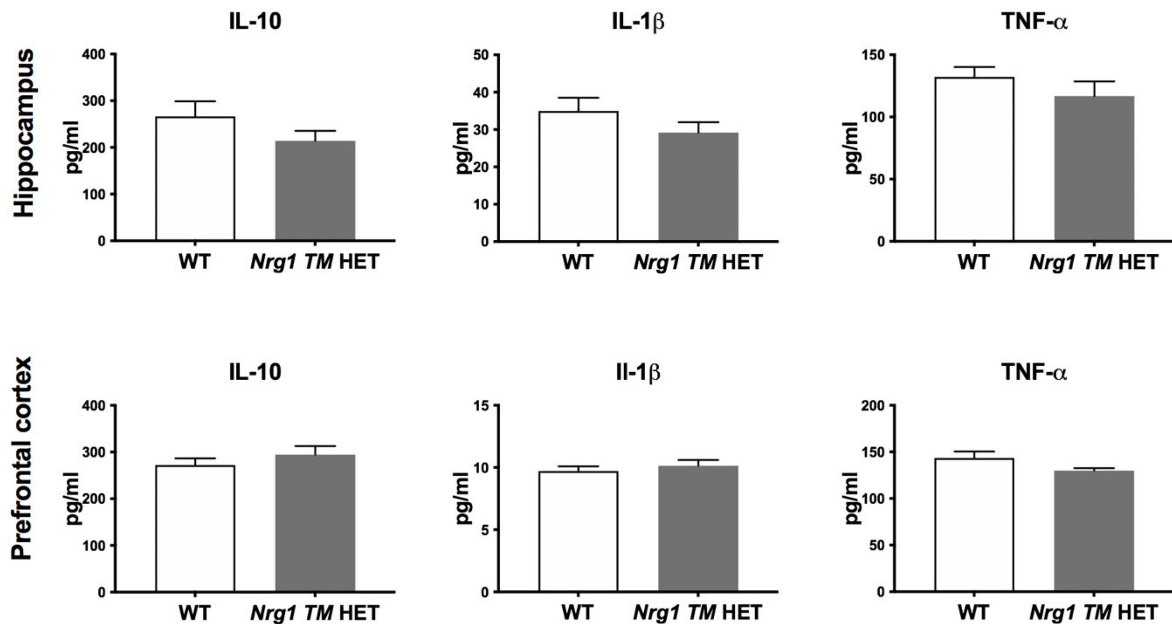

Fig. 3.9: Cytokine profiles in the hippocampus and the prefrontal cortex (PFC): Levels [pg/ml] of cytokines interleukin 10 (IL-10), interleukin 1-beta (IL-1β), and tumor necrosis factor alpha (TNF-α) of wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice. Data presented as mean ± SEM.

There were also no 'genotype' differences in expression levels of Iba1, a marker of activated microglia in brain tissue in one-way ANOVA analysis of the hippocampus ['genotype': $F(1,22) = 0.05$; $p = 0.8$], PFC ['genotype': $F(1,22) = 0.4$; $p = 0.55$], or striatum ['genotype': $F(1,22) = 2.9$; $p = 0.1$] (Fig. 3.10).

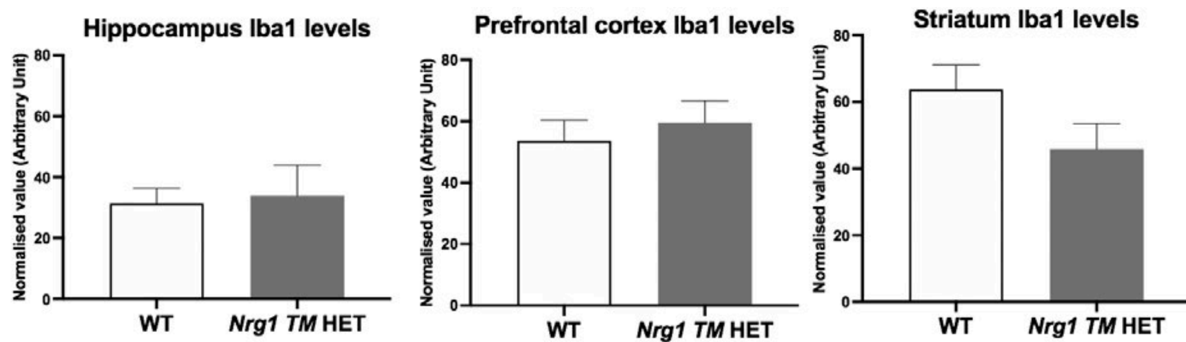

Fig.3.10: Iba1 protein levels in brain tissue: Protein levels [normalised value (arbitrary unit)] in the hippocampus, prefrontal cortex (PFC), and striatum of wild type-like (WT) or *neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice.

3.4 Discussion

This chapter investigated the baseline behavioural and neuroinflammatory phenotype of *Nrg1 TM* HET mice in the behavioural phenotyping facility at Western Sydney University. In this laboratory, male *Nrg1 TM* HET mice at 5-7 months of age displayed hyperlocomotion, decreased social interaction behaviours, decreased acoustic startle response, and a trend for reduced PPI. There were no cognitive deficits found in *Nrg1 TM* HET mice for long and short-term spatial memory, social recognition memory, or fear-associated memory (CB, FC, NORT, SPT). *Nrg1 TM* HET mice were also moderately more sensitive to the locomotor- and exploration-suppressive effects of 3 mg/kg THC. Finally, *Nrg1 TM* HET mice at 5-7 months of age exhibited WT-like levels of cytokines IL-10, IL-1β, and TNF-α in the PFC or hippocampus, and *Nrg1* mutant mice also did not show altered levels of Iba1 in either the PFC, hippocampus, or striatum.

The hyperlocomotive phenotype detected supports previous findings in this mouse model (127, 175, 177, 182). Hyperlocomotion is well documented in these mice and emerges in early-mid adulthood (177). In rodent models, hyperlocomotion induced by psychomimetic drugs is used as an indirect behavioural read-out of dopaminergic tone and ‘psychosis-like’ behaviour in animals (309). In *Nrg1 TM* HET animals, a hyperlocomotive phenotype serves as

a proxy for ‘psychosis-like’ behaviour, and provides a target for improvement in further chapters investigating pharmacological intervention in this model, where loss of this phenotype after adolescent CBD would indicate decreased psychosis-like behaviour. When analysing the antipsychotic-like effects of CBD in further chapters, the OF test will therefore be used to measure effects on reducing or altering OF behaviour, which has relevance to positive symptoms of schizophrenia.

The current study found no changes in anxiety-related behaviours in male *Nrg1 TM* HET animals. Another group has found an anxiolytic-like phenotype present in the LDT and elevated plus maze (186), but not the OF (186), suggesting test type may be relevant to anxiolytic behaviour in this mouse model. Further groups have also found this phenotype present in the LDT (173, 190) but not the OF (190). This phenotype has been reported in the OF previously in some studies (174, 177, 184); however, it has not been consistently found across test paradigms including the LDT and elevated plus maze (177, 184). While an anxiolytic-like phenotype may be present in some test settings and laboratories, it may be sensitive to environmental influences such as the testing facility differences. As the anxiety-related phenotype of this mouse model appears to be inconsistent across studies, I will not use this behavioural read out to test the effectiveness of adolescent CBD therapy on disease-relevant behaviours in the following chapters.

Social behaviour deficits were evident in *Nrg1 TM* HET mice in the SI test. This finding is an important reflection of social deficits (negative symptoms) found in schizophrenia, which have been proposed as novel targets for treatment as levels of social interaction can impact patients’ quality of life and employment (310). In line with this, many models of schizophrenia display deficits in social behaviour and this is an important measure of negative symptoms (290). In the SPT test, *Nrg1* mutant mice did not show impaired sociability or preference for social novelty. This test differs from the SI test substantially in protocol and apparatus,

however, and is not directly comparable. In SI, the mouse has direct contact with the social opponent mouse and can display or engage in a number of behaviours, and the arena is less complex (comprised of one chamber only), while in SPT testing, the social opponent mouse is behind a barrier allowing only non-contact *nosing* and the apparatus is more complex and may encourage more exploration (290). As the SI test detected reduced social behaviour in *Nrg1* mutant mice, subsequent chapters will employ this test to determine if CBD can improve social behaviours. Furthermore, considering other laboratories have reported that *Nrg1 TM* HET males and females to show increased following, and females to have a greater frequency of *climbing on/over* the social control mouse (311), it is prudent to use this test for future experiments, as the SI test can detect an array of nuanced social behaviours more easily than the SPT. While an increase in locomotion may also impact the amount of socialising mice carry out in a paradigm, it would be expected that in both the SPT and the SI test any impact locomotion may have would be consistent. Considering the SPT apparatus may encourage movement and exploration, hyperlocomotion if the cause of social deficits should have caused deficits in mutant mice in this test also. In more organic measures of sociability like in SI, deficits in sociability on their own may be more obvious, regardless of locomotion. Therefore, deficits in social interaction in the SI test likely reflect actual deficits in sociability, and not simply locomotor overactivity.

Previously, deficits in social novelty have been reported in *Nrg1 TM* HET mice (174, 181), implying that *Nrg1* mutants may lack some aspects of social recognition. While no deficits in social novelty were found in the current experiments, it is important to note that WT mice also showed no preference for the novel mouse and the test therefore did not work. This suggests this test cannot reliably demonstrate social novelty preference and will not be used in future chapters. This was surprising as SPT is established in our laboratory, and previous studies using a rodent model of Alzheimer's disease (AD) have shown successful social

novelty recognition in WT animals, at least when male mice had been tested (295, 297, 298). However, WT mice from these studies were not bred on a pure genetic background (AD transgenic mice are on a mixed C57BL/6J x C3H/HeJ background) as in the current study (*Nrg1* mutant mice are on C57BL/6J background), and their behaviour and performance in the SPT may be influenced by genetic background effects – indeed, strain differences in social memory have been found in laboratory animals (in a comparison of substrains of C57BL/6) (312).

While a multitude of PPI protocols exist in the field to test sensorimotor deficits, this protocol is based on a number of previous studies in the current laboratory and was therefore the one chosen for this and any further studies in this work. Average ASR was lower in *Nrg1 TM* HET animals at 100 and 120 dB, replicating previous findings in this mouse model (301). Interestingly, reduced ASR has been linked to anhedonia in other studies as it sometimes used as a measure for reduced reactivity (313). The current study also revealed reduced PPI at 86dB in *Nrg1 TM* HET animals, which drove a trend for overall reduced PPI. While PPI deficits have been reported in this model (127, 190), it is important to note that this depends on PPI test protocol and laboratory environment (301). PPI deficits may also be exaggerated by environmental stressors within this model (174), suggesting PPI is not only an important measure in these mice, but also an important measure by which to assess gene-environment interactions in this model. This is a relevant consideration for future experiments using CBD as a preventative treatment, and thus PPI will be included in later chapters.

Spatial learning in the CB task was intact in *Nrg1 TM* HET mice. Both genotypes showed intact learning across days and memory of the reward location at the beginning of the probe test. These findings reflect previous research showing that learning and memory in the y-maze and radial arm maze are not impaired in male *Nrg1* mutant mice (183), and acquisition and probe performance in the CB were unaffected in female *Nrg1 TM* HET mice as well (188).

Likewise, in male *Nrg1* mutants in the current study, spatial memory in the probe test was no different to WT-like mice. Reversal learning was also evident in both genotypes. Interestingly, deficits in reversal learning were found in this model at 6 months of age when utilising the Morris water maze (MWM) (314), suggesting deficits in spatial reversal learning may be test-specific. It is important to note that mice tested in the MWM experience significant stress when in the water (315), while the CB test relies on food deprivation as the motivational factor, as animals are tested on 'dry land'. This difference in test design is highly relevant as *Nrg1 TM* HET mice show altered responses to acute stress compared to control mice (169), and task acquisition could be impaired in *Nrg1 TM* HET mice when tests involve significant stressors. In the current study, spatial memory recall during the reversal probe trial was present only in *Nrg1 TM* HET animals. This is the first time that the CB has been assessed in male *Nrg1 TM* HET mice, and any differences detected in reversal learning cannot be compared with previous findings in female animals as the previous study did not assess reversal learning (188).

No deficits in short-term object recognition memory in the NORT were present in adult male *Nrg1 TM* HET animals in this facility, as all animals failed to discriminate between familiar and novel objects in both repetitions of the task. This task was not used further in this work.

Similarly, no deficits were found in fear-associated memory in the FC task, suggesting that these types of memory are not affected in male *Nrg1 TM* HET animals in adulthood. Female mice of this genotype show reduced fear-associated memory for a context associated with shock, as well as a tendency for impaired memory for a shock-associated discrete cue (188). Our laboratory has previously detected impaired recognition of a fear-associated context, as well as novel object recognition impairment in male *Nrg1* mutant mice (183); however, these results are from a different facility using protocols that varied from the current laboratory (i.e. scent cue used, apparatus used, object types), factors known to affect some

aspects of the *Nrg1 TM* HET phenotype (301). Considering the present experiments did not detect clear novel object preference, this test was not used in subsequent chapters. However, fear-associated memory to a context has been shown to be affected by CBD administration (316), and may therefore be a valuable test to measure associative memory after chronic CBD. This test was retained in further studies in order to analyse any changes to cognition caused by chronic CBD during adolescence.

Acute THC in rodents has been shown to reduce locomotion and increase anxiety behaviours (317, 318), and *Nrg1 TM* HET mice are more sensitive to the locomotor suppressant and anxiogenic effects (186, 319). In the current study, THC administration induced reduced locomotor and exploratory behaviours in both genotypes in the OF and this appeared to be more evident across THC doses in *Nrg1* mutant mice. Exploratory posthoc tests demonstrated that *Nrg1* mutant mice were susceptible to the locomotor suppressant effects of both THC doses (3 and 5 mg/kg), while WT animals were more affected by the higher 5 mg/kg dose. Similar effects of THC on *Nrg1* mutant mice were present for *rearing* and small motor movements. This confirms the behavioural susceptibility of male *Nrg1 TM* HET mice to lower dose THC. Thus, in Chapter 4 and 5, the 3 mg/kg dose was used to examine THC sensitivity in *Nrg1 TM* HET mice. Interestingly, no hyperlocomotive phenotype was seen in *Nrg1* mutants treated with vehicle, compared to WT controls of the same treatment group. It is possible that this is due to the stress of injection, as testing was carried out 30 min post-injection. Previous studies have found that chronic injection stress can mask aspects of this mouse's phenotype (such as social behaviours), and the model is more sensitive to some stressors than WT animals (169, 185). This should be considered in further chapters, as chronic injections regardless of treatment may dampen some effects due to potential stress.

Neuroinflammation in *Nrg1 TM* HET mice at 5-7 months of age was no different to WT controls, evidenced by brain levels of Iba1 and three cytokines, IL-10, IL-1β, and TNF-α.

Iba1 has not been previously analysed in this model and is an effective marker for analysing levels of microglia in the brain (320). This study was the first to analyse levels of Iba1 and therefore microglial activation in this mouse model. This was important, as while brain tissue levels of cytokines were unchanged, any alterations in microglial activation could indicate a chronic state of neuroinflammation in these animals. The three cytokines analysed in the current study have been shown to be altered in the serum of *Nrg1 TM* HET mice: IL-1β, IL-10, and TNF-α are reduced in basal serum of male *Nrg1* TM HET mice in late adolescence/early adulthood (PND85), and TNF-α tends to be higher in hippocampal tissue (173, 174). It should be noted that the levels of cytokines circulating in serum after an immune challenge compared to those found in brain tissue are very high, meaning differences in these levels may be more detectable in serum than brain tissue (321). Brain was analysed in this cohort however as it may be a more accurate representation of inflammation in each schizophrenia-relevant region individually, where serum levels only indicate systemic inflammatory response and not neuroinflammation as such. It is also possible that inflammatory markers may be elevated in *Nrg1* mutant males during early life only and that in adulthood these cytokines stabilise to WT levels. Indeed, this has been found in a Poly I:C mouse model where many cytokines including those listed above fluctuated during development before stabilising in adulthood (322). Changes across development have not been investigated in patients, and have only been analysed after some psychotic episode or risk for psychosis has been identified in patients. Interestingly, schizophrenia-relevant behavioural changes in Poly I:C mice (e.g. impaired latent inhibition and decreased sensorimotor gating) were most evident in adulthood (322) – at a time when cytokine levels of these mice were similar to control levels. Any behavioural changes are therefore more likely to be downstream of earlier cytokine elevation, and not due to concurrently altered cytokine levels. It is significant that neuroinflammatory changes could occur during late adolescence in *Nrg1 HET*

TM mice, as adolescence is a critical period for neurodevelopment, and corresponds with the prodromal period of schizophrenia in some individuals (40, 323, 324). Cytokine release is stimulated by activated microglia (reviews: (58, 62, 63)), and it is possible that behavioural changes later in life are caused by early changes in microglial activation and circulating cytokine levels that in turn cause damage to functional neurons (152, 325, 326). Furthermore, extended cytokine elevation in brain tissue leads to an increase in degenerated neurons and decreased neurogenesis, which may underlie some schizophrenia-like abnormalities especially in white matter tracts (62, 327). These effects of increased cytokines have not however been investigated in *Nrg1* mutants. Thus, it is possible that the schizophrenia-like behavioural deficits evident in adulthood are related to long-lasting effects of elevated cytokine levels during earlier developmental stages, including during synaptic development. This highlights the importance using therapeutic agents during this window, as it could reduce levels of neuroinflammation and therefore decrease schizophrenia-relevant behaviour and brain dysfunction caused by neuroinflammatory damage.

Overall, male adult *Nrg1 TM* HET animals in this laboratory displayed locomotor hyperactivity, relevant to positive symptoms of schizophrenia, decreased social interaction, relevant to negative symptoms of schizophrenia, and sensorimotor gating deficits. No deficits in cognitive domains were found. These results suggest that these mice have a schizophrenia-relevant behavioural phenotype (328) and that new treatment options can be evaluated using this mouse model. Thus, hyperlocomotion in the OF, SI deficits, and PPI deficits were therefore identified as important domains for testing the therapeutic-like effects of CBD in *Nrg1 TM* HET mice. Due to the sensitivity of contextual fear response being reduced in FC by CBD in other studies (316), this test was also retained for future batteries to investigate the effects of CBD on FC, as other cannabinoids administered during adolescence have been shown to alter fear-associated learning (329). As there was no increase in microglial activation or

neuroinflammation in adult *Nrg1 TM* HET mice, further chapters focused on the role of the endocannabinoid system and the GABA/glutamate system in the behavioural effects observed in CBD and/or THC treated *Nrg1* mutant mice, as these receptor systems are critically involved in schizophrenia pathophysiology and CBD mechanisms (41, 219, 330).

Chapter 4: Adolescent chronic cannabidiol (CBD) and acute Δ⁹-tetrahydrocannabinol (THC) in male *Neuregulin 1* mutant mice.

4.1 Introduction

Adolescence is an important period of neurodevelopment, where sensitivity to environmental factors is increased, for example, neural plasticity is vulnerable to factors such as drug abuse and stress (331, 332). This period is also a potential window for pharmacological intervention to prevent the development of schizophrenia, as either prior or concurrent treatment with neuroprotective drugs has the potential to modify the development of schizophrenia symptoms in adulthood (333). In both preclinical and clinical studies, early life interventions with environmental enrichment (such as altered housing conditions) or neuroprotective pharmacological agents can improve symptoms in schizophrenia patients and relevant behavioural domains in animal models of the disorder (153, 155, 277, 324). Current treatments for schizophrenia do not have a high adherence rate (20, 334) and can lead to several health conditions (e.g. heart disease and metabolic disease) (21, 204), demonstrating a need for novel treatments or treatment approaches.

Preventative treatment approaches are highly novel in schizophrenia research (333), and it is possible that CBD may be used to prevent the development of schizophrenia. CBD's potential as a preventative therapeutic treatment has been demonstrated *proof of principle* in other neurological models. For example, chronic CBD prevents impairment of social recognition memory in a mouse model of Alzheimer's disease, and also prevents ischemic injury in a rodent model of stroke (335-337). Adolescence may be an appropriate period for preventative CBD treatment, as some preclinical models have suggested that intervention during adolescence with CBD can mediate later schizophrenia-relevant behaviours, when

animals are tested immediately after CBD treatment (276, 278). Furthermore, clinical research has shown that short-term CBD treatment in those at risk of developing psychosis can attenuate some undesirable symptoms such as social stress (285, 286), suggesting CBD may have the ability to alter the trajectory of psychosis development. However, no studies have investigated long-term CBD in at-risk populations, and preclinical studies investigating CBD have not looked at its long-term ability to protect against schizophrenia development. Furthermore, no studies have investigated the preventative ability of CBD in a genetically relevant schizophrenia model.

Some molecular and behavioural properties of CBD also implicate its use as a preventative treatment for schizophrenia. CBD is a negative allosteric modulator of CB_1 receptors (338) and has the potential to lower the affinity and/or efficacy of these receptors in the developing brain. Importantly, CBD can decrease levels of FAAH, the enzyme responsible for anandamide degradation, therefore increasing levels of circulating anandamide (228). This is relevant to schizophrenia, as higher anandamide levels are correlated with reduced psychotic symptoms (228, 339). CBD also has anti-inflammatory properties, and can protect against damage to neurons caused by increased inflammation or oxidative stress (283, 284, 340, 341). CBD could ameliorate or prevent the development schizophrenia-relevant behaviour and brain function via its anti-inflammatory properties (283, 284), as inflammation has been reported in some schizophrenia patients (100, 342).

Preventative CBD treatment could also prevent against susceptibility to THC. Cannabis abuse during adolescence is a component risk factor for schizophrenia development (155, 158, 160). The development of first-episode psychosis is linked with cannabis of a higher potency (i.e. greater THC content), suggesting that dopaminergic changes induced by THC can alter psychosis-related pathways in the adolescent developing brain (343). Studies of cannabis composition also show that cannabis with higher levels of CBD than THC induces fewer

psychosis-related experiences than cannabis with higher levels of THC than CBD (344). The impact of cannabis consumption on schizophrenia development is hypothesised to be related to THC's short-term dopaminergic stimulation and long-term dopaminergic system blunting, where THC acts on CB_1 receptors on dopaminergic neurons (particularly in the striatum, PFC, and nucleus accumbens) and causes long-term imbalances in inhibitory and excitatory transmission (345, 346). THC also modulates the endocannabinoid system (235), which is a key regulator in brain development during adolescence (347) and a dysfunctional endocannabinoid system is linked with schizophrenia symptom severity (339). Specifically, dysregulation in CB_1 receptor levels and bindings are found in patients in brain regions that are implicated in schizophrenia symptoms than controls, e.g. striatum, hippocampus, and PFC (348-351). Chronic CBD treatment could limit sensitivity to THC, as acute CBD can reduce social withdrawal and cognitive impairment induced by THC in rats (262), and as a negative allosteric modulator of CB_1 could decrease THC's potency by reducing the ability of THC to bind to these receptors. As CBD can act to modulate CB_1 receptor binding, this is a possible mechanism by which CBD could blunt the effects of THC.

The *Nrg1 TM* HET mouse is an excellent model to investigate if CBD can prevent or limit schizophrenia-relevant behavioural and brain dysfunction, as well as susceptibility to THC. *NRG1* is a well-established genetic risk factor for schizophrenia (352), and a mutation in the transmembrane domain region of *NRG1* is found in patients with schizophrenia (167). *Nrg1 TM* HET mice show high face, construct, and predictive validity for the disease (175) and exhibit schizophrenia-relevant behavioural deficits (e.g. hyperlocomotion, impaired sensorimotor gating, reduced social behaviour) (173, 177, 182) as shown in Chapter 3. Male *Nrg1 TM* HET mice show a stronger disease-relevant phenotype than females (173, 175), reflecting findings in clinical cohorts (189). *Nrg1* mutant mice also show brain changes relevant to schizophrenia (i.e. altered glutamatergic/GABAergic system and inflammatory

markers) that are relevant to CBD's mechanisms of action (171, 174, 179, 190). Furthermore, as outlined in Chapter 3, sensitivity to THC in *Nrg1 TM* HET mice may reflect the association between mutant *NRG1* and increased risk for cannabis abuse in humans (353). Chronic vehicle injections can also affect the phenotype of these mice in adolescence, suggesting this is an important period for investigating pharmacological intervention on the behavioural phenotype (185).

Here, I investigated the effects of adolescent CBD treatment on neuro-behavioural deficits of *Nrg1 TM* HET mice. I also determined the potential of adolescent CBD to reduce the behavioural effects of an acute THC challenge in early adulthood. In addition, I also examined how CBD affected endocannabinoid and glutamatergic protein levels as well as the inflammatory marker Iba1 in the hippocampus.

Aim Two:

CBD has the potential to protect against neural changes to the *Nrg1 TM* HET mouse during adolescent development that cause later behavioural deficits, possibly by anti-inflammatory or endocannabinoid modulatory effects. We therefore aim to investigate the neuro-behavioural effects of chronic adolescent CBD in schizophrenia-relevant domains immediately post-treatment period at baseline and after acute THC challenge in *Nrg1 TM* HET mutant mice.

4.2 Materials and methods

4.2.1 Animals

Male *Nrg1 TM* HET and wild type-like littermates were bred and group housed in individually ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at Animal BioResources (Moss Vale, Australia). At approximately 21-30 days old mice were transported to the mouse holding and test facilities at the School of Medicine, Western Sydney University (WSU), and were transferred to group-housing in filter top cages (1144B: Techniplast, Rydalmere Australia) with corn cob bedding (Tecniplast Australia, Rydalmere, Australia) and tissues for nesting. Mice were kept in a 12:12 h light:dark schedule [light phase: white light (illumination: 124 lx), dark phase: red light (illumination: < 2 lx; light phase from 0900-2100]. Mice were fed *ad libitum* with mouse feed pellets (Gordon's Specialty Stockfeeds Pty Ltd., Yanderra, Australia) and water. Bodyweight was monitored every 48 h and significant changes across time and between condition were not detected throughout the experiment (data not shown). Age-matched adolescent male A/J mice from Animal Resources Centre (Canning Vale, Australia) were used in the social-based tests. All research projects were approved by the WSU Animal Care and Ethics Committee (#A13298) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.2.2 Drug preparation and administration

Powdered cannabidiol (CBD: THC Pharm GmbH, Frankfurt/Main, Germany) was dissolved in equal parts of Tween 80 (Sigma-Aldrich Co, St Louis, USA) and 100% ethanol. It was then diluted with 0.9% sodium chloride to the final concentration (5% ethanol, 5% Tween 80, 90% saline). A vehicle control (VEH) was prepared by mixing all components minus CBD. VEH and CBD 30 mg/kg were injected daily intraperitoneally (i.p.); the injection volume was 10

ml/kg (190). Treatment started on PND35 (±5 days), where mice were injected once daily in the afternoon (1200-1500) for three weeks. Injections continued for another three weeks while behavioural testing was performed. During the behavioural testing period, animals were still injected after the relevant tests concluded for the day. CBD or VEH treatment ended approximately 24 hr before animals were euthanized. Mice were weighed every four days and dosage was adjusted accordingly.

For the THC challenge, THC and VEH (THC Pharm GmbH, Frankfurt/Main, Germany) were prepared similarly to CBD preparation. An acute dose of 3 mg/kg was injected intraperitoneally 30 minutes before behavioural testing commenced, at approximately 77 days (±5 days) of age. The THC dose was based on previous research in our laboratory (186) and the results from Chapter 3.

4.2.3 Behavioural testing

Behavioural tests were conducted in the first half of the light phase between 0930 and 1400 in the Behavioural Neuroscience Facility at WSU. All tests were separated by an inter-test interval of at least 48 h and equipment and apparatus were cleaned with 80% ethanol between test animals unless specified otherwise. Behavioural testing order and duration are outlined below in Table 4.1. Tests used are outlined in the General methods section 2.3 Statistics were performed as per the General Methods section 2.7.

<table>
<tr><th colspan="4">Test Order</th><th colspan="4">Postnatal age (days)</th></tr>
<tr><td colspan="4">Adolescent CBD Treatment</td><td colspan="4">35-84 (±5 days)</td></tr>
<tr><td colspan="4">Open Field (OF)</td><td colspan="4">56 (±5 days)</td></tr>
<tr><td colspan="4">Social Interaction (SI)</td><td colspan="4">58-59 (±5 days)</td></tr>
<tr><td colspan="4">Prepulse Inhibition (PPI)</td><td colspan="4">60-64 (±5 days)</td></tr>
<tr><td colspan="4">Fear Conditioning (FC)</td><td colspan="4">65-68 (±5 days)</td></tr>
<tr><td colspan="4">THC Challenge (OF, SI, PPI)</td><td colspan="4">77 (±5 days)</td></tr>
<tr><td colspan="4">Tissue collection</td><td colspan="4">85 (±5 days)</td></tr>
<tr><th colspan="8">Animal Numbers</th></tr>
<tr><td colspan="4">WT</td><td colspan="4">Nrg1 TM HET</td></tr>
<tr><td colspan="2">VEH</td><td colspan="2">CBD</td><td colspan="2">VEH</td><td colspan="2">CBD</td></tr>
<tr><td>VEH</td><td>THC</td><td>VEH</td><td>THC</td><td>VEH</td><td>THC</td><td>VEH</td><td>THC</td></tr>
<tr><td>7</td><td>7</td><td>7</td><td>8</td><td>11</td><td>12</td><td>9</td><td>10</td></tr>
</table>

Table 4.1: Testing timeline and animal numbers: Test order, age (days), and animal numbers per group (*n*). Treatments included 30 mg/kg cannabidiol (CBD), 3 mg/kg Δ^9-tetrahydrocannabinol (THC), or vehicle controls (VEH).

4.2.4 Acute THC challenge

Seven days after the initial behavioural testing ceased, mice were injected with either 3 mg/kg of THC or vehicle control, and 30 min later, each mouse was placed into the OF apparatus for 10 min. Following OF testing, mice were then placed into the SI arena with an opponent mouse as described in the general methods (185). After completion of the SI test, mice were placed into the startle chamber apparatus to test them for sensorimotor gating (Fig. 4.1). This left a ~1

min intertest interval between behavioural tests. These experimental methods are in line with previous studies on acute THC effects in this mouse model (184, 186).

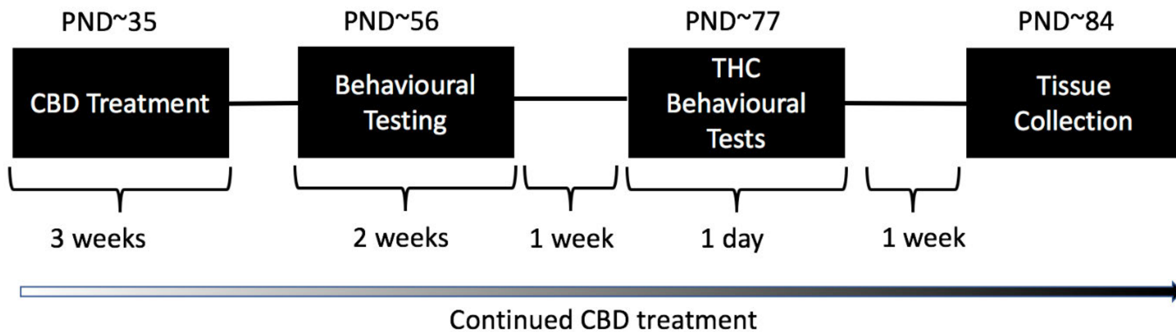

Fig. 4.1: Treatment and testing schedule for test animals: Wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice were treated chronically with either vehicle (VEH) or 30 mg/kg cannabidiol (CBD). Daily treatment commenced on post-natal day (PND) 35 ± 5. Mice commenced behavioural evaluation (starting PND 56 ± 5 days) while CBD treatment continued. Effects of chronic adolescent CBD treatment on behaviour were evaluated between PND56-70 ± 5. An acute THC challenge (3 mg/kg or VEH) was administered on PND77 ± 5 followed by behavioural testing 30 min later. Mice were euthanized one week later, on approximately PND84.

4.2.5 Western blot

One-week post-behavioural testing mice were humanely euthanised and the right hippocampus was collected and stored as per the General methods section 2.4 for western blotting outlined in section 2.5. Targets analysed were levels of CB_1, GAD_{67}, and Iba1 in the hippocampus.

4.2.6 Statistical analysis

Data for behavioural and molecular analyses were analysed using two-way or three-way analysis of variance (ANOVA) to investigate main effects and interactions between experimental factors ‘genotype’, ‘CBD’, and ‘THC’. Four-way repeated measures (RM) ANOVAs were used where the within factor was ‘time’, ‘cue’, prepulse’, ‘1-min block’, or ‘block’, and the between factors ‘genotype’, ‘THC’, and ‘CBD’. Where interactions were detected, ANOVAs were split by corresponding factor and further ANOVA conducted. Group differences were regarded as significant if $p < 0.05$. F-values and degrees of freedom are presented for all ANOVAs and data are shown as means ± standard error of means (SEM).

In figures and tables, significant ‘genotype’ effects are shown by ‘*’ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Significant ‘CBD’ effects are reported by ‘#’ (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). Significant ‘THC’ effects are shown by ‘^’ (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$). RM effects are shown by ‘+’ (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$), and any trend effects are reported with the corresponding p value. Statistical analyses were conducted using SPSS 27 for Mac and GraphPad Prism 8 for Mac.

4.3 Results

4.3.1 Chronic adolescent CBD and locomotion, exploration and anxiety

Total distance in the OF was increased by 'genotype' [two-way ANOVA: 'genotype': $F(1,68) = 6.7$; $p = 0.01$; Fig. 4.2A], whereby the *Nrg1 TM* HET genotype increased locomotion. Locomotor habituation was not different between groups and locomotor activity was not elevated in *Nrg1* mutants at any point in the test (data not shown). Centre entries were higher in *Nrg1* mutant mice [$F(1,68) = 8.0$; $p = 0.006$; Fig. 4.2B]. There was no effect of 'genotype' on distance ratio ($F(1,68) = 2.6$; $p = 0.1$; Fig. 4.2C), and centre time was unchanged in *Nrg1 TM* HET animals as well [$F(1,68) = 3.3$; $p = 0.07$; Fig. 4.2D]. There was no effect of 'genotype' for small motor movements ['genotype' $F(1,68) = 3.6$; $p = 0.063$; Fig. 4.2E], and *rearing* was unchanged by 'genotype' [$F(1,68) = 0.1$; $p = 0.3$; Fig. 4.2F]. There were no effects of 'CBD' on any of these measures except for a CBD-induced increase in total distance travelled across genotypes [$F(1,68) = 5.07$; $p = 0.03$; Fig. 4.2A], and no interactions between 'CBD' and genotype were identified (all *p's* > 0.05).

4.3.2 Chronic adolescent CBD and social behaviours

No 'genotype' effects were detected for total SI time or frequency, or the duration or frequency of individual socio-positive behaviours (Table 4.2, all *p's* > 0.05). There was no effect of 'CBD' on total SI time [$F(1,68) = 3.6$; $p = 0.067$; Fig. 4.3A] or total SI frequency [$F(1,68) = 1.2$; $p = 0.3$; Fig. 4.3B]. *Nosing* time [$F(1,68) = 3.4$; $p = 0.07$; Table 4.2], *climbing on/over* time [$F(1,68) = 3.2$; $p = 0.076$; Table 4.2], and *climbing on/over* frequency [$F(1,68) = 3.5$; $p = 0.07$; Table 4.2] were also unaffected by chronic CBD (main effects of 'CBD', two-way ANOVA). There were no interactions between 'genotype' and 'CBD' for socio-positive behaviours (all *p's* > 0.05).

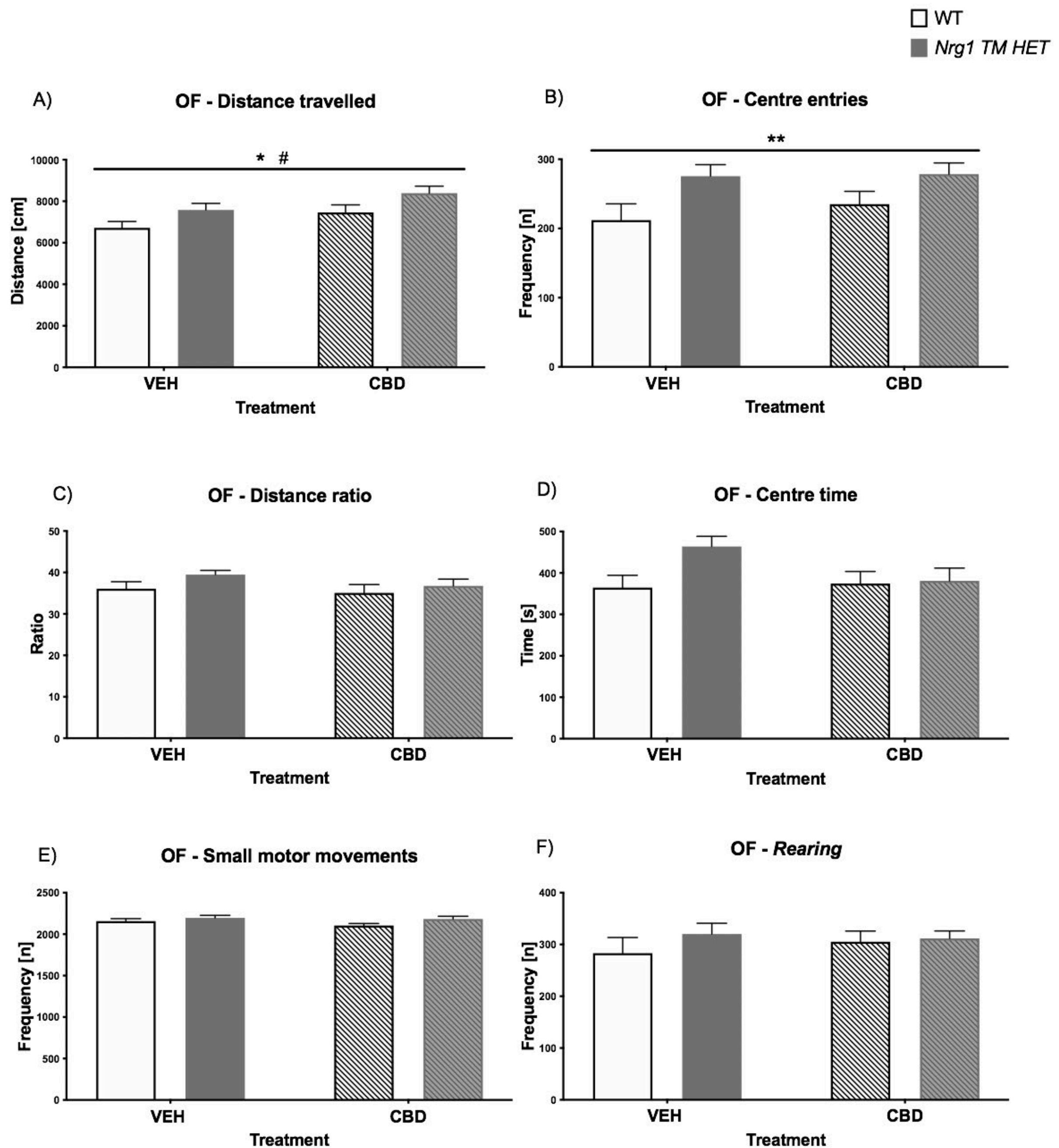

Fig. 4.2A-F: Open field (OF) - locomotion, exploration, and small motor movements after adolescent CBD treatment: Data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either vehicle (VEH) or cannabidiol (CBD). A) distance travelled [cm]; B) centre entries frequency [n]; C) distance ratio [%]; D) centre time [s]; E) small motor movement frequency [n]; and F) *rearing* frequency [n]. Significant two-way ANOVA main effects of CBD are indicated by $^{\#}p < 0.05$, and main effects of genotype are shown as $^{*}p < 0.05$.

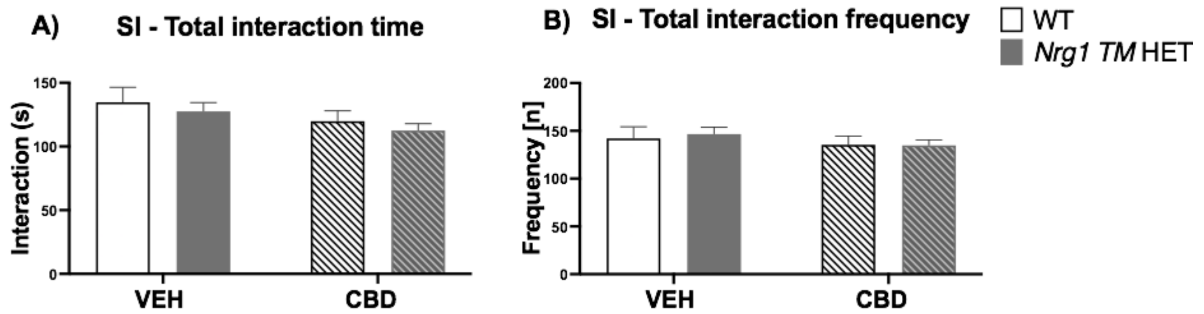

Fig. 4.3A-B: Social interaction (SI) after adolescent CBD treatment: Total interaction time [s] and frequency [n] expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either vehicle (VEH) or cannabidiol (CBD); A) total interaction time [s] after CBD; B) total interaction frequency [n] after CBD.

Genotype	**WT**		***Nrg1 TM* HET**	
Chronic Treatment	VEH	CBD	VEH	CBD
Nosing [s]	84.7 ± 5.6	77.9 ± 4.7	79.6 ± 3.8	71.2 ± 2.7
Anogenital Sniffing [s]	30.2 ± 4	27.2 ± 2.8	30.3 ± 2.6	26.8 ± 2
Climbing On/Over [s]	15.1 ± 3	12 ± 2.1	13.3 ± 1.7	9.3 ± 0.9
Following [s]	4.7 ± 1.3	3.3 ± 0.9	4.3 ± 0.9	5.2 ± 0.9
Nosing [n]	87.6 ± 5.8	86.7 ± 5.3	91.3 ± 4	85.1 ± 3.2
Anogenital Sniffing [n]	29.8 ± 3.1	29 ± 2.3	32 ± 2.1	30.8 ± 1.6
Climbing On/Over [n]	17.1 ± 3	14.5 ± 2.3	16.2 ± 1.6	11.6 ± 1
Following [n]	7.6 ± 2.2	5.2 ± 1.3	6.9 ± 1.2	7.2 ± 1

Table 4.2: Socio-positive behaviours after adolescent CBD treatment: Duration [s] and frequency [n] of *nosing*, *anogenital sniffing*, *climbing on/over*, and *following* the A/J mouse in the social interaction (SI) test. Data expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either vehicle (VEH) or cannabidiol (CBD).

4.3.3 Chronic adolescent CBD and prepulse inhibition

'Genotype' did not affect startle intensity regardless of treatment condition [three-way RM ANOVA 'genotype' main effect: $F(3,68) = 1.9$; $p = 0.4$; Fig. 4.4A], and adolescent CBD also had no overall effect on average startle across startle intensities [$F(1,68) = 3.6$; $p = 0.063$; Fig. 4.4A]. No interactions between 'genotype', 'CBD', or 'startle pulse intensity' were present (all *p's* > 0.05). As expected, higher startle pulse intensities elicited greater startle across experimental test conditions [three-way RM ANOVA for 'startle pulse intensity': $F(2,136) = 496.9$; $p < 0.0001$; Fig. 4.4A].

At 100 dB *Nrg1 TM* HET animals startled less than WT controls regardless of treatment [two-way ANOVA 'genotype' main effect: $F(1,68) = 14.3$; $p = 0.0003$; no 'genotype' by 'CBD' interaction, $p > 0.05$; Fig. 4.4A]. At 70 dB and 120 dB, no significant main effects or interactions between factors were present (all *p's* > 0.05).

Startle habituation was present in all animals regardless of 'genotype' or 'CBD' [three-way RM ANOVA for 'block': $F(2,136) = 25.2$; $p < 0.0001$; Fig. 4.4B] (no interactions of 'genotype' and/or 'CBD' with 'block': all *p's* > 0.05).

Nrg1 TM HET animals had significantly higher %PPI at all prepulse intensities compared to WT controls [$F(1,68) = 8.5$; $p = 0.005$; Fig. 4.4C]. At individual prepulses, *Nrg1* mutants showed increased %PPI at all prepulse intensities compared to WT mice [74 dB: $F(1,68) = 7.3$; $p = 0.009$; 82 dB: $F(1,68) = 7.5$; $p = 0.008$; 86 dB: $F(1,68) = 7.1$; $p = 0.01$]. There was no effect of 'CBD' on %PPI, and no interactions between prepulse intensity and either 'CBD' or 'genotype' (all *p's* > 0.05). As expected, %PPI increased with increasing prepulse intensities [three-way RM ANOVA for 'prepulse': $F(2,136) = 501.7$; $p < 0.0001$; Fig. 4.4C].

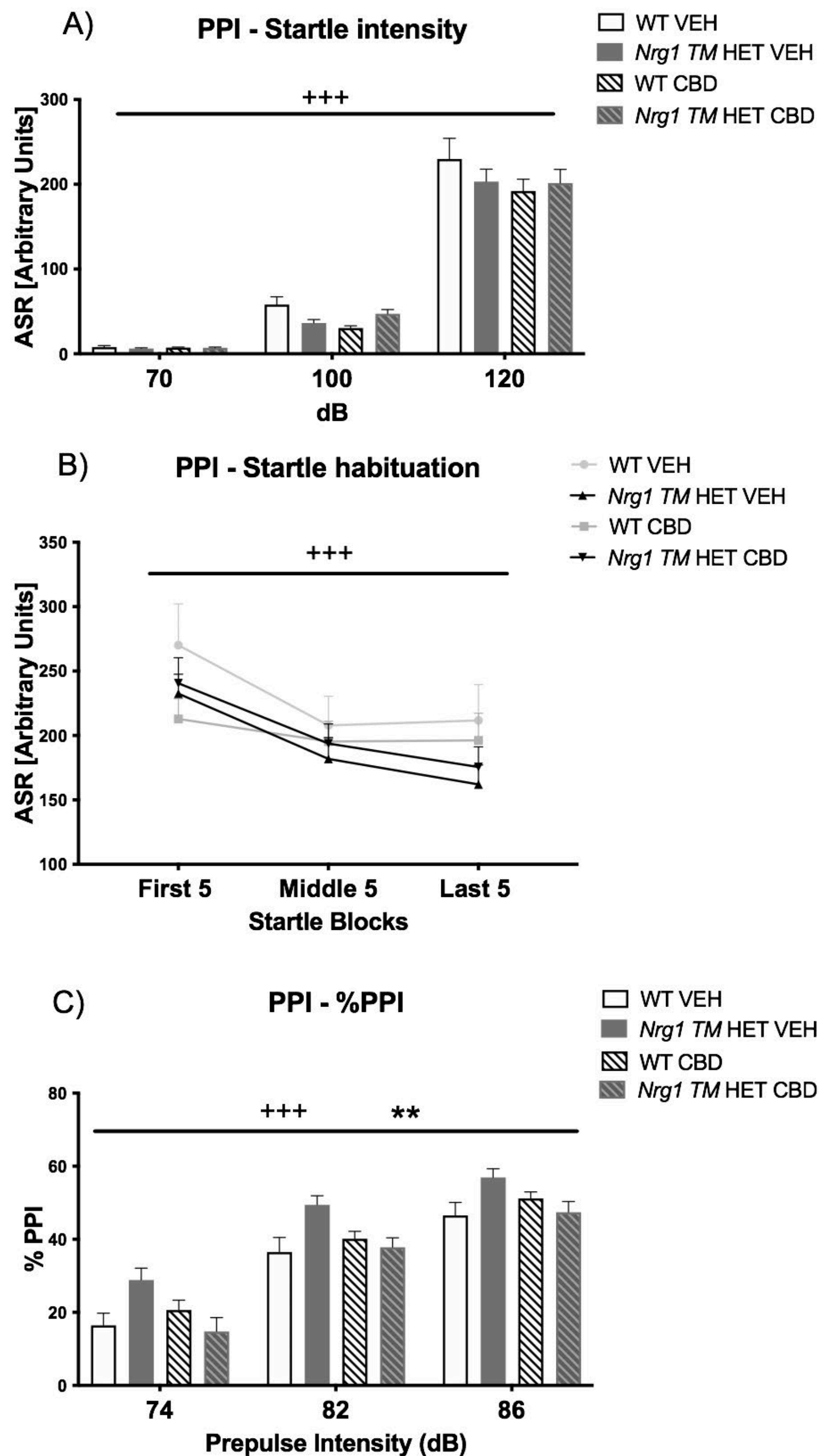

Fig. 4.4A-C: Acoustic startle response and prepulse inhibition (PPI) after adolescent CBD treatment: A) average startle [ASR], B) average first, middle, and last 5 startle responses (startle habituation), and C) percentage PPI (% PPI) across a 74dB, 82dB, and 86dB prepulse intensities, expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with vehicle (VEH) or cannabidiol (CBD). Main effects of 'genotype' indicated by ** $p < 0.01$, RM effects indicated with $^{+++}p < 0.001$.

4.3.4 Chronic adolescent CBD and fear-associated memory

Conditioning: *Freezing* at baseline in the first two minutes of conditioning was unaffected by 'genotype' [$F(1,68) = 0.6$; $p = 0.4$] or 'CBD' treatment [$F(1,68) = 0.9$; $p = 0.3$]. *Freezing* during conditioning increased across the test [three-way RM ANOVA for 'time': $F(6,408) = 71.9$; $p < 0.0001$] and *Nrg1 TM* HET mice *froze* less than WT mice ['genotype' $F(1,68) = 4.13$; $p = 0.05$; Fig. 4.5A]; this was unaffected by CBD treatment (no 'CBD' main effect or interactions, *p*'s > .05). A 'time' x 'genotype' interaction [RM three-way ANOVA $F(6,408) = 4.18$; $p = 0.0004$] when split by 'genotype' showed both *Nrg1 TM* HET animals [$F(6,246) = 33.9$; $p < 0.0001$] and WT animals [$F(6,162) = 34.1$; $p < 0.0001$] altered *freezing* across the test.

To check that all mice formed an association with the conditioned stimuli, *freezing* in the first two minutes was compared across all three experimental conditions: *freezing* in the first two minutes of the context and cue tests was significantly higher than during the first two minutes of conditioning [three-way RM ANOVA for 'days': $F(2,136) = 30.3$; $p < 0.0001$; Fig. 4.5E]. This indicates all mice remembered the tone-context-shock association; and no 'genotype' or 'CBD' effects or interactions were present (all *p's* > 0.05).

Context Test: Total *freezing* in the context test was reduced in *Nrg1 TM* HET animals compared to WTs [$F(1,68) = 9.8$; $p = 0.003$; Fig. 4.5B]. No interactions were present between 'time' and 'genotype' or 'CBD' (all *p's* > 0.05), suggesting that how the *freezing* response adjusted across time was similar between genotype and treatment groups.

Cue Test: Across the entire test, all mice increased their *freezing* behaviour across 1-min blocks [$F(8,536) = 66.2$; $p < 0.0001$] but *Nrg1 TM* HET animals *froze less* overall than WT counterparts ['genotype' $F(1,67) = 7.9$; $p = 0.006$; Fig. 4.5C]. There were no interactions between 'time' and 'CBD' or 'genotype', and no main effects of 'CBD' (all *p's* > 0.05). *Freezing* response analysed during cue presentation only was reduced in mutant animals as

well [two-way ANOVA main effect of 'genotype': $F(1,67) = 7$; $p = 0.01$]. Comparing average *freezing* before cue presentation with during cue presentation, all mice displayed increased *freezing* to the cue [three-way RM ANOVA for 'cue': $F(1,67) = 263.1$; $p < 0.0001$; Fig. 4.5D]. No other main effects or interactions were found (all *p's* > 0.05).

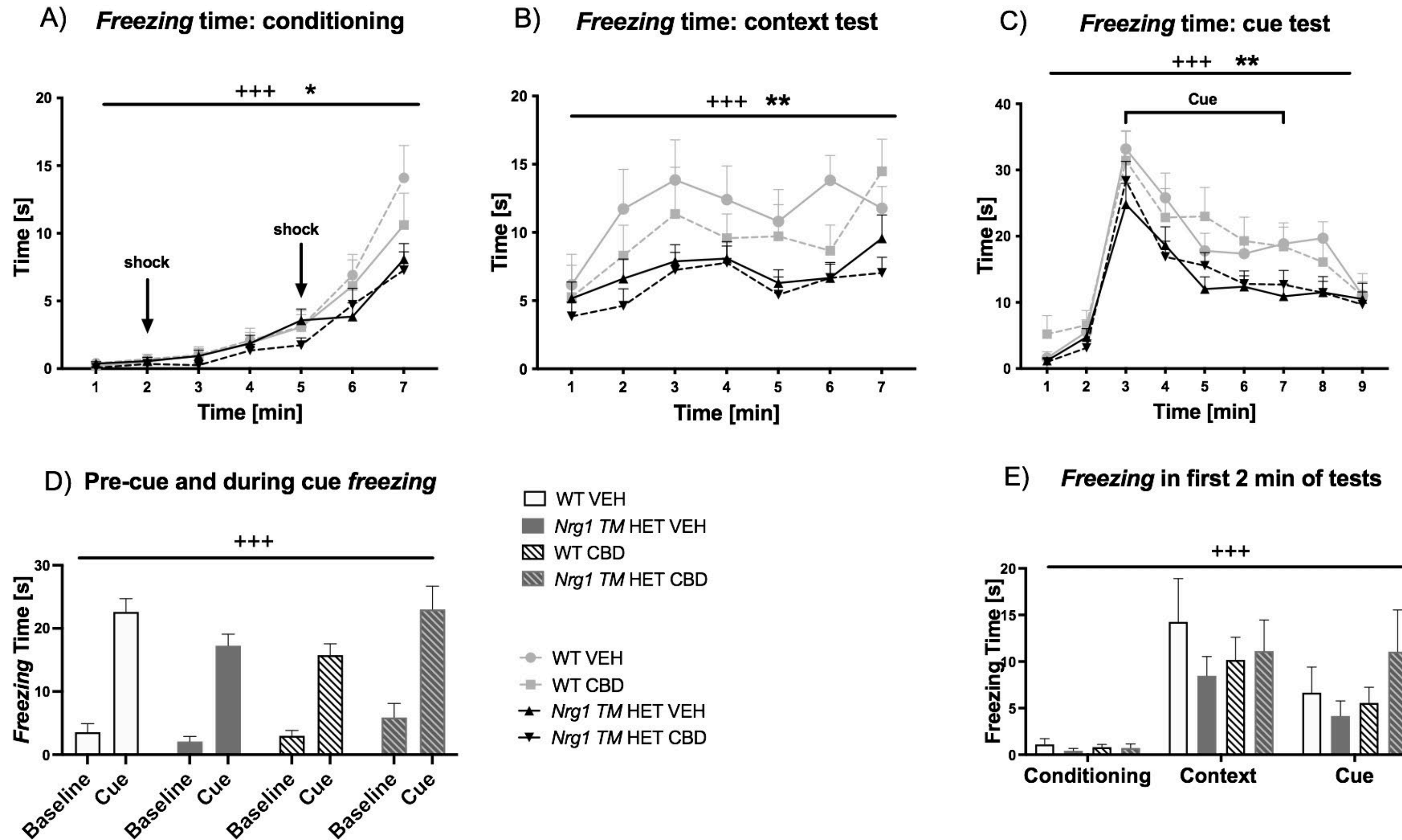

Fig. 4.5A-E: Time spent *freezing* in fear conditioning (FC) after adolescent CBD treatment: Duration [s] of *freezing* expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either vehicle (VEH) or cannabidiol (CBD). A) *freezing* time across conditioning; B) *freezing* time across context test; C) *freezing* time across cue test; D) average baseline *freezing* vs during the cue; and E) baseline *freezing* across test days. Genotype main effects indicated by * $p < 0.05$ and ** $p < 0.01$, RM effects indicated by $^{+++}$ $p < 0.001$.

4.3.5 Acute adolescent THC and locomotion, exploration and anxiety

There were no effects of acute treatment with 3 mg/kg THC on the total distance travelled in the OF and no other main effects or interactions (three-way ANOVA for 'THC', 'genotype' and 'CBD'; all *p's* > 0.05, no interactions; Fig. 4.6A). THC significantly decreased small motor movement frequency [main effect of 'THC': $F(1,64) = 50.5$; $p < 0.0001$; Fig. 4.6B] and *rearing* frequency [main effect of 'THC': $F(1,64) = 43.4$; $p < 0.0001$; Fig. 4.6C] across groups; importantly, effects of acute THC challenge were not affected by adolescent CBD treatment or genotype (no interactions: all *p's* > 0.05). Acute THC also decreased distance ratio [main effect of 'THC': $F(1,64) = 5.8$; $p < 0.0001$; Fig. 4.6D] and time in the centre of the OF compared to VEH controls [main effect of 'THC': $F(1,64) = 4.9$; $p = 0.03$; Fig. 4.6E]. There were no effects of 'genotype' or 'CBD' for all parameters measured in the OF (all *p's* > 0.05). No interactions between factors were present (all *p's* > 0.05).

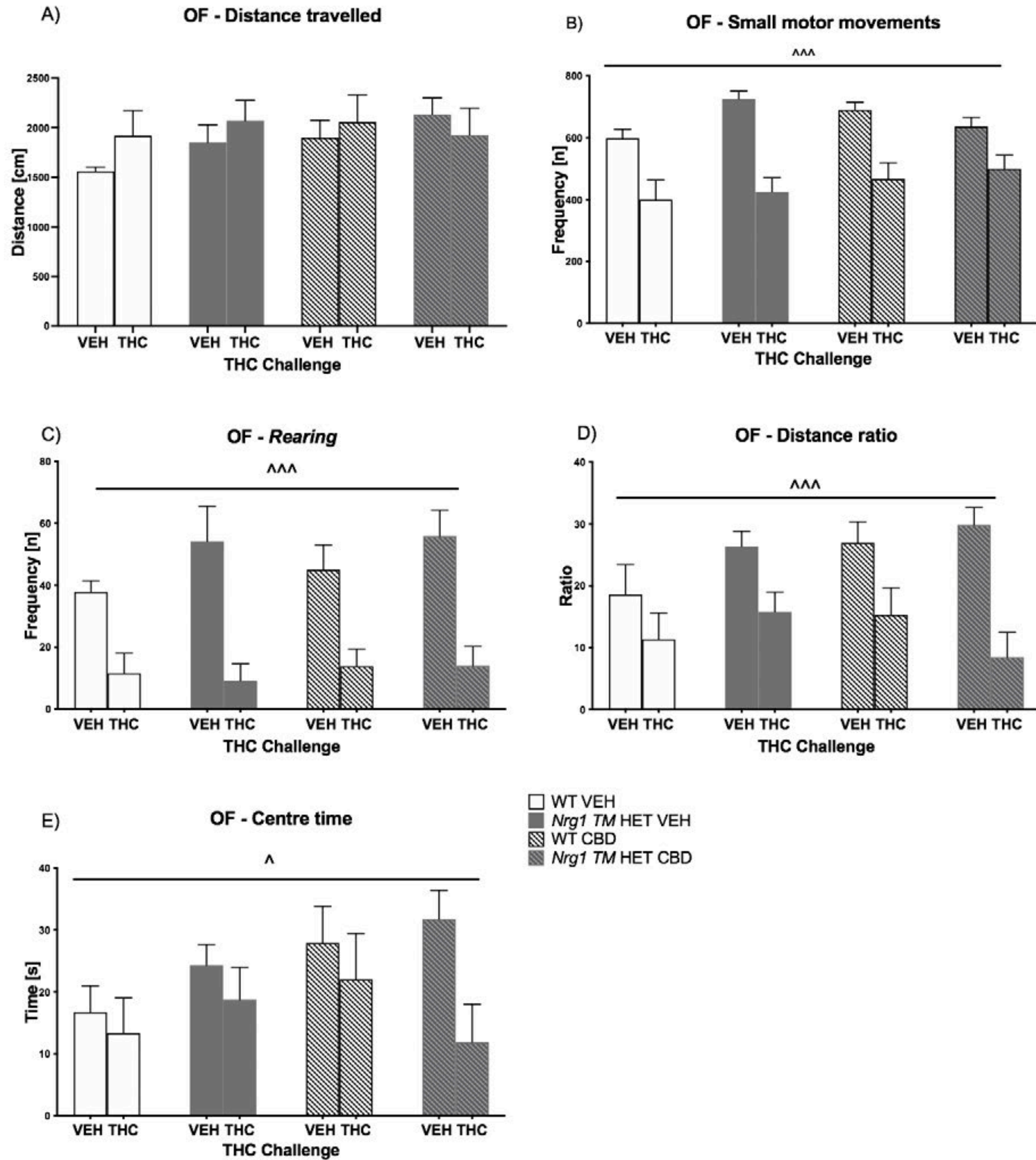

Fig. 4.6A-E: Open field (OF) - locomotion, exploration, and small motor movements after adolescent CBD and then later THC treatment: Data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated with chronically with either vehicle (VEH) or cannabidiol (CBD), then later vehicle or Δ^9-tetrahydrocannabinol (THC). A) distance travelled [cm]; B) small motor movement frequency [n]; C) *rearing* frequency [n]; D) distance ratio; E) centre time [s]. Significant two-way ANOVA main effects of 'THC' are shown as ^ $p < 0.05$ and ^^^ $p < 0.001$.

4.3.6 Acute adolescent THC and social behaviours

THC increased total SI time [$F(1,64) = 8.3$; $p = 0.005$; Fig. 4.7A], as did chronic adolescent CBD [$F(1,64) = 4.5$; $p = 0.04$; Fig. 4.7A]. Total social interaction frequency also tended to be increased by CBD treatment [$F(1,64) = 3.8$; $p = 0.054$], but there were no interactions between 'CBD' and 'genotype' or 'THC' (all *p's* > 0.05). There was no interaction between 'THC' and 'genotype' for total SI time [$F(1,64) = 3.5$; $p = 0.064$; Fig. 4.7B].

Within individual SI behaviours, THC increased *nosing* time [$F(1,64) = 7.6$; $p = 0.007$; Table 4.3], but there was no effect of 'CBD' or 'genotype' and no interactions were present for *nosing* time or *nosing* frequency (all *p's* > 0.05).

THC did not affect any measures of *anogenital sniffing* (all *p's* > 0.05) and no 'genotype' effect was found for *anogenital sniffing* frequency [$F(1,64) = 3.2$; $p = 0.079$; Table 4.3] whereas CBD increased *anogenital sniffing* frequency [$F(1,64) = 6.2$; $p = 0.01$; Table 4.3.2]. Interestingly, an interaction between 'THC' and 'genotype' for *anogenital sniffing* frequency was evident [$F(1,64) = 5.3$; $p = 0.02$; Table 4.3]. When split by 'THC', an effect of 'genotype' was only found in VEH-treated animals [$F(1,34) = 7.4$; $p = 0.01$], and not THC-treated animals [$F(1,36) = 0.2$; $p = 0.7$], indicating that *Nrg1 TM* HET animals had lower *anogenital sniffing* at baseline but not after THC treatment. When split by 'genotype', an effect of 'THC' was found in *Nrg1 TM* HET animals [$F(1,43) = 5.5$; $p = 0.02$] but not WT animals [$F(1,29) = 1.2$; $p = 0.3$], confirming that mutant mice appear to be more susceptible to some of the behavioural effects of THC. THC did not affect time *anogenital sniffing* [$F(1,64) = 2.2$; $p = 0.1$], but *Nrg1 TM* HET animals spent less time engaged in *anogenital sniffing* [$F(1,64) = 4$; $p = 0.049$; Table 4.3]. No effects of 'CBD' or interactions were present (all *p's* > 0.05).

THC increased *climbing on/over* time [$F(1,64) = 4.7$; $p = 0.03$; Table 4.3], and tended to increase *climbing on/over* frequency [$F(1,64) = 3.9$; $p = 0.051$; Table 4.3] (no interactions with 'genotype' or 'CBD', all *p's* > 0.05). CBD also increased time *climbing on/over* [F(1,64)

= 5.09; $p = 0.03$; Table 4.3]. A ‘THC’ by ‘genotype’ interaction for *climbing on/over* frequency was found [$F(1,64) = 5.5$; $p = 0.02$; Table 4.3]. When split by ‘THC’, only *Nrg1* mice of the VEH group showed a reduced frequency [$F(1,34) = 4.4$; $p = 0.04$] but not the THC-treated group [$F(1,36) = 4.6$; $p = 0.04$]. When split by ‘genotype’ instead, the inhibiting effect of ‘THC’ was only found in *Nrg1 TM* HET animals [$F(1,43) = 12$; $p = 0.001$] and not WT animals [$F(1,29) = 0.05$; $p = 0.8$], again suggesting that *Nrg1* mutant mice appear to be more susceptible to some of the behavioural effects of THC.

THC did not affect the time spent *following* or frequencies *following* the AJ mouse (all p's > 0.05). Similarly, ‘genotype’ and ‘CBD also did not alter this behaviour, and no interactions between factors were present (all p's > 0.05, Table 4.3).

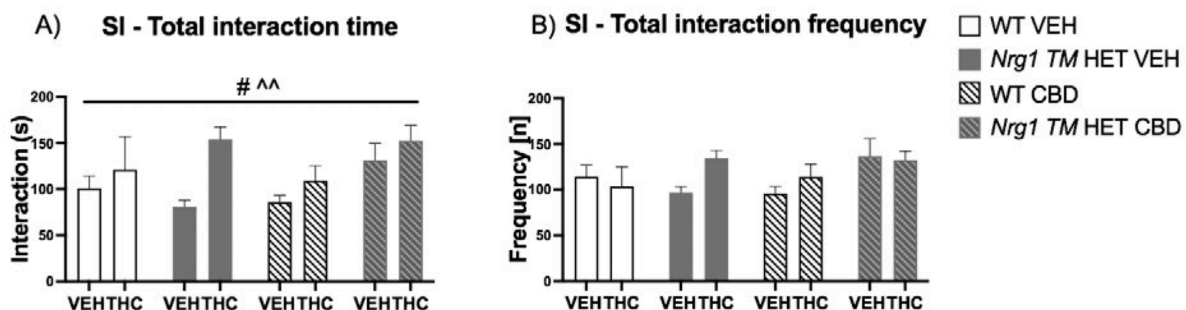

Fig. 4.7A-B: Social interaction (SI) after adolescent CBD and then later THC treatment: Total interaction time [s] and frequency [n] expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD), then later vehicle or Δ^9-tetrahydrocannabinol (THC). A) total interaction time [s] after THC; B) total interaction frequency [n] after THC. Significant effects of ‘CBD’ are indicated by $^{\#}$ $p < 0.05$, and significant effects of ‘THC’ are indicated by ^^ $p < 0.01$.

Genotype	**WT**				***Nrg1 TM* HET**			
Chronic Treatment	VEH		CBD		VEH		CBD	
THC Challenge	VEH	THC	VEH	THC	VEH	THC	VEH	THC
Nosing [s]	68.9 ± 8.5	90.4 ± 27.4	85.8 ± 10.6	95.4 ± 13.8	59.3 ± 5.15	73.4 ± 10.7	59.2 ± 5.2	107.3 ± 8.9
Anogenital Sniffing [s]	20.9 ± 5.9	17.5 ± 5.9	28 ± 6.7	33.7 ± 4.2	15.5 ± 2.6	21.6 ± 4.7	13.6 ± 2.3	24.1 ± 3.6
Climbing On/Over [s]	7.9 ± 1.4	8.23 ± 3	13.3 ± 3.8	16.1 ± 5.6	7.7 ± 1.5	10.8 ± 2.1	6.2 ± 1.34	20.2 ± 5.4
Following [s]	3.2 ± 1.5	4.61 ± 3.3	3.9 ± 1.2	7.4 ± 1.9	3.1 ± 1.4	3.1 ± 1.7	1.5 ± 0.6	2.2 ± 1.3
Nosing [n]	77.8 ± 7.5	75.3 ± 14.1	88.8 ± 10.7	84 ± 6.9	68.4 ± 5.7	76.5 ± 8.6	71.5 ± 4.5	91 ± 5.3
Anogenital Sniffing [n] †	22.8 ± 4.5	15.1 ± 3.7	27.8 ± 6.3	26.2 ± 1.3	15.6 ± 2.3	21.8 ± 3.7	15.3 ± 1.9 *	22.1 ± 1.3
Climbing On/Over [n] †	9 ± 1.9	7.85 ± 2.3	13.6 ± 3.1	13.5 ± 3.2	7.4 ± 1.2	11.5 ± 2	7.6 ± 1.2 **	18.3 ± 3.6
Following [n]	4 ± 1.7	5.1 ± 3.7	6 ± 1.7	8.4 ± 1.7	4.2 ± 1.8	3.7 ± 1.5	2.1 ± 0.6	2.5 ± 1.1

Table 4.3: Socio-positive behaviours after adolescent CBD and then later THC treatment: Duration [s] and frequency (fq) [n] of *nosing*, *anogenital sniffing*, *climbing on/over*, and *following* the A/J mouse in the social interaction (SI) test following acute THC exposure. Data expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD) then later vehicle or Δ^9-tetrahydrocannabinol (THC). Interactions between 'THC' and 'genotype' were found for *anogenital sniffing* [† $p = 0.02$] and *climbing on/over* [† $p = 0.02$] frequency. Split by effects of 'genotype' within these interactions are indicated with * $p < 0.05$ and ** $p < 0.01$.

4.3.7 Acute adolescent THC and prepulse inhibition

The startle response increased with higher startle pulse intensities [four-way RM ANOVA for 'startle pulse intensity': $F(2,128) = 364.6$; $p = < 0.0001$; Fig. 4.8A]. There was also a trend for 'THC' to decrease the startle response compared to VEH-treated animals [$F(1,64) = 3.7$; $p = 0.058$; Fig. 4.8A; no interactions with 'CBD' or 'genotype' and no other main effects].

All animals habituated to the startle stimulus ['startle block': $F(2,128) = 37.1$; $p < 0.0001$; Fig. 4.8B]. A 'THC' trend indicated that 'THC' tended to reduce startle [$F(1,64) = 3.5$; $p = 0.06$; Fig. 4.8B]. An interaction between 'startle block', 'genotype', 'CBD', and 'THC' [$F(2,128) = 7.3$; $p = 0.003$] was also present, whereby it appeared *Nrg1* mutants treated with CBD and THC did not habituate to the startle stimulus. To further investigate this, data were split by all factors ('genotype', 'CBD', and 'THC') to investigate the RM effect of 'startle block' in each individual group. This analysis showed that while every other group habituated (all *p's* < 0.05), *Nrg1 TM* HET mice treated with chronic CBD then acute THC had no RM effect of 'startle block' [$F(2,16) = 1.1$; $p = 0.3$] and habituation was therefore impaired (Fig. 4.8B).

No effects of 'THC', 'genotype', or 'CBD' were found for % PPI (all *p's* > 0.05). There was an interaction between 'prepulse' and 'THC' [$F(2,64) = 3.1$; $p = 0.049$], however, when split by 'THC' no further main effects or interactions were present (all *p's* > 0.05), and both THC-treated ['prepulse': $F(2,66) = 208.7$; $p < 0.0001$] and VEH-treated groups ['prepulse': $F(2,62) = 165.7$; $p < 0.0001$] showed increasing PPI with higher prepulse intensities. No other effects or interactions were found (all *p's* > 0.05). %PPI overall increased with increasing prepulse intensities [four-way RM ANOVA for 'prepulse': $F(2,64) = 371.7$; $p = < 0.0001$; Fig. 4.8C].

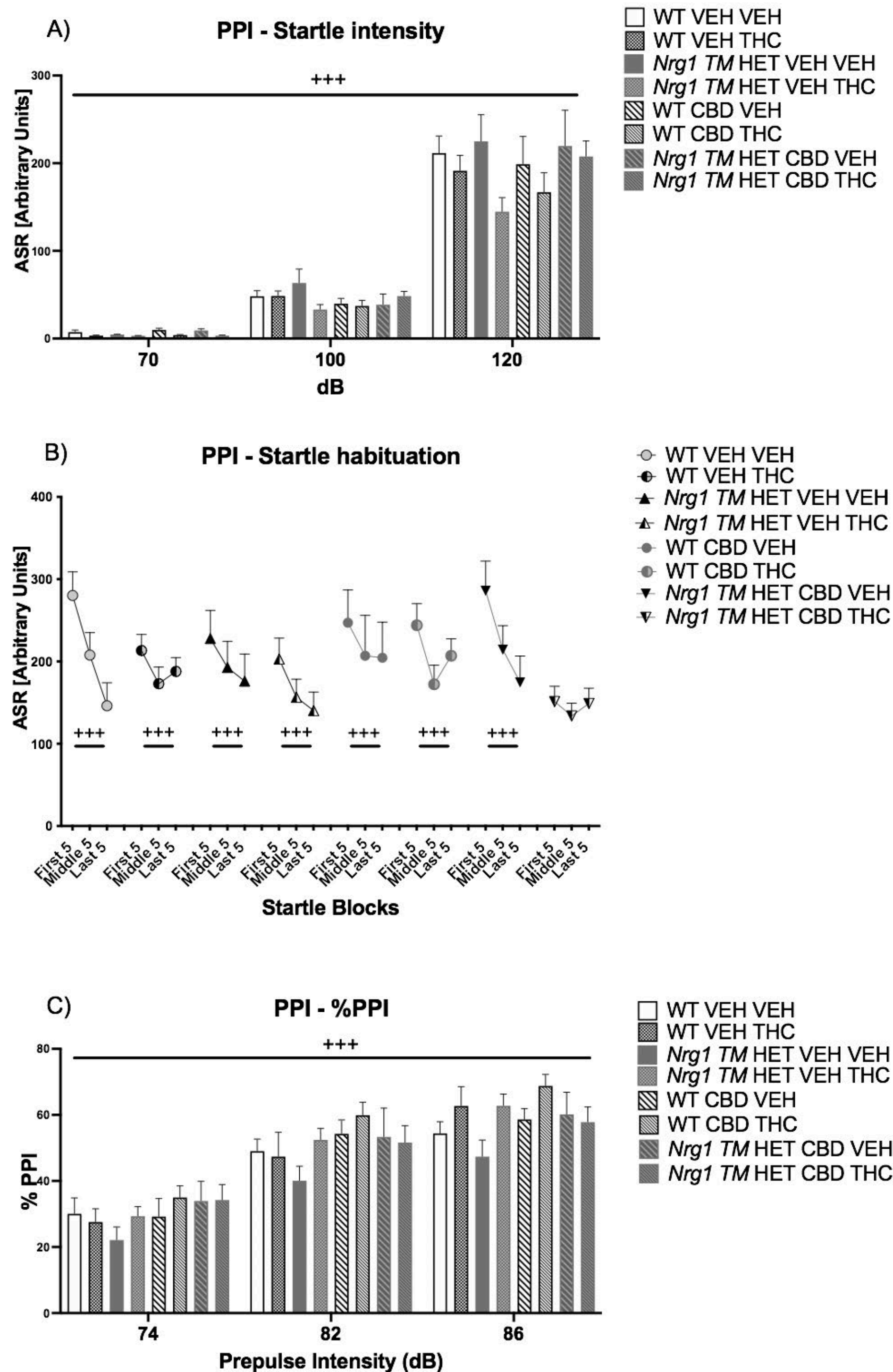

Fig. 4.8A-C: Acoustic startle response and prepulse inhibition (PPI) after adolescent CBD and then later THC treatment: A) average startle [ASR], B) average first, middle, and last 5 startle responses (startle habituation), and C) percentage PPI (% PPI) using 74dB, 82dB, and 86dB prepulses expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD), then later vehicle or Δ⁹-tetrahydrocannabinol (THC). RM effects are indicated as $^{+++}$ $p < 0.001$. An interaction between all factors was present [p = 0.003] that when split revealed a 'genotype' effect indicated with * $p < 0.05$.

4.3.8 Molecular changes in the hippocampus

CB_1 receptor levels in the HPC of animals were not altered by genotype, adolescent CBD exposure (two-way ANOVA main effects, all *p's* > 0.05; Fig. 4.9A). GAD_{67} levels were significantly decreased by CBD treatment [F(1,44) = 9; *p* = 0.004; Fig. 4.9B]. No main effects of 'genotype' or interactions were found (all *p's* > 0.05). No significant main effects of 'CBD' or 'genotype' were found for Iba1 (all *p's* > 0.05, Fig. 4.9C).

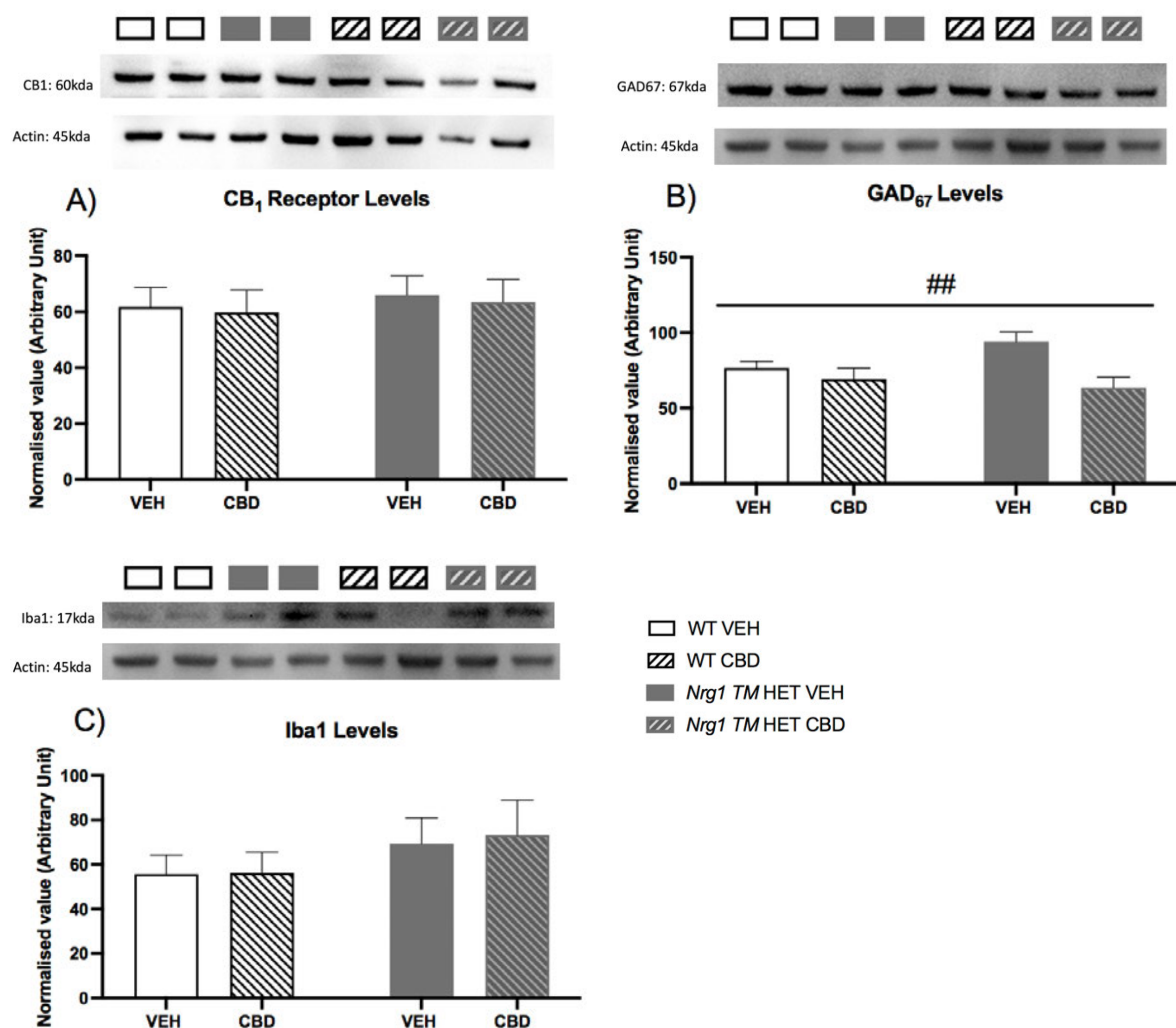

Fig. 4.9: Protein levels in the hippocampus after adolescent CBD treatment: Normalized value (arbitrary units) for A) cannabinoid receptor 1 (CB_1), B) glutamate decarboxylase 67 (GAD_{67}), and C) Iba1 expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD). Effects of CBD treatment indicated with $^{\#\#}$ *p* < 0.01.

4.4 Discussion

The present study investigated if chronic adolescent CBD treatment could protect against the development of schizophrenia-relevant behaviours and modulate behavioural susceptibility to acute THC in late adolescent/early adult *Nrg1 TM* HET males. At baseline in this study, *Nrg1 TM* HET mutants showed behavioural deficits similar to those found in Chapter 3 including increased OF locomotion, reduced ASR, reductions in some social behaviours, and impaired fear behaviour, however they also showed increased PPI in adolescence that disappeared with further testing during the THC battery in early adulthood. CBD increased locomotion in late adolescence and social interaction in early adulthood in the THC battery in all mice, but did not alter the behavioural phenotype of *Nrg1* mutant mice. Acute THC increased anxiety-like OF behaviour and overall social interaction, and decreased exploration across genotypes and treatment groups, and also increased some social behaviours in *Nrg1* mutants. Combined, chronic CBD and acute THC reduced startle habituation in *Nrg1 TM* HET mice, but there were no other combined effects of chronic CBD and acute THC treatment. Interestingly, CBD treatment from adolescence onwards decreased levels of GAD_{67} in all mice. There were no effects of either drug or genotype on hippocampal CB_1 receptor or Iba1 levels. While CBD was hypothesised to impact the early adulthood phenotype of *Nrg1 TM* HET mice and mediate responses to acute THC exposure, it did not have any effects on these mutant mice exclusively, and indeed had few effects overall.

VEH-treated *Nrg1* mutants in Chapter 4 showed a similar behavioural phenotype to that found in Chapter 3, and this Chapter aimed to assess whether chronic adolescent CBD could reverse this phenotype. Hyperlocomotion (found in Chapter 3) was present in late adolescent/early adult *Nrg1* mutant mice irrespective of treatment group, as were some social interaction deficits in the SI test of the THC battery. *Freezing* in the cue and context tasks was decreased in FC. Decreased startle at 100 dB was also present in mutant mice, similar to

Chapter 3 findings. Interestingly, PPI was elevated in not yet adult *Nrg1* mutant mice in the first PPI test. It is possible that this was an artefact of injection stress experienced by these animals, as *Nrg1* animals at 3-4 months of age are sensitive in some aspects of chronic restraint stress (i.e. decreased distance ratio) (169). Adult *Nrg1* mutants show altered social behaviours and locomotor activity in chronic stress paradigms (174). While the above study did not find that stress alters PPI in *Nrg1* mutant mice, mice were assessed at a later age than the current study and this effect on PPI may be present earlier in adolescence (173). Importantly, chronic physical stress (induced with repeated foot shocks) has been found to increase PPI and decrease startle in rats (354, 355). This stress-related modulation of sensorimotor gating is hypothesised to be linked to dopaminergic pathway dysfunction, as animals in the study by Piljman and colleagues showed decreased sensitivity to the locomotion-increasing effects of a dopaminergic agonist (354). Furthermore, increased dopamine activity in the nucleus accumbens caused by amphetamine is related to decreases in prepulse inhibition in rats (356). Indeed, imbalances found in dopaminergic receptor expression of the striatum have been detected in these animals in adulthood (179). It is possible that in the current study, *Nrg1* mutants were more sensitive than WT mice to the stress induced by repeated injections and this altered dopaminergic system function and increased PPI.

CBD alone in the first OF test induced a mild hyperlocomotive phenotype in all animals regardless of genotype. This is in contrast with other literature, that suggests acute and chronic CBD can protect against hyperlocomotion induced by other psychoactive compounds, but does not induce hyperlocomotion itself (264, 317, 357). Indeed, another study investigating chronic CBD found a six-week chronic treatment course of 20 mg/kg lowered locomotion in the OF test in C57BL/6 mice (279). It is possible that the age of the animal tested after CBD treatment may be an important factor for the discrepancies found across studies; previous studies reporting that CBD has no effect on, or even reduces locomotor activity have been conducted

in adult animals (264, 279). Unfortunately, studies investigating effects of chronic CBD treatment during adolescence have not examined effects on locomotion (276, 278). It is possible that chronic CBD increases locomotor activity only in adolescence and that this change does not last: however, future research needs to investigate this question. In the current study this may be demonstrated by the effects of CBD between OF trials, as any effects of CBD are found in the first OF test and not the second, suggesting that as mice age this effect is lost.

CBD also had no other effects on the *Nrg1 TM* HET phenotype in early adulthood after chronic treatment, suggesting it was not protective against the earlier development of behavioural deficits similar to those found in adult animals (5-7 mo) in Chapter 3. After three weeks of chronic 30 mg/kg CBD, startle deficits in mutant animals were unaltered, and hyperlocomotive behaviours were not ameliorated by CBD. Lower *freezing* in *Nrg1* animals explored in Chapter 3 was also unaffected by CBD. These data suggest that the CBD treatment design chosen is not effective for preventing against development of behavioural phenotypes in early adulthood caused by mutant *Nrg1 TM*. These findings are dissimilar to literature where acute 30 mg/kg of CBD reduced hyperlocomotion induced by a combination of ketamine- and D-amphetamine in adult mice (264). It may be that this dose is more efficacious as a remedial treatment in adult animals displaying a schizophrenia-relevant phenotype. It should also be noted that social interaction deficits ameliorated in other models (e.g. Poly I:C) with 10 mg/kg adolescent CBD (276) were not present at baseline in the initial testing of the present cohort. There could be other experimental designs that may result in CBD having stronger preventative effects, whereby dose and time course are adjusted for increased efficacy.

Acute THC had some anxiogenic properties, as found previously in other studies which used both acute and chronic dosing regimes (317, 358, 359). Surprisingly, acute THC increased sociability in all animals, much like the effects of chronic CBD on the same day. The ability for low dose THC to acutely increase sociability in control mice has not previously been

reported. Indeed, acute THC has previously been shown to decrease social behaviours in rats at 1 mg/kg (262), and in mice decreases social interaction behaviour frequency at 3 and 10 mg/kg (317). It is possible that in this earlier period of adulthood, animals are less sensitive to these specific effects of THC.

In the SI test of the THC battery, CBD increased social behaviours in both genotypes. This CBD-induced upregulation of SI behaviours may be explained by the length of CBD treatment at the time of testing, or the age of development at the time of testing in relation to CBD exposure. At the first SI test, animals had been treated for just over three weeks with CBD, and animals were still in the adolescent period. The second SI was performed during the THC battery ~ 20 days later, thus animals had been administered CBD for almost six weeks at this point and had reached early adulthood. It is possible that CBD treatment takes longer than three weeks to improve social behaviours but to date, limited investigation of the impact of longer-term CBD treatment on social behaviours has occurred. Studies have either evaluated long-term CBD treatment but ignored social behaviours (279) or used a shorter treatment paradigm of three weeks only and found no increase in social behaviours in control animals (190, 277, 360). It is possible the increase in social behaviours in both WT and *Nrg1 TM* HET mice in the present study was due to the extended CBD treatment period.

Few changes in THC sensitivity by chronic CBD were found in this study. However, direct interactions between these cannabinoids were not expected due to the extended time window between their administration (361). Indeed, the last dose of CBD was 20-24 h prior to acute THC, and CBD is eliminated within 24 h (362). Considering this, it is possible that the CBD-THC interaction in the current study, where THC had stronger effects on startle habituation in *Nrg1* mice which had been chronically treated with CBD, is not a direct result of acute CBD-THC interactions and is due to long-term changes induced by CBD. Any predisposition by CBD to later THC effects would however need to be more clearly

understood, and neural changes made by CBD that affect THC susceptibility have not been investigated in other studies to date. Increased sensitivity to THC by prior CBD has important implications for future clinical research also, as this could limit the use of CBD as a chronic treatment in cases where individuals may later be exposed to cannabis use.

A decrease in GAD_{67} in the hippocampus caused by chronic CBD may be related to the effects of CBD on increasing locomotion, although it should be considered that alterations to protein levels do not rule out possible changes to receptor functionality by chronic adolescent CBD. Other brain regions were also not explored in the current study due to limitations of time and resources, and therefore it is only speculation that these two factors may be linked. While the molecular analysis of the current study was limited in scope, it does suggest potential hippocampal involvement for some behavioural changes seen, and it does show that CBD can have long term effects on some systems when given during adolescence. The reduction of GAD_{67} protein levels in the hippocampus suggests a potentially hyper-excitatory environment in this brain region caused by 30 mg/kg CBD. The synthesis of GABA by GAD_{67} is an important step in inhibitory transmission in the hippocampus as inhibition is mediated by newly synthesised GABA, especially when the hippocampus is being repeatedly activated (363). Furthermore, while short-term neural transmission can rely on free GABA, neural activation will deplete this and inhibitory transmission is no longer possible when GABA is depleted (363). Hyperexcitability in the hippocampus is only seen when GABA levels are low due to lack of GABA synthesis (363), suggesting the reduction in GAD_{67} observed here could be a driver for hyperactivity in the hippocampus. This reduction could be related to the increase in locomotion in CBD-treated animals, as GABAergic dysfunction in regions implicated in activity-relevant pathways has been linked with hyperlocomotion (364-366). The current study therefore suggests a decrease in inhibitory drive in the hippocampus by CBD as potentially relevant for the observed effect of chronic adolescent CBD increasing locomotion in mice.

While alterations to this one marker may impact alterations in behaviour seen in the current study, a hyperexcitatory environment in the hippocampus could also be due to functional or protein alterations in NMDA and AMPA receptors. Other alterations to other regions could also be causing these behavioural changes.

In light of this, it is unlikely that a decrease in GAD_{67} would result in increased social behaviours, as a decrease in GABA levels in some brain regions is associated with decreased social behaviour and a decrease in GAD_{67} levels is also associated with social interaction deficits (367, 368). Furthermore, any action by CBD on CB_1 receptors may also not result in increased social interaction, as previous studies have found CB_1 receptor depletion in outbred mice decreases social interaction in a novel setting (369), and in a phencyclidine-induced model of schizophrenia CB_1 receptor depletion does not impact social behaviours (370). It is possible that an increase in social behaviour by CBD may be due to increases in anandamide and 2-AG availability via reduction of their hydrolysis, as this has been shown to increase sociopositive behaviours in adolescent mice and rats (371). CBD's elevation of anandamide and 2-AG has been linked to amelioration of psychotic symptoms in patients (228). It is possible that this mechanism may be involved in the increase of social behaviours induced by chronic CBD in the current study.

While no effects of CBD alone were found in *Nrg1* mutants, a combination of chronic CBD and acute THC impaired startle habituation in these mutant mice. Impaired startle habituation is theorised to be caused by the inhibition of glutamate release from the auditory caudal pontine reticular nucleus through the activation of inhibitory presynaptic metabotropic glutamate receptors (372). Startle habituation is also impaired by the administration of psychomimetic drugs such as phencyclidine (373), suggesting a role for blocking NMDA receptors, therefore reducing binding of glutamate in regions involved to the startle response pathway, such as the nucleus accumbens, caudal pontine reticular nucleus, and amygdala. *Nrg1*

TM HET animals show region-specific glutamatergic receptor imbalance, with decreased NMDA receptors in the thalamus at 20 weeks (179). The thalamus is involved in the regulation of acoustic stimulus habituation: reduced excitatory stimulation from the thalamus may decrease startle habituation through decreased input to the amygdala and thus reduced excitatory input to the pontine reticular nucleus (374). It is possible that decreased thalamic excitation could be a factor in startle habituation deficits in this model. Perhaps *Nrg1* mutant animals have increased sensitivity to the effects of CBD on regions involved in ASR habituation due to prior changes to glutamatergic pathways, and THC exposure even at a low dose can exacerbate this. Chronic CBD may therefore potentiate some effects of THC in adolescent/early adult animals of this genotype, rather than protecting against them. It is also possible that there is a threshold effect, where *Nrg1* mutation alone is not sufficient for eliciting this response in habituation to a stimulus, but with the additive effects of both CBD and THC this is seen.

In summary, this study suggests chronic adolescent CBD does not limit the development of schizophrenia-relevant behaviours in *Nrg1* mouse model in young adulthood, and may not be a preventative therapeutic candidate for patients harbouring this mutation. Furthermore, in control mice, some effects of long-term exposure to CBD during adolescence were the opposite to what was expected considering the potential antipsychotic effects of CBD, e.g. increased locomotion and decreased GAD_{67} in the hippocampus. Nonetheless, an increase in social behaviours instigated by adolescent CBD does suggest some therapeutic potential when social impairment is present. Our data promotes investigation of chronic CBD treatment in adolescence in different models of schizophrenia as CBD continues to show promise as an intervention for schizophrenia-relevant behaviours in other preclinical studies (276, 278), as well as further investigation of mechanisms by which chronic CBD could act to alter schizophrenia-relevant behaviour. The current study also did not investigate whether changes

elicited in late adolescent/early adulthood are permanent. This will be investigated in Chapter 5.

Chapter 5: Chronic adolescent cannabidiol and later acute adult Δ^9-tetrahydrocannabinol interact to produce some bio-behavioural deficits in *Neuregulin 1* mutant mice.

5.1 Introduction

In Chapter 4, the effects of chronic adolescent 30 mg/kg CBD were investigated in *Nrg1 TM* HET mice, and CBD did not prevent the behavioural phenotype outlined in Chapter 3 from emerging in *Nrg1* mutants. Chronic CBD also did not protect against the effects of acute THC shortly after this adolescent treatment period, and indeed potentiated its effects on startle habituation in *Nrg1* mutant mice, suggesting a role for chronic CBD in modulating some effects of THC. This study did not, however, investigate whether behavioural and neurochemical changes caused by chronic CBD could persist into adulthood. This is important, as the behavioural phenotype of *Nrg1 TM* HET animals develops around 5-6 months of age in adulthood (177), and may be affected by earlier chronic treatment.

Due to its sensitivity to environmental insult and stage of neural development (155, 331, 375), adolescence could be targeted as a window for pharmacological intervention in order to improve later prognosis in life. Early life interventions can also have the potential to decrease the severity of later symptoms in schizophrenia, which has been shown in both animal studies and clinical cohorts (153, 155, 277, 324). There has been limited research into CBD as a candidate for early pharmacological intervention in schizophrenia; however, there is precedence for this, as the endocannabinoid system is a key regulator in brain development

during adolescence (347) and endocannabinoid system dysfunction is linked with schizophrenia symptomology (339). CBD is a negative allosteric modulator of CB_1 receptors (338), and high concentrations of CB_1 receptors are found in schizophrenia symptom-relevant brain regions, e.g. hypothalamus, amygdala, thalamus, hippocampus, and PFC (214, 215). CBD can decrease neuroinflammation (283, 284), which is a factor in schizophrenia development (152). Considering this, it is possible CBD could have a role as an early intervention in schizophrenia, but this has not been investigated. Importantly, CBD also has a low side-effect profile compared to current treatments for schizophrenia (228), which suggests there could be a low risk of unwanted side effects from the compound.

I therefore aimed to investigate the potential for CBD to permanently alter the behavioural phenotype of *Nrg1 TM* HET animals, as well as its long-term neurochemical effects on CB_1 receptors, GAD_{67}, and Iba1 in the hippocampus. I also investigated how chronic CBD administration during adolescence would interact with later adult THC exposure, after a long washout period of three months, when adult *Nrg1* mutants display the behavioural phenotype outlined in Chapter 3.

Aim Three:

CBD may alter neural systems long-term that cause persisting effects on animal behaviour relevant to schizophrenia. We therefore aim to investigate the neuro-behavioural effects of chronic adolescent CBD in schizophrenia-relevant domains in later adulthood in *Nrg1 TM* HET mice, and investigate whether a history of CBD treatment may modulate the response to later THC exposure.

5.2 Materials and methods

5.2.1 Animals

Male *Nrg1 TM* HET and wild type-like littermates were bred and group housed in individually ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) at Animal BioResources (Moss Vale, Australia). At approximately 21-30 days old mice were transported to the mouse holding and test facilities at the School of Medicine, WSU, and were transferred to group-housing in filter top cages (1144B: Techniplast, Rydalmere Australia) with corn cob bedding (Tecniplast Australia, Rydalmere, Australia) and tissues for nesting. Mice were kept in a 12:12 h light:dark schedule [light phase: white light (illumination: 124 lx), dark phase: red light (illumination: < 2 lx; light phase from 0900-2100]. Mice were fed *ad libitum* with mouse feed pellets (Gordon's Specialty Stockfeeds Pty Ltd., Yanderra, Australia) and water. Bodyweight was monitored every 48 h and significant changes across time and between condition were not detected throughout the experiment (data not shown). Adult male A/J mice from Animal Resources Centre (Canning Vale, Australia) were used in the social-based tests in all studies. All research projects were approved by the WSU Animal Care and Ethics Committee (#A13298) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.2.2 Drug preparation and administration

Powdered cannabidiol (CBD: THC Pharm GmbH, Frankfurt/Main, Germany) was dissolved in equal parts of Tween 80 (Sigma-Aldrich Co, St Louis, USA) and 100% ethanol. It was then diluted with 0.9% sodium chloride to the final concentration (5% ethanol, 5% Tween 80, 90% saline). A vehicle control (VEH) was prepared by mixing all components minus CBD. VEH and CBD were injected daily intraperitoneally at a dose of 30 mg/kg body weight using an

injection volume of 10 ml/kg body weight. Treatment started on PND35 (±5 days), and mice were injected once daily in the afternoon (1200-1500) for three weeks. Mice were weighed every four days and the dosage adjusted accordingly. After treatment completion, animals were left in group housing to await behavioural testing at 5-6 months.

For the THC challenge, THC and VEH (THC Pharm GmbH, Frankfurt/Main, Germany) were prepared similarly to CBD. An acute dose of 3 mg/kg of THC or VEH was injected intraperitoneally 30 min before behavioural testing commenced, at approximately 180 days (±5 days) of age (experimental schedule outlined in Fig. 5.1).

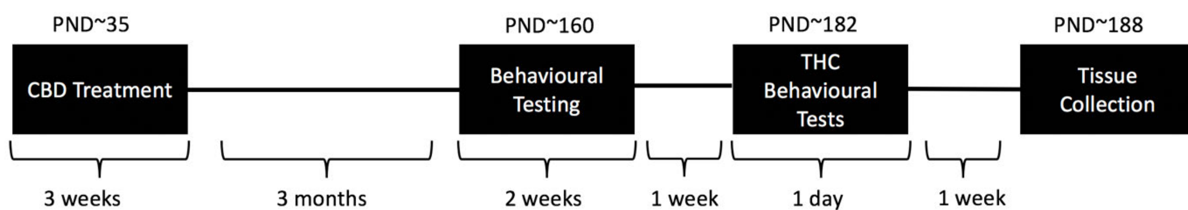

Fig. 5.1: Treatment and testing schedule for test animals: Wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice were treated chronically in adolescence with either vehicle (VEH) or 30 mg/kg of cannabidiol (CBD). Daily treatment commenced on post-natal day (PND) 35 ± 5 and continued for 3 weeks. After this period, mice were left undisturbed and untreated in their home cages until 5-6 months of age (160 ± 5 days) when behavioural testing started (see Table 1). Long-term effects of chronic adolescent CBD were evaluated between PND160-174 ± 5. An acute THC challenge was carried out on PND180 ± 5 followed by behavioural testing 30 min later. Mice were euthanized at approximately PND188.

5.2.3 Behavioural testing

Behavioural tests were conducted in the first half of the light phase between 0930 and 1400 in the Behavioural Neuroscience Facility at WSU. All tests were separated by an inter-test interval of at least 48 h and equipment and apparatus were cleaned with 80% ethanol between test runs unless specified otherwise. Behavioural testing order and duration are outlined below in Table 5.1. Tests used are outlined in the General methods section 2.3. Statistics were performed as per the General Methods section 2.7 and as per *Chapter 4*.

<table>
<tr><th colspan="4">Test Order</th><th colspan="4">Postnatal age (days)</th></tr>
<tr><td colspan="4">Adolescent CBD Treatment</td><td colspan="4">35-56 (±5 days)</td></tr>
<tr><td colspan="4">Open Field (OF)</td><td colspan="4">160 (±5 days)</td></tr>
<tr><td colspan="4">Social Interaction (SI)</td><td colspan="4">161-162 (±5 days)</td></tr>
<tr><td colspan="4">Prepulse Inhibition (PPI)</td><td colspan="4">164-167 (±5 days)</td></tr>
<tr><td colspan="4">Fear Conditioning (FC)</td><td colspan="4">169-172 (±5 days)</td></tr>
<tr><td colspan="4">THC Challenge (OF, SI, PPI)</td><td colspan="4">180 (±5 days)</td></tr>
<tr><td colspan="4">Tissue collection</td><td colspan="4">188 (±5 days)</td></tr>
<tr><th colspan="8">Animal Numbers</th></tr>
<tr><td colspan="4">WT</td><td colspan="4">Nrg1 TM HET</td></tr>
<tr><td colspan="2">VEH</td><td colspan="2">CBD</td><td colspan="2">VEH</td><td colspan="2">CBD</td></tr>
<tr><td>VEH</td><td>THC</td><td>VEH</td><td>THC</td><td>VEH</td><td>THC</td><td>VEH</td><td>THC</td></tr>
<tr><td>11</td><td>11</td><td>9</td><td>12</td><td>9</td><td>8</td><td>6</td><td>12</td></tr>
</table>

Table 5.1: Testing timeline and animal numbers: Test order, age (days), and animal numbers per group (*n*). Treatments included 30 mg/kg cannabidiol (CBD), 3 mg/kg Δ^9-tetrahydrocannabinol (THC), or vehicle control (VEH).

5.2.4 Acute THC challenge

Seven days after behavioural testing ceased, mice were injected with either 3 mg/kg THC or vehicle control, and 30 min later were placed into the OF apparatus for 10 min (186). Mice were then placed into the SI arena with an opponent mouse as described in the general methods. After this, mice were tested for PPI (Fig. 1). The ITI between these tests was ~ 1 min.

5.2.5 Western blot

One week post-behavioural testing mice were humanely euthanised and the right hippocampus was collected and stored as per the General methods section 2.4 for western blotting outlined in section 2.5. Targets analysed were CB_1 levels, GAD_{67} levels, and Iba1 levels in the hippocampus of all test animals.

5.2.6 Statistical analysis

Data for behavioural and molecular analyses were analysed using two-way or three-way analysis of variance (ANOVA) to investigate main effects and interactions between experimental factors 'genotype', 'CBD', and 'THC'. Four-way repeated measures (RM) ANOVAs were used where the within factor was 'time', 'cue', prepulse', '1-min block', or 'block', and the between factors 'genotype', 'THC', and 'CBD'. Where interactions were detected, ANOVAs were split by corresponding factor and further ANOVA conducted. Group differences were regarded as significant if $p < 0.05$. F-values and degrees of freedom are presented for all ANOVAs and data are shown as means ± standard error of means (SEM).

In figures and tables, significant 'genotype' effects are shown by '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Significant 'CBD' effects are reported by '#' (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). Significant 'THC' effects are shown by '^' (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$). RM effects are shown by '+' (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$), and any trend effects are reported with the corresponding p value. Significant interactions between factors were reported in tables as †$p < 0.05$, †† $p < 0.01$, and ††† $p < 0.001$. Statistical analyses were conducted using SPSS 27 for Mac and GraphPad Prism 8 for Mac.

5.3 Results

5.3.1 Chronic adolescent CBD and locomotion, exploration and anxiety

Testing locomotion in the OF, two-way ANOVA revealed there were no differences between any experimental groups for total distance travelled (no 'genotype' or 'CBD' main effects or interactions: all p's > 0.05; Fig. 5.2A).

Rearing frequency was also not affected by 'genotype' or 'CBD' treatment, but a significant interaction between 'genotype' and 'CBD' was detected [$F(1,74) = 7.8$; $p = 0.007$; Fig. 5.2B]. When split by 'genotype', an effect of 'CBD' was only found in WT mice [$F(1,42) = 6.2$; $p = 0.02$; Fig. 5.2B] and not *Nrg1 TM* HET mice [$F(1,34) = 2.3$; $p = 0.1$], suggesting CBD reduced *rearing* in WT mice only. When the data was instead split by 'CBD', a 'genotype' effect was found only in VEH-treated animals [$F(1,39) = 7.6$; $p = 0.009$] and not CBD-treated animals [$F(1,39) = 1.4$; $p = 0.2$], indicating *rearing* was greater in VEH-treated WTs than VEH-treated *Nrg1 TM* HET mice. The frequency of small motor movements was significantly higher in *Nrg1 TM* HET mice [two-way ANOVA for 'genotype': $F(1,74) = 10.5$; $p = 0.002$; Fig. 5.2C] and this was not affected by 'CBD' treatment (no main effect of 'CBD' or 'CBD' by 'genotype' interaction: all p's > 0.05).

Evaluating anxiety-relevant behaviours in the OF, no 'genotype' effects were detected (all p's > 0.05), but two-way ANOVA revealed a main effect of 'CBD' on distance ratio [$F(1,74) = 5.7$; $p = 0.02$; Fig. 5.2D] with adolescent 'CBD' treatment increasing OF centre locomotion of adult mice, compared to vehicle-treated adult mice. Time spent in the OF centre was not affected by either factor, and no interactions were present (all p's > 0.05; Fig. 5.2E).

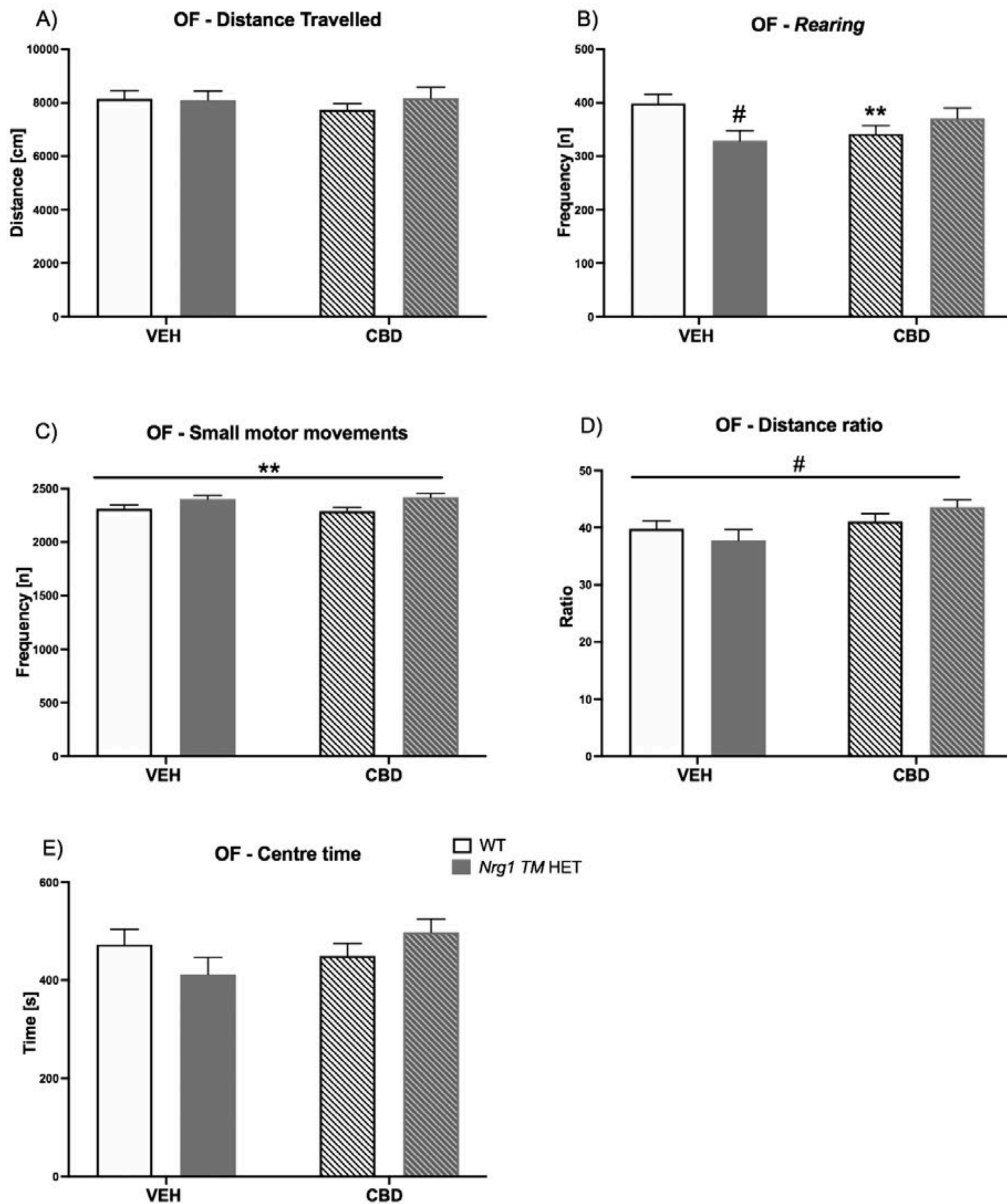

Fig. 5.2A-E: Open field (OF) - locomotion, exploration, and small motor movements in adulthood after adolescent CBD treatment: Data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either cannabidiol (CBD) or vehicle (VEH); A) distance travelled [cm]; B) rearing frequency [n]; C) small motor movement frequency [n]; D) distance ratio; E) centre time [s]. A 'genotype' by 'CBD' interaction for *rearing* [$p = 0.007$] when split shows significant effects of 'genotype' in VEH-treated animals ** $p < 0.01$ and 'CBD' in WT animals $^{\#}$ $p < 0.05$. Main effects of 'genotype' are indicated by ** $p < 0.01$. Main effects of CBD are indicated with $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$.

5.3.2 Chronic adolescent CBD and social behaviours

‘Genotype’ had no effect on any social parameters investigated (all *p’s* > 0.05). Adolescent CBD treatment did not affect total social interaction time [‘CBD’: F(1,75) = 1.4; *p* = 0.2; Fig. 5.3A] but it reduced the total frequency of socio-positive behaviours regardless of ‘genotype’ [F(1,75) = 6.3; *p* = 0.01 – no significant ‘CBD’ by ‘genotype’ interaction, *p* < 0.05; Fig. 5.3B].

When analysing individual social behaviours (Table 5.2), there were no main effects of ‘genotype’ on any behaviour (all *p*’s > 0.05). However, a significant main effect of ‘CBD’ on *nosing* frequency indicated CBD reduced *nosing* frequency [F(1,75) = 5.4; *p* = 0.02], but time spent *nosing* was not affected by ‘CBD’ (*p* > 0.05). Adolescent CBD treatment reduced the frequency of *following* [F(1,75) = 8.2; *p* = 0.005], time spent *following* [F(1,75) = 6.5; *p* = 0.01], and frequency of *climbing on/over* [F(1,75) = 4.7; *p* = 0.03]. ‘CBD’ had no effect on time spent on *climbing on/over* or time spent / frequency of *anogenital sniffing* (all *p’s* > 0.05). No interactions between ‘genotype’ and ‘CBD’ were present (all *p’s* > 0.05) (Table 5.2).

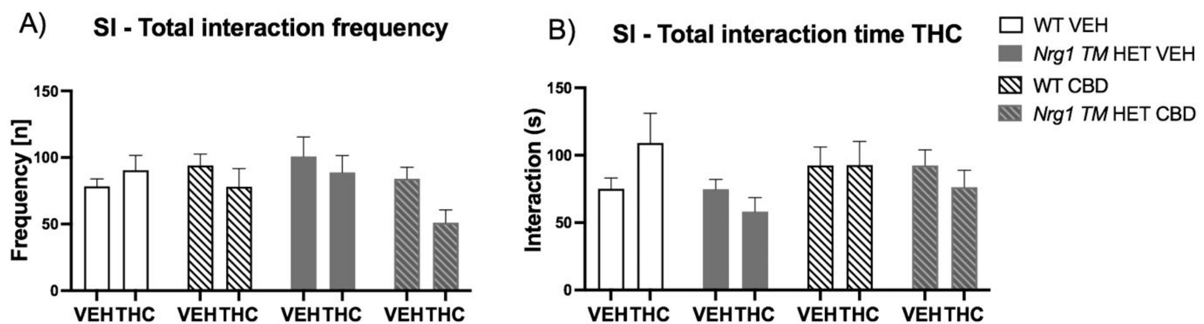

Fig. 5.3A-B: Social interaction (SI) after adolescent CBD treatment: Total interaction time [s] and frequency [n] expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either cannabidiol (CBD) or vehicle (VEH); A) total interaction frequency [n] after CBD treatment; B) total interaction time [s] after CBD. Significant main effects of CBD treatment are indicated by $^{\#}$ *p* < 0.05.

Genotype	**WT**		***Nrg1 TM* HET**	
Chronic Treatment	VEH	CBD	VEH	CBD
Nosing [s]	76.9 ± 3.6	74.2 ± 3.9	75.5 ± 4.5	73.02 ± 3.6
Anogenital Sniffing [s]	26.4 ± 2.2	28.6 ± 4.3	25.3 ± 3.9	22.7 ± 2.6
Climbing On/Over [s]	22.3 ± 1.9	19.02 ± 1.9	21.9 ± 2.2	19.7 ± 2.7
Following [s] #	4.4 ± 0.9	3.5 ± 0.6	6.5 ± 1.2	2.9 ± 0.6
Nosing fq [n] #	77.4 ± 4.6	67.09 ± 3.8	76.4 ± 2.8	67.7 ± 4.3
Anogenital Sniffing fq [n]	27.05 ± 2.01	26.4 ± 4.03	22.7 ± 3.4	21.8 ± 2.5
Climbing On/Over fq [n] #	22.9 ± 1.9	18.2 ± 1.2	22 ± 1.7	18.9 ± 2.05
Following fq [n] #	6.2 ± 0.9	4.8 ± 0.8	7.5 ± 1.2	3.6 ± 0.6

Table 5.2: Socio-positive behaviours after adolescent CBD treatment: Duration [s] and frequency (fq) [n] of *nosing*, *anogenital sniffing*, *climbing on/over*, and *following* the A/J mouse in the social interaction (SI) test. Data expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD). Significant main effects of 'CBD' indicated with # $p < 0.05$.

5.3.3 Chronic adolescent CBD and prepulse inhibition

Nrg1 TM HET mice regardless of 'CBD' treatment tended to exhibit a lower average ASR across startle pulse intensities than WT littermates [$F(1,75) = 3.6$; $p = 0.06$; no effects of 'CBD' or interactions: *p's* > 0.05; Fig. 5.4A]. Three-way RM ANOVA detected a main effect of startle stimulus intensity (i.e. 'startle pulse intensity') on average ASR, with animals startling more with increasing startle stimulus intensities [$F(2,150) = 119.1$; $p < 0.0001$; Fig. 5.4A]. At individual startle pulses, no significant effects of 'genotype' or 'CBD' or interactions were detected (all *p's* > 0.05).

All mice regardless of experimental group habituated to a 120dB startle stimulus across

startle blocks, and this was not different between groups [RM effect of 'startle block': $F(2,139) = 23.7$; $p < 0.0001$ - no main effects of 'CBD' or 'genotype' and no interactions; all *p's* > 0.05; Fig. 5.4B].

Sensorimotor gating was not affected by 'genotype' or 'CBD' and there were no interactions (all *p's* > 0.05; Fig. 5.4C). % PPI increased as prepulse intensity increased across all mice [three-way RM effect of 'prepulse intensity': $F(2,150) = 381.8$; $p < 0.0001$ - no interactions, all *p's* > 0.05; Fig. 5.4C]. Likewise, at each prepulse intensity no effects of 'genotype' or 'CBD' or interactions were present (all *p's* > 0.05).

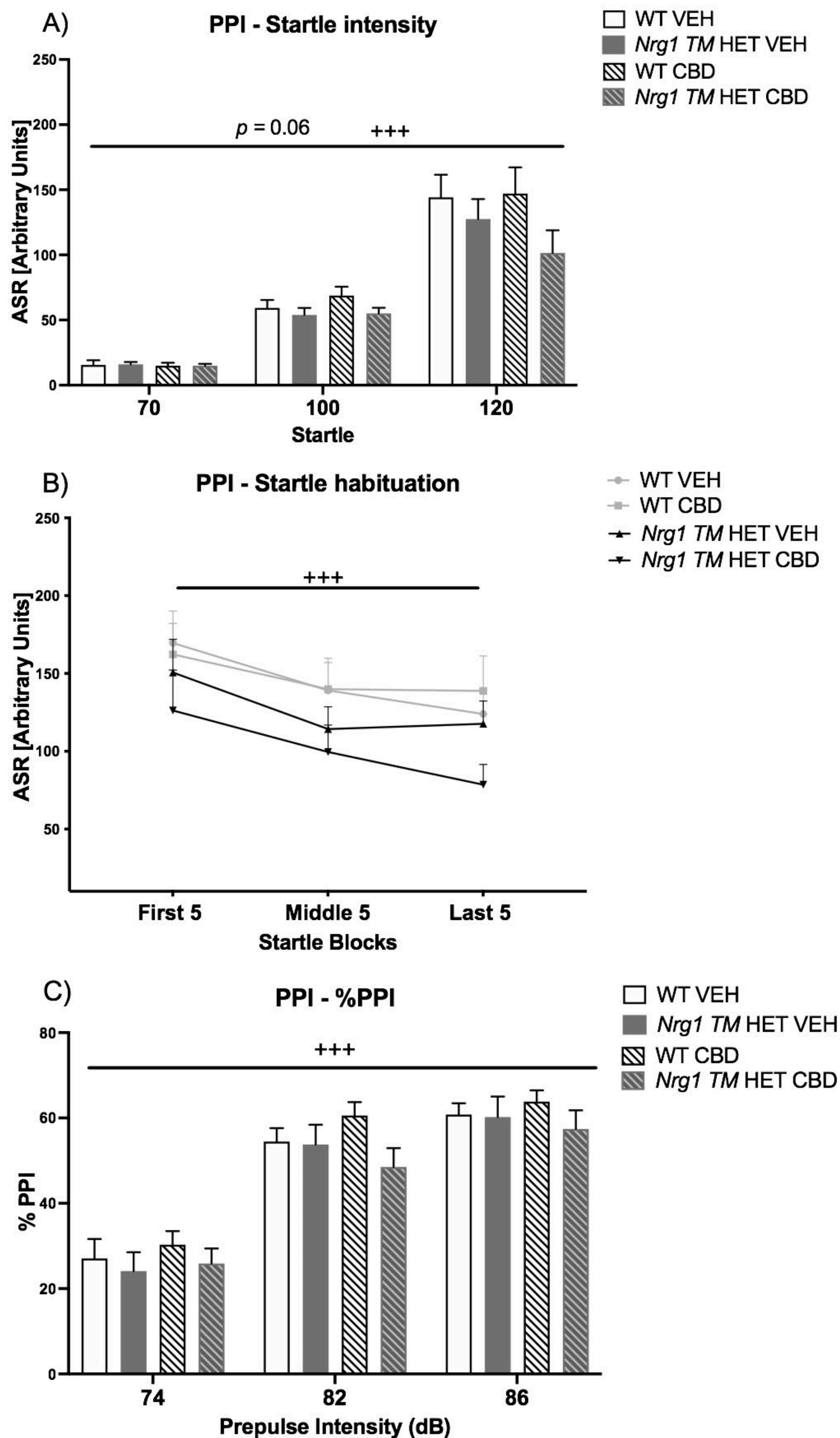

Fig. 5.4A-C: Acoustic startle response and prepulse inhibition (PPI) after adolescent CBD treatment: A) average startle [ASR], B) average first, middle, and last 5 startle responses (startle habituation), and C) percentage PPI (% PPI) with a 74dB, 82dB, and 86dB prepulse expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD). Repeated measures (RM) effects are indicated with $^{+++}$ $p < 0.001$. A trend effect of 'genotype' is indicated with the relevant p value.

5.3.4 Chronic adolescent CBD and fear associated memory

Baseline *Freezing*: Analysing baseline *freezing* (i.e. first 2 min of *freezing* during conditioning prior to the presentation of CS / US), no significant 'genotype' or 'CBD' differences or interactions in *freezing* time were detected (all *p's* >0.05). Comparing *freezing* in the first two minutes of each trial using three-way RM ANOVA revealed differences between test sessions [$F(2,219) = 38.8$; $p < 0.0001$; Fig. 5.5E] with *freezing* in the first 2 min being highest in the context test and lowest prior to conditioning indicating the animals associated the US and CS.

Conditioning: During conditioning, there was no 'genotype' effect [$F(1,69) = 2.7$; $p = 0.1$] on *freezing* and all animals *froze* more after receiving foot shocks, confirming the acquisition of the foot shock-context association [three-way RM ANOVA for '1-min block': $F(6,414) = 77.9$; $p < 0.0001$; Fig. 5.5A]. There were no effects of 'CBD' or interactions (all *p*'s > 0.05).

Context Test: *Freezing* in the context test was unaffected by 'genotype' or 'CBD' (all *p's* > 0.05) and increased across the test ['1 min block' $F(6, 450) = 10.9$; $p < 0.0001$; Fig. 5.5B], indicating expression of learned fear.

Cue Test: Average *freezing* across the test was not different between groups (all *p's* > 0.05 for 'CBD' and 'genotype' main effects, no interactions; Fig. 5.5C), though an RM effect of '1-min block' was present across groups [$F(8,600) = 122.3$; $p < 0.0001$; no interactions]. Genotype and CBD treatment had no impact on *freezing* during the cue test (no interactions, all *p's* > 0.05). All animals increased average *freezing* during cue presentation compared to the first 2 min of the test [three-way RM ANOVA main effect of 'cue': $F(4,300) = 57$; $p < 0.0001$; Fig. 5.5D].

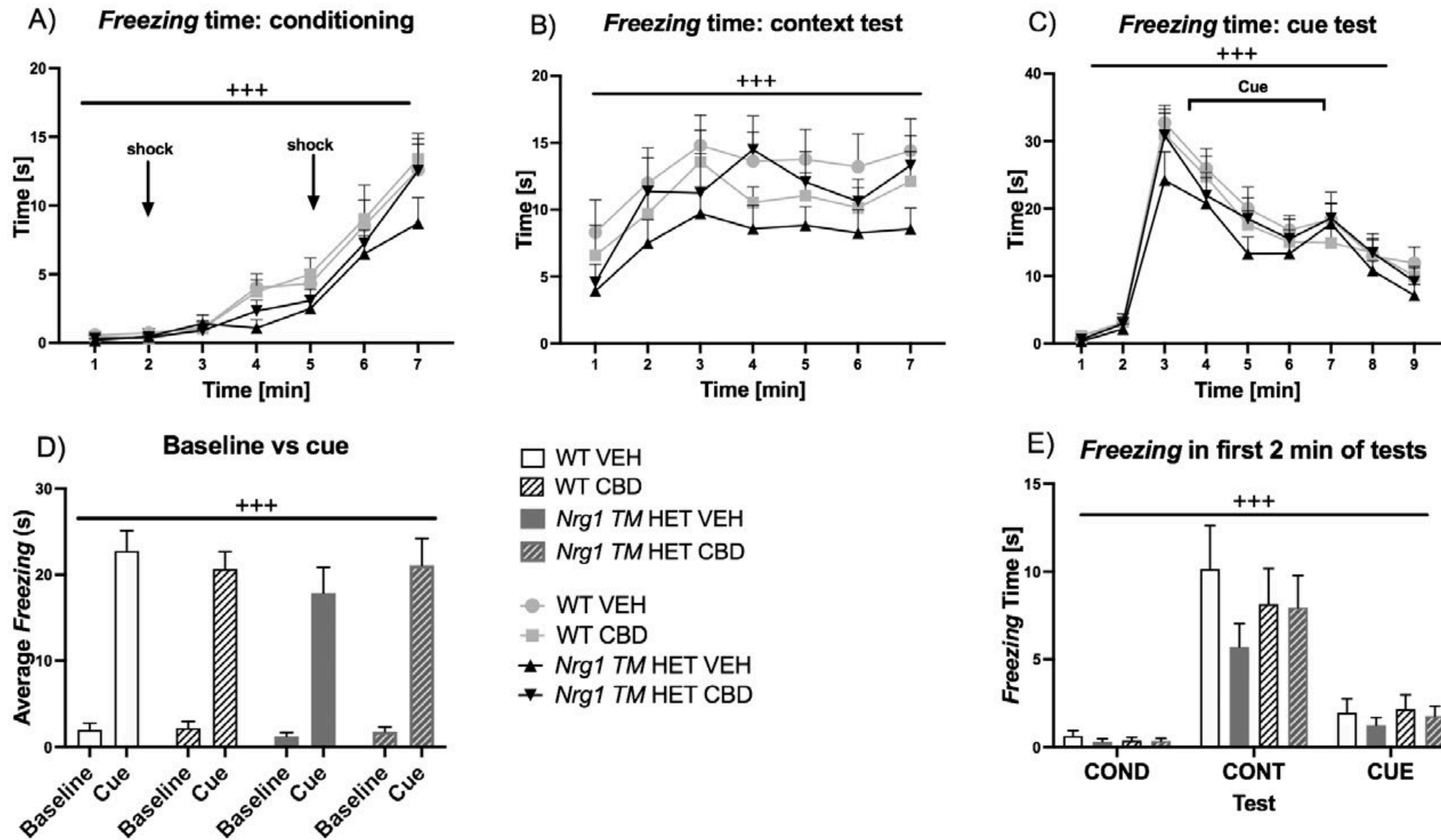

Fig. 5.5A-E: Time *freezing* in fear conditioning (FC) after adolescent CBD treatment: *freezing* time [s] expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD). A) *freezing* time across conditioning; B) *freezing* time across context test; C) *freezing* time across cue test; D) average baseline *freezing* vs during the cue; and E) baseline *freezing* across test days. Repeated measures (RM) effects indicated with $^{+++}$ $p < 0.001$.

5.3.5 Acute THC and locomotion, exploration and anxiety

Total distance travelled in the OF tended to be reduced by 'THC' [three-way ANOVA: $F(1,71) = 3.9$; $p = 0.051$], but was unaffected by 'genotype' [$F(1,71) = 2.6$; $p = 0.1$]. Total distance was also significantly reduced by 'CBD' [$F(1,71) = 4.7$; $p = 0.03$; Fig. 5.6A]. An interaction was present between 'THC' and 'genotype' [$F(1,71) = 4.2$; $p = 0.04$], which when split by 'genotype' revealed THC only decreased locomotion in *Nrg1 TM* HET animals [$F(1,35) = 5.1$; $p = 0.03$] but not in WT animals [$F(1,44) = 0.003$; $p = 0.9$], replicating the sensitivity to THC in mutant mice of similar age seen in *Chapter 3*. No other interactions were found for locomotion (all *p's* > 0.05).

Rearing frequency was decreased by 'THC' [main effect of 'THC': $F(1, 71) = 54$; $p < 0.0001$; Fig. 5.6B], however this behaviour was unaffected by 'genotype' [$F(1,71) = 1.4$; $p = 0.3$]. Adolescent CBD treatment decreased *rearing* frequency across groups [$F(1,71) = 4.9$; $p = 0.04$; Fig. 5.6B]. A trend for an interaction was present between 'THC' and 'genotype' [$F(1,71) = 3.7$; $p = 0.058$], suggesting THC tended to decrease *rearing* more in *Nrg1* mutant mice than in WTs. No other interactions were found (all *p's* > 0.05).

'THC' significantly reduced small motor movements [$F(1,71) = 33.6$; $p < 0.0001$; Fig. 5.6C] whereas 'genotype' and 'CBD' had no effect, and no interactions were present (all *p's* > 0.05).

Looking at the impact of an acute 'THC' challenge on anxiety-related behaviours, 'THC' decreased distance ratio in the OF [$F(1,71) = 19.2$; $p < 0.0001$; Fig. 5.6D] and reduced OF centre zone time [$F(1,71) = 7.6$; $p = 0.007$; Fig. 5.6E] but there were no other main effects or interactions (all *p's* > 0.05).

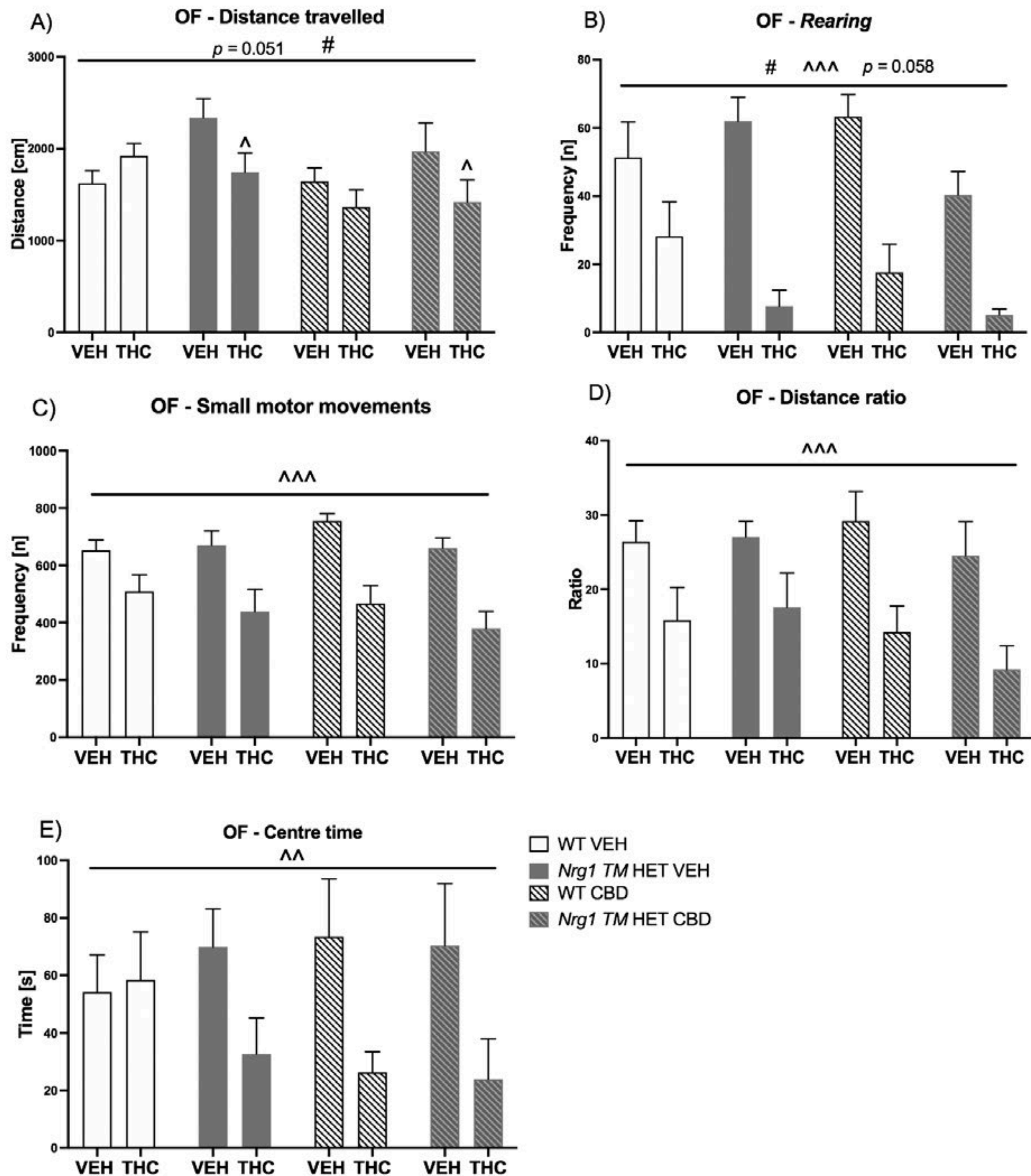

Fig. 5.6A-E: Open field (OF) - locomotion, exploration, and small motor movements after adolescent CBD and then later THC treatment: Data expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated during adolescence with either cannabidiol (CBD) or vehicle (VEH), then later THC or VEH; A) distance travelled [cm]; B) *rearing* frequency [n]; C) small motor movement frequency [n]; D) distance ratio; E) centre time [s]. Main effects of 'CBD' are indicated by $^{\#}$ $p < 0.05$, and main effects of 'THC' are shown as ^^ $p < 0.01$ and ^^^ $p < 0.001$. An interaction between 'genotype' and 'THC' for distance travelled [$p = 0.04$] was split by THC, and significant effects of 'THC' are indicated with ^ $p < 0.05$. A trend for an interaction between 'genotype' and 'THC' for *rearing* [$p = 0.058$] is indicated with the relevant p value.

5.3.6 Acute THC and social behaviours

Time spent in socio-positive behaviours was not affected by any experimental test condition and no interactions were present (all *p's* > 0.05; Fig. 5.7A). No main effects of 'THC', 'genotype' or 'CBD' were found for total frequency of socio-positive behaviours (all *p's* > 0.05). However, total frequency of socio-positive behaviours was affected by an interaction between 'genotype' and 'CBD' [F(1,71) = 5.2; *p* = 0.03; Fig. 5.7B], which when split by 'genotype' showed CBD reduced social interaction frequency in *Nrg1 TM* HET animals only [F(1,35) = 0.4; *p* = 0.01 - WT animals: F(1,44) = 0.09; *p* = 0.7; Fig. 5.7B]. No further interactions were present.

While there was no main effect of THC on *nosing* frequency, a main effect of 'CBD' on *nosing* frequency indicates 'CBD' decreased *nosing* [F(1,71) = 4; *p* = 0.049]. There was an interaction between 'genotype' and 'CBD' [F(1,71) = 5; *p* = 0.03], which when split by 'genotype', showed that CBD reduced *nosing* frequency only in *Nrg1* mutants [*Nrg1*: F(1,35) = 8.02; *p* = 0.008; Table 5.3] and not WT littermates [F(1,44) = 0.03; *p* = 0.9]. A main effect of 'THC' was found for *anogenital sniffing* frequency [F(1,71) = 6.3; *p* = 0.01; Table 5.3], with 'THC' reducing *anogenital sniffing* in all mice (no interactions, *p* > 0.05). A main effect of 'THC' was found for *following* time [F(1,71) = 4.6; *p* = 0.04; Table 5.3] with 'THC' reducing this behaviour in all mice (no interactions, *p* > 0.05). No other significant effects or interactions were found for parameters investigated (all *p's* > 0.05).

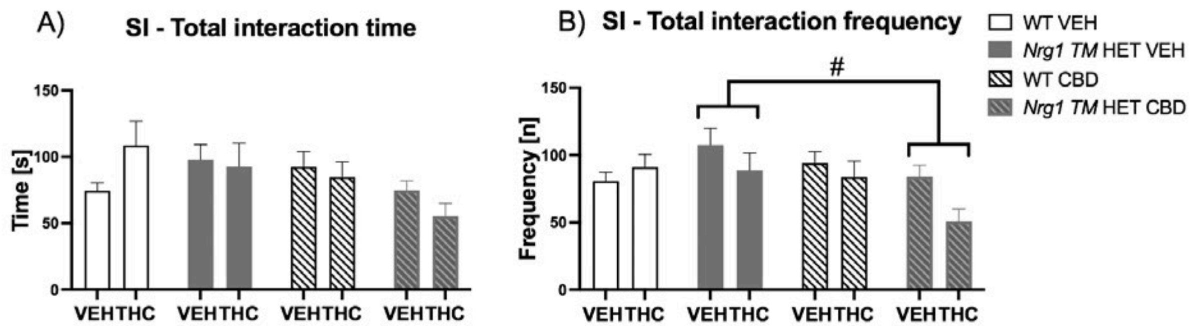

Fig. 5.7A-B: Social interaction (SI) after adolescent CBD and then later THC treatment: total interaction time [s] and frequency [n] expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice; A) total interaction frequency [n] after cannabidiol (CBD) or vehicle (VEH) then acute THC; B) total interaction time [s] after CBD then acute THC. An interaction between 'genotype' and 'CBD' (p = 0.03), which when split shows CBD reduced SI frequency compared to VEH controls in *Nrg1 TM* HET mice only ($^{\#}$ p < 0.05).

Genotype	WT				*Nrg1 TM* HET			
Chronic Treatment	VEH		CBD		VEH		CBD	
THC Challenge	VEH	THC	VEH	THC	VEH	THC	VEH	THC
Nosing [s]	50.6 ± 4.2	71.9 ± 10.3	60.6 ± 9.2	55.7 ± 7	62.4 ± 5.4	63.04 ± 11.1	47.9 ± 3	42.2 ± 6.7
Anogenital Sniffing [s]	13.5 ± 2.1	12.9 ± 2	17.8 ± 2.9	13.4 ± 4.07	17.1 ± 4.1	16.8 ± 5.4	16.1 ± 2.1	5.6 ± 1.3
Climbing On/Over [s]	9.07 ± 1.5	22.4 ± 10.9	11.7 ± 1.1	14.3 ± 2.1	15.8 ± 2.06	11.6 ± 3.8	8 ± 2.3	7.4 ± 2.5
Following [s] ^	1.3 ± 0.7	1.4 ± 0.5 ^	2.3 ± 0.8	1.2 ± 0.6 ^	2.6 ± 1.4	1.9 ± 0.6 ^	2.7 ± 1.4	0.15 ± 0.06 ^
Nosing fq [n] #	52.8 ± 5.1	59.4 ± 6.7	61 ± 6.4	53.4 ± 6.7	71.1 ± 6.7	60.2 ± 8.5	54.2 ± 4.3 ##	36.7 ± 6.3 ##
Anogenital Sniffing fq [n] ^	15.5 ± 1.4	14.5 ± 1.8 ^	18.4 ± 2.3	14.2 ± 3.7 ^	18.4 ± 3.7	15.2 ± 3.1 ^	17 ± 2.6	6.08 ± 1.6 ^
Climbing On/Over fq [n]	9.6 ± 1.4	15.3 ± 4.4	11.7 ± 1.2	13.8 ± 1.9	15.2 ± 2.2	11.6 ± 3.2	9 ± 2.5	7.6 ± 2.1
Following fq [n]	2.7 ± 1.1	2 ± 0.7	2.9 ± 0.9	2.2 ± 1	2.8 ± 1	1.6 ± 0.7	3.8 ± 1.6	0.4 ± 0.2

Table 5.3: Socio-positive behaviours after adolescent CBD and then later THC treatment: Duration [s] and frequency (fq) [n] of *nosing*, *anogenital sniffing*, *climbing on/over*, and *following* the A/J mouse in the social interaction (SI) test. Data expressed as mean ± SEM for wild type-like (WT) and *Neuregulin 1* transmembrane domain heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD) then later vehicle or Δ^9-tetrahydrocannabinol (THC). Main effects of 'THC' indicated with ^ $p < 0.05$, and main effects of 'CBD' are indicated with # $p < 0.05$. An interaction between 'genotype' and 'CBD' [$p = 0.049$] for *nosing* frequency was split by 'CBD', and effects of CBD vs VEH controls in *Nrg1 TM* HET mice are indicated with ## $p < 0.01$.

5.3.7 Acute THC and prepulse inhibition

A main effect of 'genotype' across startle stimulus intensities indicated that *Nrg1 TM* HET animals startled less than WT littermates, irrespective of 'CBD' or 'THC' treatment [$F(1,70) = 4.2$; $p = 0.04$; Fig. 5.8A]. No other main effects or interactions were found (all *p's* > 0.05). A RM effect of 'startle pulse intensity' confirmed that animals startled more with increasing startle stimulus intensities [$F(2,140) = 189.8$; $p < 0.0001$; Fig. 5.8A].

Looking at individual startle stimulus intensities, THC reduced startle at 70dB (i.e. background noise) [no effects of 'genotype' or 'CBD'; three-way ANOVA main effect for 'THC': $F(1,78) = 30.04$; $p < 0.0001$; Fig. 5.8A]. There was an interaction between 'THC' and 'genotype' [$F(1,78) = 5.2$; $p = 0.02$], but when split by 'THC', no 'genotype' effect was evident in either treatment group (p's > 0.05). When split by 'genotype' instead, THC effects were present in both the WT group [$F(1,43) = 5.9$; $p = 0.02$] and *Nrg1* mutant group [$F(1,35) = 26.7$; $p < 0.001$], indicating THC reduced startle in both genotypes.

At 100dB, *Nrg1 TM* HET animals startled less than WTs [main effect of 'genotype': $F(1,78) = 4.5$; $p = 0.04$; no main effects of 'THC' or 'CBD'; Fig. 5.8A]. There was also a significant 'CBD' by 'THC' by 'genotype' interaction [$F(1,78) = 4.1$; $p = 0.046$]. When split by 'genotype', a 'THC' by 'CBD' interaction was present in *Nrg1* mutants [$F(1,35) = 5.9$; $p = 0.02$] but not in WT animals [$F(1,43) = 0.4$; $p = 0.5$]. When this data were further split by 'CBD', THC reduced startle only in CBD-treated *Nrg1 TM* HET animals [$F(1,18) = 12.2$; $p = 0.003$] but not VEH-treated *Nrg1* mutant mice [$F(1,17) = 0.3$; $p = 0.6$], and no effects were found in WT treatment groups (all p's > 0.05). This suggests that adolescent CBD potentiated the effects of THC-induced reductions in ASR in *Nrg1 TM* HET animals only (Fig. 5.8A).

At 120dB there were no significant group differences or interactions (all p's > 0.05).

Startle habituation was evident in mice from all genotypes and treatments [four-way RM effect of 'startle block' $F(2,140) = 22.8$; $p = 0.0001$; Fig. 5.8B]. An interaction between 'startle block' and 'THC' [$F(2,140) = 5.1$; $p = 0.01$; Fig. 5.8B] was present, however, further ANOVAs split by 'startle block' showed no significant effects of 'THC' at any individual startle block (all p's > 0.05). When data were instead split by 'THC', startle habituation was present in both treatment groups (all p's < 0.05).

For %PPI, there were no main effects of 'CBD', 'THC', 'genotype', or interactions (all p's > 0.05). A main effect of 'prepulse' in a four-way RM ANOVA [$F(2,140) = 511.2$; $p >$

0.0001; Fig. 5.8C] demonstrated increased %PPI at higher prepulse intensities in all mice. Separate ANOVA were conducted to analyse effects of experimental factors at individual prepulse intensities, but no main effects or interactions were present at individual prepulse intensities (all p's > 0.05).

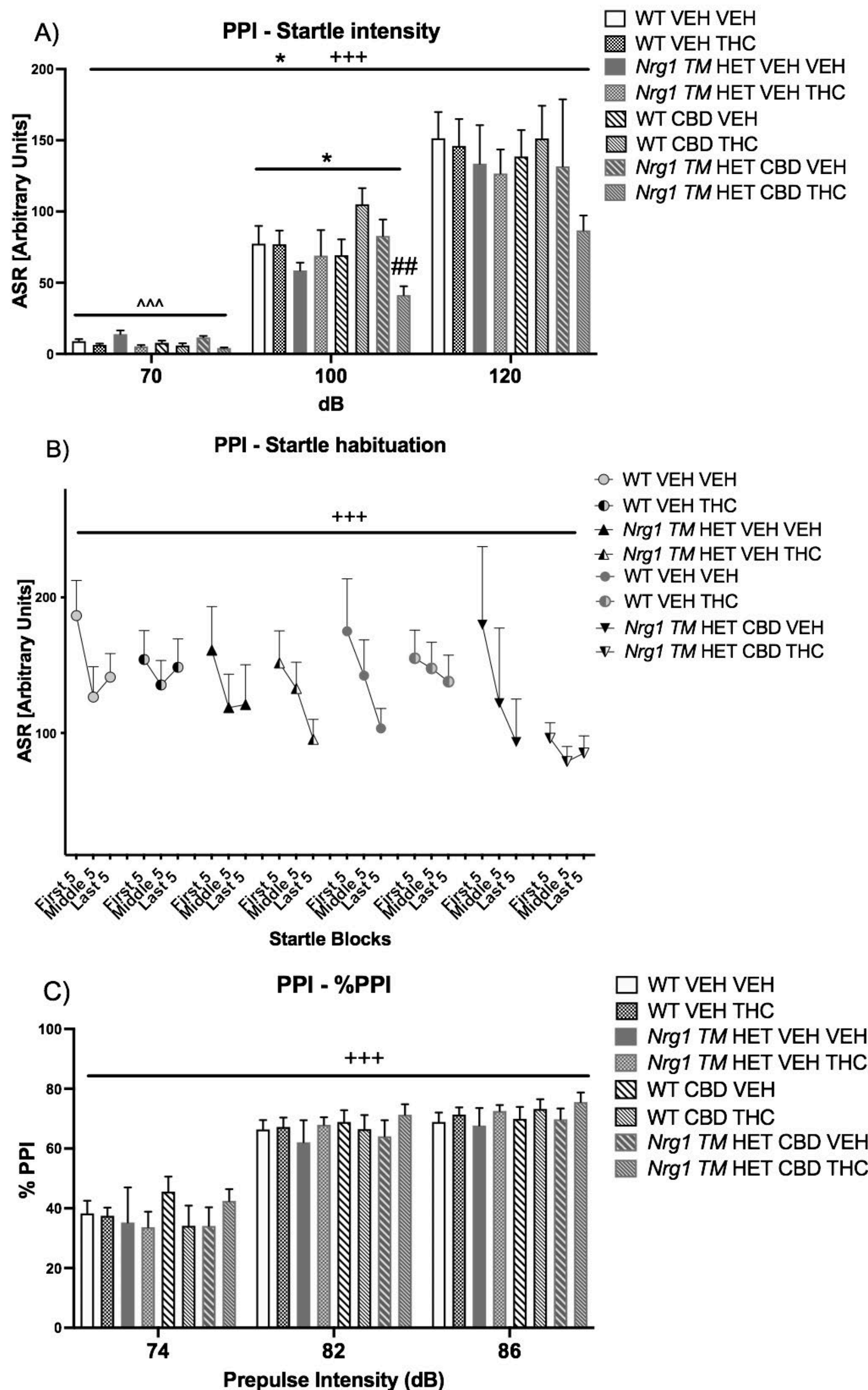

Fig. 5.8A-C: Acoustic startle response and prepulse inhibition (PPI) after adolescent CBD and then later THC treatment: A) average startle (ASR), B) average first, middle, and last 5 startle responses (startle habituation), and C) percentage PPI (% PPI) across a 74dB, 82dB, and 86dB prepulse expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD), then later vehicle or Δ^9-tetrahydrocannabinol (THC). Effects of genotype are indicated with * $p < 0.05$ and effects of THC are indicated with ^^^ $p < 0.001$. A significant 'genotype' by 'THC' by 'CBD' interaction [$p = 0.046$], which when split further revealed 'CBD' effects in THC-treated *Nrg1 TM* HET mice only ($^{\#\#}$ $p < 0.01$). RM effects are indicated with $^{+++}$ $p < 0.001$.

5.3.8 Western blot

There was a main effect of 'genotype' [$F(1,44) = 4.1$; $p = 0.047$] on CB_1 receptor protein levels, whereby *Nrg1* mutants showed increased CB_1 receptor levels in the hippocampus. Chronic adolescent 'CBD' treatment increased CB_1 protein levels in the hippocampus of adult animals [$F(1,43) = 6.2$; $p = 0.02$; Fig. 5.9A]; no 'CBD' x 'genotype' interaction was present [$F(1,44) = 0.9$; $p = 0.3$]. There was no effect of 'genotype' on levels of GAD_{67} in the hippocampus of mice [$F(1,44) = 0.6$; $p = 0.4$], and 'CBD' treatment did not affect GAD_{67} levels [$F(1,44) = 2.8$; $p = 0.09$]. No interaction was present between factors [$F(1,44) = 1.3$; $p = 0.3$]. Finally, no main effects of 'genotype' [$F(1,44) = 0.8$; $p = 0.4$] were found for Iba1 protein levels in the hippocampus, nor did CBD alter Iba1 [$F(1,44) = 0.1$; $p = 0.7$], with no interaction [$F(1,44) = 0.02$; $p = 0.9$].

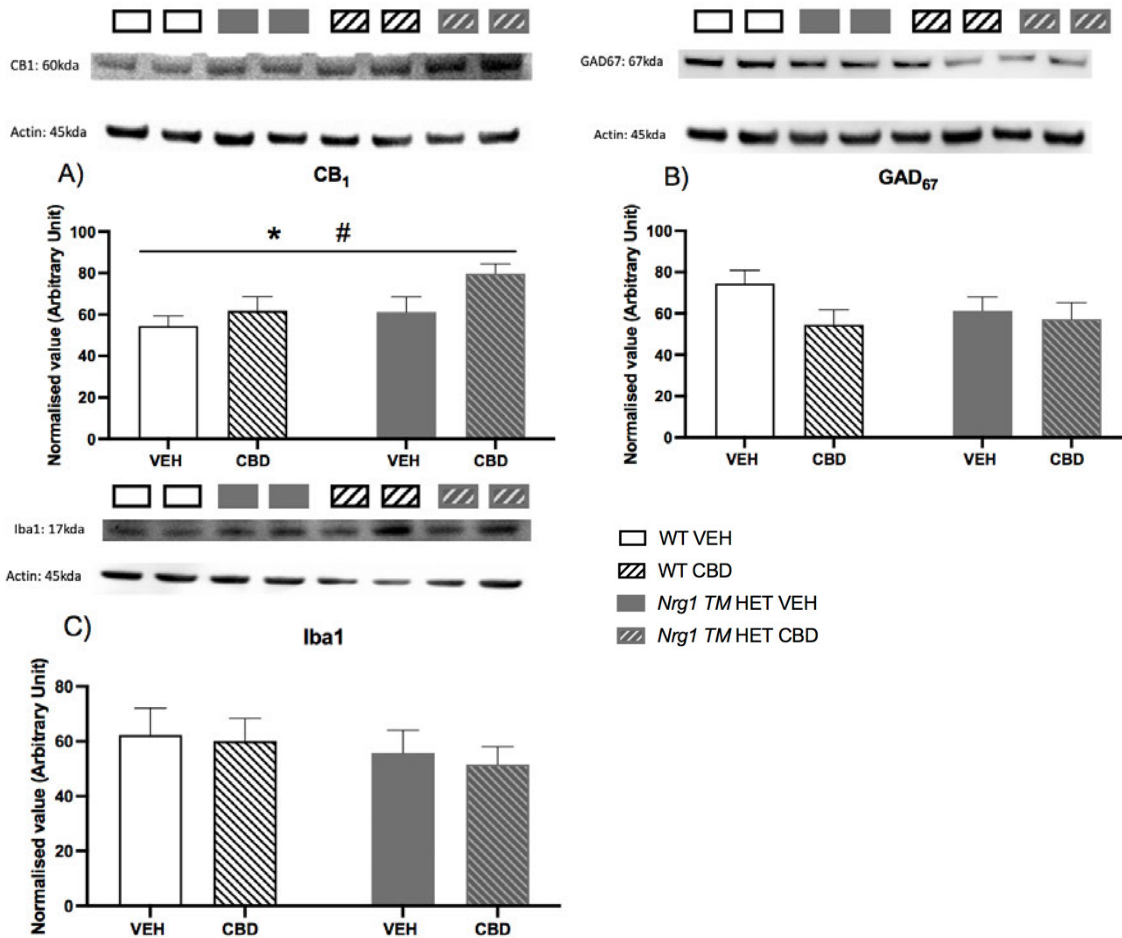

Fig. 5.9: Protein levels in the hippocampus after adolescent CBD treatment: normalized value (arbitrary unit) expressed as mean ± SEM for either wild type-like (WT) or *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET) mice treated chronically with either vehicle (VEH) or cannabidiol (CBD), then later vehicle or Δ^9-tetrahydrocannabinol (THC). Main effects of 'CBD' treatment indicated with # $p < 0.05$, and main effects of 'genotype' treatment indicated with * $p < 0.05$.

5.4 Discussion

Adolescence encapsulates an important neural 'growth spurt' where individuals are sensitive to environmental stressors such as drugs or stress, which can damage emerging neural pathways (107). Chronic exposure to a neuroprotective and low side-effect profile drug could therefore limit the development of some of the later pathology in schizophrenia. We examined this question by investigating persistent effects of chronic adolescent CBD treatment in a genetic mouse model of schizophrenia risk.

In this study, very few schizophrenia-relevant behaviours were evident in *Nrg1 TM* HET mice. *Nrg1 TM* HET males showed increased small motor movements in the OF, as well as a trend for reduced ASR in PPI, a finding that has been reported previously (177, 301). When challenged with an acute low dose of THC, *Nrg1 TM* HET mice showed increased behavioural susceptibility to THC: THC significantly reduced distance travelled, and tended to reduce exploration (*rearing*) in *Nrg1* mutants compared to control mice. Other than these behavioural differences, *Nrg1* mutants did not show any of the previously described behavioural phenotypes described in Chapter 3. The hyperlocomotive phenotype found in the previous two experiments outlined in chapters 3 and 4 and reported previously (177, 182) was not present in VEH-treated mutants in this study. It was interesting that this study did not detect the previously published *Nrg1* male phenotype, as male *Nrg1* mutants have previously been reported to exhibit hyperlocomotion in the OF by 4 months of age, as well as social interaction deficits, and sometimes deficits in startle and PPI (177, 181, 183, 300) . This will be further discussed in Chapter 6 in comparison to previous literature and other chapter findings in this work, as schizophrenia-relevant behaviours in this model have been found to be sometimes elusive depending on the experimental setting (186, 190, 301).

CB_1 receptor protein levels were increased in *Nrg1 TM* HET animals in the current study. While no hyperlocomotive phenotype was found, it may be that increased

endocannabinoid activity by CBD paired with increased CB_1 in the hippocampus at this age may have masked the hyperlocomotor phenotype of *Nrg1 TM* HET mice, as an increase in CB_1 receptor agonism can result in decreased locomotion (376). There have been no changes to hippocampal CB_1 mRNA receptor expression from postnatal day 7-161 in *Nrg1 TM* HET mice (377), and CB_1 binding density is unaffected in *Nrg1* mutants in several brain regions including the hippocampus at 14 and 20 weeks of age (though a trend for increased CB_1 in *Nrg1* mutants independent of age was found for the substantia nigra) (179). CB_1 binding in adult male *Nrg1 TM* HET mice was unchanged both after both VEH and 50-100 mg/kg CBD in several brain regions including the hippocampus (190). It is worth noting that changes to binding may not reflect changes to overall receptor availability, and that all of the studies above only measured one of these factors (i.e. binding or protein expression), as did the current study. This study is the first to find alteration to these receptors in *Nrg1 TM* HET mutants in adulthood, following an adolescent treatment regime. Considering there were few behavioural impairments in *Nrg1* mutants in this study to relate the increased CB_1 levels to, it is possible that some factors such as injection stress during adolescence may affect endocannabinoid system regulation in these mice (378). CB_1 receptor changes may not necessarily be found in the absence of stressors (e.g. (377)), and may not develop immediately after injection stress occurred (e.g. (185)).

Chronic adolescent CBD did not alleviate the schizophrenia-relevant behavioural phenotype of adult *Nrg1 TM* HET animals but had long-term effects on the behaviours of both adult WT and *Nrg1 TM* HET mice. CBD caused a long-term deficit in *rearing* behaviour in the OF in WT mice but not mutants, suggesting persistent effects of adolescent CBD on exploration behaviour later in adulthood. Chronic adolescent CBD also decreased anxiety-like behaviour in the OF in all mice, suggesting that neurochemical changes caused by adolescent CBD may drive anxiolytic-like behaviour in adulthood. CBD has shown acute anxiolytic potential previously (379-382), and even chronic CBD at a higher dose (50 mg/kg) decreased

anxiety in adult male C57BL/6JArc mice when tested during CBD administration (184). Importantly, the present data suggests that anxiolytic effects of CBD can be long-lasting and not limited to effects during CBD treatment. Indeed, it seems the anxiolytic effects of CBD can persist for several months after treatment, as CBD had not been administered since adolescence which was 3 months before testing. This is a novel finding as other studies have not investigated the anxiolytic potential of CBD following an extended washout period. This is also disease-relevant, as anxiety has a strong co-morbidity with schizophrenia and is often overlooked when evaluating new treatment candidates (383). The findings suggesting a potential role for adolescent CBD in reducing anxiety-related behaviours in later life.

Interestingly, frequencies but not duration of social interaction behaviours were reduced in all groups in adulthood by adolescent CBD treatment in the first SI test. This is similar to a recent finding, where chronic adolescent CBD (10 mg/kg) reduced social interaction duration in healthy female rats, when rats were tested during CBD treatment (278). However, previous work from our laboratory found chronic 50 mg/kg CBD in adulthood selectively increased social behaviours during treatment course in *Nrg1 TM* HET mice (190), and another recent study showed chronic adolescent CBD compared to VEH ameliorated social interaction deficits during treatment course in male Poly I:C-treated rats (276). It is possible that age of administration and washout period play a part in how CBD affects social behaviours, as the studies cited above did not investigate persistent effects of adolescent CBD and did not use an extended washout period between treatment and testing. Interestingly, in the THC test battery adolescent CBD treatment decreased social behaviours in *Nrg1* mutants only, suggesting this genotype may be more sensitive to the social suppressant effects of adolescent CBD treatment. This difference in results between tests could be due to retest familiarity (384), whereby animals may be less stressed as in the second battery they have previously been familiarised with equipment.

It is possible that some behavioural effects of CBD found may relate to neurochemical changes found in this study. CBD significantly increased levels of CB_1 receptors in the hippocampus compared to VEH-treated mice of both genotypes, and under VEH-treatment these receptors are already upregulated in adult *Nrg1* mutant animals in the current study. The increase in CB_1 levels by CBD could alter anxiety-like and social behaviours. Indeed, other studies have found that an increase in CB_1 receptor activation in the hippocampus of rats has anxiolytic effects in the elevated plus maze (385, 386). Social deficits have also been related to increased expression of CB_1 receptors in the PFC (387) and activation of CB_1 receptors in the ventral hippocampus (388). Research to date has provided mixed findings regarding the effects of chronic CBD treatment on CB_1 receptor expression in the brain, as repeated CBD treatment was associated with increased CB_1 receptor expression in the hippocampus in one study (389) but not another (190). Furthermore, chronic adolescent CBD rescues Poly I:C-induced deficits in CB_1 receptor binding in the PFC of male adolescent rats while not affecting controls (277), and conversely reducing PFC CB_1 receptor binding in healthy female rats (278). Binding in these two Osborne et al. (2019) studies was measured in late adolescence/early adulthood, immediately after CBD cessation, and it is unknown what long-term effects adolescent CBD had on CB_1 receptor expression. Interestingly, elevated CB_1 receptor binding has been observed in multiple brain regions of patients with schizophrenia, including the hippocampus (349), and increased CB_1 activity has been associated with disrupted sensory gating and aberrant schizophrenia-relevant neuronal oscillations (390). This suggests that CB_1 receptor expression in both the PFC and hippocampus may be involved in the anxiolytic and social behaviour effects of CBD in the current study, as animals tested showed alterations in both factors. Due to the limited scope of the molecular analysis in the current study, we can only imply possible involvement of this system in the results found.

It was hypothesised that adolescent CBD may modify behavioural responses to an acute THC challenge in adulthood in *Nrg1* mutant mice; however, this effect was not observed. Acute THC reduced distance, exploration and anxiety in the OF regardless of genotype, and also inhibited selected social behaviours in all mice, replicating previous findings using acute THC in mice (262, 317, 359). In *Nrg1* mutants, THC also had more of a locomotor suppressant effect than in WT mice, replicating previous findings (186) and Chapter 3 that these animals are more sensitive to THC-induced locomotor suppression. Prior adolescent treatment with CBD enhanced the startle-reducing effects of THC in *Nrg1 TM* HET mice only, with CBD and THC-treated *Nrg1* mutants displaying lower startle at 100 dB compared to VEH and THC-treated animals of the same genotype. The impact of adolescent CBD on later THC effects has not been explored or reported previously, and this result represents a potential risk that adolescent CBD may potentiate some effects of later THC. This interaction requires further exploration in order to understand the mechanism of CBD's earlier action during brain development, and how this affects THC-relevant pathways. This will be further discussed in Chapter 6.

The modulation of CB_1 receptors may be a potential mechanism by which CBD modifies THC effects. The expression of CB_1 receptors affects how much THC can bind, and the upregulation of hippocampal CB_1 receptors in the current study could be the mechanism by which the behavioural effects of THC are increased in *Nrg1* mutants, as more binding sites are available for THC. In the current study, *Nrg1* mutants showed increased CB_1 expression under VEH treatment, suggesting they may have elevated potential for THC binding in the absence of CBD treatment. While this is the case, functionality and accessibility of these receptors was not assessed in the current study, meaning that upregulation on its own may not be responsible for behavioural changes seen, and more investigations into long-term alterations by CBD in this system are pertinent.

The current study did not find that chronic CBD reversed the *Nrg1* adult behavioural phenotype; however, as the schizophrenia-relevant phenotype of *Nrg1 TM* HET mice was not prominent in this study, it was difficult to detect ameliorative effects of CBD. Importantly, chronic CBD potentiated the effects of THC on startle in *Nrg1* mutant mice, and CBD also increased CB_1 receptors in both genotypes. These findings are important as they suggest adolescent CBD may cause long-lasting changes to the endocannabinoid system that may affect later interactions with exogenous cannabinoids. This is relevant for clinical populations, as CBD is currently in clinical trials for schizophrenia (see: ClinicalTrials.gov Identifier: NCT04411225, NCT02088060, NCT02504151, etc.). While CBD shows promise as a potential antipsychotic in adults, more must be known about its interactions with the endocannabinoid system before being used during periods of important neurological development. More research is needed before adolescent CBD treatment is considered for schizophrenia.

Chapter 6. General Discussion

The present thesis aimed to: 1) establish the baseline behavioural schizophrenia-relevant phenotype and neuroinflammatory state of male adult *Nrg1 TM* HET animals; 2) investigate if adolescent CBD could prevent the development of this phenotype and previously reported increased susceptibility to acute THC in *Nrg1 TM* HET mice; and 3) investigate whether adolescent CBD would have long-term effects on the adult behavioural phenotype of *Nrg1* mice and also reduce sensitivity to THC in later adulthood.

In Chapter 3, at 5-7 months the behavioural phenotype of *Nrg1 TM* HET mice was found to be hyperlocomotion in the OF, decreased SI, reduced ASR and PPI, and increased sensitivity to the locomotor suppressant effects of low dose acute THC (Table 6.1). These findings are in line with previously published work. While most characteristics of this behavioural phenotype were present in early adulthood at 2-3 months of age in Chapter 4, very few of these behavioural characteristics were present in mutant animals in later adulthood (5-6 months of age) in Chapter 5 (Table 6.1).

In Chapter 4, chronic adolescent CBD did not prevent the development of the schizophrenia-relevant phenotype in *Nrg1 TM* HET mice during early adulthood, but CBD increased both social behaviour and locomotion (Table 6.2), and reduced levels of GAD_{67} in the hippocampus in all animals. Interestingly, chronic CBD also potentiated the acute THC-mediated decrease in startle habituation during the PPI test.

In Chapter 5, when animals were tested in the absence of CBD in later adulthood, chronic adolescent CBD reduced the frequency of several social behaviours in *Nrg1* mutants during the THC challenge and did not alleviate any aspects of the schizophrenia-relevant phenotype (though these were few) (Table 6.2). Indeed, adolescent CBD increased susceptibility of *Nrg1* mutants to the THC-induced reduction of startle behaviour. CBD also

had an anxiolytic-like effect in the OF in all animals, and reduced CB_1 receptor expression in the hippocampus of adult animals.

The discussion below will outline potential reasons for inconsistencies in the behavioural phenotype of *Nrg1 TM* HET mice across experiments, the lack of effects of adolescent CBD on the schizophrenia-relevant behavioural phenotype of mutant mice, and the more general effects of adolescent CBD on animal behaviour and hippocampal neurochemical profiles.

Behavioural deficits in *Nrg1 TM* HET male mice by age			
	Chapter / Age (months)		
Behavioural Domain	**Chapter 4: 2-3 mo (VEH)**	**Chapter 5: 5-6 mo (VEH)**	**Chapter 3: 5-7 mo (no treatment)**
Locomotion	Hyperlocomotion in OF	No deficits	Hyperlocomotion in OF
Exploration	No deficits	Increased small motor movements	No deficits
Anxiety	Decreased anxiety in OF	No deficits	No deficits
Object recognition	*No data*	*No data*	*No data*
Fear behaviour	Decreased *freezing* in conditioning and context / cue tests	No deficits	No deficits
Fear-associated memory	No deficits	No deficits	No deficits
Spatial memory	*No data*	*No data*	No deficits
Social interaction	Deficits in some social behaviours	No deficits	Deficits in total social interaction time and some social behaviours
Social recognition	*No data*	*No data*	No deficits
Social preference	*No data*	*No data*	No deficits
Startle	Startle deficit at 100 dB	Trend for overall startle deficits deficit at 100 dB	Startle deficit at 100 dB and 120 dB
Sensorimotor gating	Increased PPI	No deficits	PPI deficits at 86 dB, trend for overall PPI deficit
Startle habituation	No deficits	No deficits	No deficits
THC sensitivity	Mild sensitivity to THC in SI	Increased sensitivity to THC for locomotion and exploration	Increased sensitivity to THC for locomotion and exploration

Table 6.1: Behavioural deficits in baseline or vehicle-treated *Nrg1 TM* HET male mice by age: "No data" describes tests that were not conducted or did not work. Red text indicates where deficits are present, green text indicates where behaviour is different to controls but not a deficit, and grey text indicates where no data was available to score deficits. Abbreviations: *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET), open field (OF), social interaction (SI), prepulse inhibition (PPI), decibel (dB), vehicle (VEH), Δ^9-tetrahydrocannabinol (THC), months of age (mo).

Effects of chronic Cannabidiol in *Nrg1 TM* HET and WT mice				
Behavioural Domain	**Genotype / Age (months)**			
	WT 2-3 mo	***Nrg1* 2-3 mo**	**WT 5-6 mo**	***Nrg1* 5-6 mo**
Locomotion	Increased locomotion	Increased locomotion	Reduced locomotion	Reduced locomotion
Exploration	No effects	No effects	Decreased *rearing*	No effects
Anxiety	No effects	No effects	Decreased anxiety-like behaviour	Decreased anxiety-like behaviour
Fear behaviour	No effects	No effects	No effects	No effects
Fear-associated memory	No effects	No effects	No effects	No effects
Social interaction	No effect after 3 weeks / Increased SI time after 6 weeks	No effect after 3 weeks / Increased SI time after 6 weeks	Reduced most SI frequencies	Reduced most SI frequencies, more than WT
Startle	No effects	No effects	No effects	No effects
Sensorimotor gating	No effects	No effects	No effects	No effects
Startle habituation	No effects	No effects	No effects	No effects
THC sensitivity	No effects	Further decreased startle habituation	No effects	Further decreased startle at 100 dB

Table 6.2: Effect of chronic 30 mg/kg cannabidiol (CBD) in WT and *Nrg1 TM* HET male mice: Effects per study at different ages (2-3 versus 5-6 months of age). Red text indicates where deficits are present, green text indicates where behaviour is different to controls but not a deficit, and grey text indicates where no data was available to score deficits. Abbreviations: *Neuregulin 1 transmembrane domain* heterozygous (*Nrg1 TM* HET), wild type-like (WT), social interaction (SI), decibel (dB), months of age (mo), Δ^9-tetrahydrocannabinol (THC).

6.1 *Nrg1 TM* HET mouse behavioural phenotype across studies

The schizophrenia-relevant behaviours found in *Nrg1* mutant mice in Chapter 3 are similar to those described before in this model: locomotor hyperactivity (177, 182, 299), reduced social

interaction (181, 300), and impaired sensorimotor gating (301) (Table 6.1). These behaviours are relevant to schizophrenia symptoms in patients, whereby patients experience sensorimotor gating deficits, psychosis, and social withdrawal (5, 391). In the current study, *Nrg1* mutant males showed this phenotype in Chapter 3 at 5-7 months of age (Table 6.1), similar to previous work demonstrating an age-dependent phenotype (177). In Chapter 4, male mutants already displayed some aspects of this phenotype at 2-3 months of age, after chronic VEH injections, such as hyperactivity in the OF, reduced startle, and a reduction in selected social behaviours but not overall sociability. This contrasts somewhat with previous research where these animals did not show this behavioural phenotype compared to WT animals at 3 months of age (177). However, no chronic VEH injections were administered in those mice (177). This protocol difference is important as hyperactivity has also been detected in adolescent *Nrg1* mutants after chronic VEH treatment (185), although other schizophrenia-relevant behaviours, such as social withdrawal and sensorimotor gating deficits, were not present in the same paradigm, similar to the current study. These findings across studies suggests chronic injections may advance the development of some behaviours in these mutant mice. It is likely that many aspects of this phenotype are age-dependent and become more prominent after adolescence, with robust social deficits and reduced sensorimotor gating and startle being strongest in adulthood after 5 months of age (Table 6.1). For sensorimotor gating in particular, an increased stress response may be involved (169), which will be discussed further below.

PPI was increased in *Nrg1* mutants in early adulthood (i.e. 2-3 months) in Chapter 4, a finding not reported across a number of studies (177, 185) but found in some studies where animals were tested using a variable ISI protocol (184, 301). Alterations to sensorimotor gating of *Nrg1 TM* HET mice appear to be highly dependent on test location and PPI test protocol (301), making this phenotype less robust than other behavioural characteristics of this model. Importantly, PPI can be increased by physical stress in rodents (354), and daily handling and

injections may have been a physical stressor in the current study. While all animals underwent the same levels of stress, it is possible that *Nrg1* mutants' increased sensitivity to stress (169-172, 174) resulted in more pronounced effect of stress on PPI of mutant mice, and not WT animals (but also see (185) where VEH-treated mutants did not show altered PPI from WT animals). It is also possible that sensorimotor gating of *Nrg1 TM* HET mice is more sensitive to environmental impacts such as facility, experimenter, and general cohort differences (such as season testing took place, conditions with the breeding facility, etc.) than some of the other behavioural characteristics of this model such as hyperactivity in the OF, which has been consistently reported across several studies in our laboratory (177, 186, 190). Stress may also explain why *Nrg1* mutants in Chapter 4 showed a more prominent schizophrenia-relevant phenotype (hyperlocomotion, reduced startle at 100 dB, some SI deficits) than those in Chapter 5 (increased small motor movements and reduced *rearing* in the OF, trend for reduced startle, THC sensitivity). This is despite being younger by almost 3 months, as in Chapter 4 these injections were ongoing and in Chapter 5 injections ceased for up to 3 months before behavioural testing. Thus, inconsistencies in the behavioural phenotype of *Nrg1* mutant mice across chapters may be influenced by stress, in line with findings showing that the behavioural phenotype of *Nrg1 TM* HET mutants is sensitive to social defeat and restraint stress (169, 174). Interestingly, however, body weights did not differ between genotypes in any study (data not shown). It should be noted that body weight was monitored for significant changes across time also, and no decrease in body weight was found in any animals treated with either CBD or VEH across time (data not shown). Therefore any stress-related changes in bodyweight were not identified in any condition.

One key difference between Chapter 4 and 5 is the frequency, duration and recency of when animals were handled. In Chapter 4, animals were handled daily before and after behavioural testing, as chronic injections continued during behavioural testing (in the afternoon

post-testing). In contrast, Chapter 5 animals were only handled during chronic injections for 21 days in adolescence, then treatment-related handling ceased for three months. Considering handling prior to behavioural testing can increase locomotor activity and exploration, and decrease anxiety-like behaviours in rats and mice (392, 393), it is possible that the continued treatment-related handling of *Nrg1* mice during the testing period in Chapter 4 may have influenced the increased expression of their behavioural phenotype in comparison to adult animals in Chapter 5. The absence of regular handling for months prior to behavioural testing in Chapter 5 may have led to a diminished phenotype compared with that reported in other chapters as mice may have been acutely stressed with the sudden onset of handling, where mice were handled more extensively in the weeks prior to behavioural testing. Prior chronic injection stress in adolescence could also have impacted this. This implies that standardisation of procedures is important for observing the behavioural phenotype found in Chapter 3 in *Nrg1* mutant mice, in line with what has been previously discussed regarding the PPI phenotype of this mouse model.

Results of Chapter 5 confirmed the increased sensitivity of *Nrg1 TM* HET male mice to acute THC challenge (186) (Table 6.1). This sensitivity was not found in early adulthood in Chapter 4 animals (Table 6.1), suggesting sensitivity to THC in *Nrg1* mutants may be a characteristic of animals later in life, and that some brain changes during neurodevelopment between adolescence and adulthood may enhance susceptibility to behavioural effects of THC. Indeed, hippocampal CB_1 receptor levels were increased in adult *Nrg1* mutant animals of Chapter 5 (179), and as expression of CB_1 receptors affects how much THC can bind (214), greater CB_1 activation by THC could therefore be why *Nrg1* mutants show increased sensitivity to THC. Age-dependent alterations to receptor expression levels in the hippocampus could be why sensitivity to THC was not found in *Nrg1* mutants Chapter 4, as CB_1 receptor expression levels were not yet altered in these animals. It may therefore be pertinent for future studies to

investigate in more detail the role of CB_1 receptor expression in THC sensitivity in *Nrg1* mutants across age.

NMDA receptor development could also be involved with age-related sensitivity to THC in *Nrg1 TM* HET mice. Research using zebrafish has shown that NMDA agonists reverse the stereotypic effects of THC (394), suggesting some effects of THC may be mediated indirectly via NMDA receptor-related pathway alterations. Importantly, the NMDA antagonist MK-801 causes more severe behavioural deficits in adult control C57BL/6J mice (12 months of age) than adolescent animals (1 month of age), and in rats this increased sensitivity to MK-801 is evident after adolescence (45 days of age) (395, 396), suggesting the sensitivity of this system develops with age and does not develop fully until adulthood. In *Nrg1* mice, glutamatergic system alterations may contribute to this age-dependent sensitivity, as these animals show decreased expression levels of NMDA receptors in the thalamus at 20 but not 14 weeks of age, suggesting changes to the glutamatergic system develop with age in *Nrg1* mice (179). Together, this implicates the CB_1 and NMDA receptor systems as potential targets for further investigation in *Nrg1 TM* HET mice, both to understand baseline phenotypes as well as THC-induced behavioural responses.

6.2 Cannabidiol as a preventative for schizophrenia-relevant behaviour

CBD treatment during adolescence was hypothesised to prevent or at least ameliorate the development of schizophrenia-relevant behaviours in adolescent *Nrg1 TM* HET mice in Chapter 4, and perhaps reduce the phenotype in later adulthood in Chapter 5. Chronic CBD treatment did not affect the development of schizophrenia-relevant behaviours at either age in these animals (Table 6.2). It is possible that the null effects of CBD on schizophrenia-relevant behaviour is because there is minimal interaction with systems that regulate behavioural deficits in adult *Nrg1* mice.

In adult *Nrg1* mutants, previous work from our laboratory found that chronic CBD at 1, 50 and 100 mg/kg after 13 days had no effects on a hyperlocomotive phenotype, but at 1 and 100 mg/kg did act as an anxiolytic in WT mice (190). Furthermore, 19 days of 50 and 100 mg/kg CBD treatment increased selected social behaviours in both WT and *Nrg1* mutants, some more in *Nrg1* mice (190), suggesting positive effects of chronic CBD at these doses on social domains. In previously published work from another laboratory investigating chronic CBD during adolescence, 10 mg/kg CBD twice daily for 21 days prevented the development of prenatal Poly I:C-induced behavioural deficits in rats including social interaction deficits as well as object recognition and working memory impairments (276). The rat Poly I:C model of schizophrenia is however that of schizophrenia development and not genetic predisposition, and presents differently to the *Nrg1 TM* HET mouse model due to differences in both pathological and behavioural factors (275, 397). Chronic CBD also attenuated Poly I:C-induced deficits in CB_1 receptor binding in the PFC and GAD_{67} levels in the hippocampus (277). In poly I:C rats, the CBD-induced increase in GAD_{67} in the hippocampus may be at least partly responsible for the increase in SI in animals, as decreased GAD_{67} levels in the PFC and hippocampus are associated with social interaction deficits (367, 368). CBD did not affect NMDA receptors in either region in the same study (277). The effects of chronic CBD on social interaction behaviours may differ strongly depending on the age of animals, and the model used, and need to further be explored in a schizophrenia-relevant context.

Perhaps the reason CBD did not ameliorate deficits of *Nrg1* mutants in the current study is because the mechanism causing schizophrenia-relevant deficits in these animals is unaffected by chronic CBD treatment. Hyperlocomotion can be linked to NMDA hypofunction in several regions including the PFC, as NMDA antagonists can trigger this behaviour (366, 398), and is also linked to a lack of dopamine transporter activity resulting in increased extracellular dopamine and decreased dopamine receptors (399). Sensorimotor deficits can also

be related to NMDA hypofunction in multiple brain regions (400, 401), and *Nrg1* mutants show decreased NMDA receptors in the thalamus at 20 weeks and reduced D_2 receptors in the striatum (179). NMDA receptor hypofunction in the thalamus impairs cortico-thalamo-cortical connectivity and in turn disrupts sensorimotor pathways (402). It is possible that CBD alters the glutamatergic system by facilitating CB_1 receptor-mediated decreases in glutamate release in regions related to these deficits (403), or perhaps via crosstalk between CB_1 receptors and D_2 receptors (404). Sensorimotor deficits and hyperlocomotion may therefore not be affected by CBD if chronic treatment is not altering NMDA binding or receptor levels (190, 277). Deficits in *Nrg1* mice may therefore not be attenuated by adolescent CBD for this reason. Alterations to dopamine were not explored in any chronic CBD study cited and therefore effects on these pathways remain unknown. NMDA hypofunction is however linked to social interaction deficits also (400) and may be the cause of reduced social interaction of adult *Nrg1* mice described in Chapter 5, while in the Poly I:C study above SI deficits and their attenuation by CBD may occur via increases GAD_{67} levels, which are reduced in Poly I:C rats (276, 277). In both chapters, some aspects of the behavioural phenotype were also not detected, which would limit the ability of CBD to minimise these.

CBD had long-term effects on social and anxiety-like behaviours in both mutant and WT mice, and this discussion will focus on these due to the social withdrawal evident in schizophrenia patients, and high rates of comorbid anxiety disorders in patients (383). Chronic adolescent CBD treatment had divergent effects on social behaviour depending on at which age animals were tested, and how long they had been treated with CBD (Table 6.2). While CBD increased sociability in young adult mice during chronic treatment (Chapter 4), this effect was not present in later adulthood and CBD even reduced social behaviours (Chapter 5) (Table 6.2). Effects of CBD on social behaviour are not likely to be associated with anxiolytic-like effects of CBD, as in Chapter 4, CBD did not exert anxiolytic-like effects in the OF, and in

Chapter 5, CBD-treated animals showed less anxiety-like behaviour but also less social behaviour (discussed further below). Effects of chronic adolescent CBD on social interaction have been explored previously, with mixed results. Previous studies have found doses from 10 – 100 mg/kg can increase social interaction in male Poly I:C rats and *Nrg1* mutant mice, as well as WT mice, during treatment course (190, 276), however has the opposite effects on female Poly I:C rats (278). Interestingly, these effects were found during the chronic treatment course, much like in Chapter 4. Concurrent treatment of CBD during testing male animals may account for the difference between CBD being beneficial or detrimental to social behaviours in the current study. It is possible that pro-social effects only occur during CBD treatment but not after an extended washout period, and it is unknown whether CBD is fully metabolised when this effect occurs.

The mechanism by which CBD increases social behaviours in Chapter 4 may be related to the GABAergic system. Increased binding at the $GABA_A$ α2 receptor subtype may be involved in this effect, as CBD is known to bind to this part of the receptor as a positive allosteric modulator (405) and activity at this site has been linked with anxiolytic-like and socio-positive effects in rodents (406, 407). Indeed, the α2 subtype has been shown to be key for the pro-social and anti-anxiety effects of other typical anxiolytic drugs (408). Interestingly, there is a uniformly high concentration of this subtype of receptor in the rat hippocampus from birth to later adulthood (409). Alternatively, it is also possible that a short-term effect of CBD may be responsible for the increase in SI seen as described in Chapter 4. CBD is an antagonist of G protein-coupled receptor 55 (GPR55), a cannabinoid receptor found in the CNS (410). Increased levels of GPR55 receptors have been found in the hippocampus of a rodent model of Rett syndrome, which is categorised by cognitive, social, and motor deficits (411). Furthermore, Cannabidivarin, another cannabinoid from the cannabis plant and also a GPR55 antagonist, reverses social deficits in the same Rett syndrome rat model (411). Thus, it is

possible that this elevation of GPR55 in the hippocampus contributes to some aspects of these social deficits. Indeed, other research indicates that GPR55 agonists administered in the ventral hippocampus strongly disrupt social interaction and social recognition memory (412), suggesting that CBD-induced alterations to GPR55 receptor levels could be at least partially responsible for some behaviours affected by CBD treatment. If CBD is binding to these receptors as an antagonist, this may be how CBD increases social interaction behaviours in Chapter 4 animals. That this effect was not found after a long washout in adult animals could also be explained by the fact that CBD may have caused altered GPR55 receptor sensitivity or receptor upregulation following chronic administration, or conversely has no effects on plasticity and changes in this receptor expression. No studies have yet looked at the regulation of GPR55 receptors in the brains of individuals with schizophrenia or preclinical models of the disorder. This could be a pertinent receptor to analyse in future studies in terms of the CBD effects on behaviour found in the current study.

While CBD treatment during adolescence may not exert beneficial effects on social behaviours in later adulthood (Chapter 5), it did increase distance ratio in the OF in all animals, demonstrating some long-term anxiolytic properties. The acute anxiolytic-like effects of CBD in rodents have been well documented in doses from 1 to 100 mg/kg, including at 30 mg/kg ((381, 382); reviews: (379, 380)). However, it appears that no studies have investigated whether adolescent CBD can modulate anxiety-like behaviours long-term, i.e. following an extended washout period. To date, only a limited number of studies investigated CBD effects on anxiety domains after a short washout period (<24 h) (190, 276, 278). Thus, CBD's potential as a long-term anti-anxiety preventative treatment is relatively unknown and this is the first study providing some insights into this field.

CBD's anxiolytic-like effects in Chapter 5 may be related to the increase in hippocampal CB_1 receptors following chronic CBD. The increase in hippocampal CB_1 receptor

protein levels corresponds with previous reports: repeated CBD treatment increases hippocampal CB_1 receptor expression in early adult mice (PND 50-60) at 20 mg/kg (389) and increases CB_1 receptor binding in the adolescent rat PFC at 10 mg/kg (277). Importantly, increased CB_1 receptor activation in the hippocampus decreases anxiety-related behaviours (385, 386), and this may explain the decreased anxiety-like behaviour of all CBD-treated animals described in Chapter 5. While increased expression does not necessarily predict increased activation, it is possible that these changes to CB_1 receptor profiles regardless of genotype may have long-lasting effects on anxiety.

It is clear that CBD does not prevent the onset of the *Nrg1* mutant behavioural phenotype. Perhaps a lack of preventative effects of CBD is due to animals desensitising to this dose over the period of administration. Indeed, tolerance to cannabinoids such as THC has been demonstrated previously in mice, who display increased tolerance to 10 mg/kg of THC after only 6.5 days of treatment, and 13 injections (413). While in the Chapter 5 study, tolerance may not factor into changes seen in later adulthood after months of washout, in Chapter 4 an increased tolerance to CBD may be why few effects were seen. While this is the case, tolerance to CBD has not yet been explored in the field, and it is unknown whether animals desensitise to the compound's effects over a time course of three weeks. It should be considered, however, that other studies have demonstrated effects of CBD on cognition in mice after weeks of treatment (335, 414), which questions whether tolerance to CBD is built up in a similar manner to THC.

6.3 Chronic cannabidiol and later acute THC

Experiments outlined in Chapters 4 and 5 did not reveal any potential of adolescent chronic CBD to limit behavioural responses to acute THC, either in early adulthood during concurrent treatment (Chapter 4), or in later adulthood after a three-month washout period (Chapter 5).

Indeed, at both ages CBD potentiated the impairment of startle habituation and ASR by THC in *Nrg1 TM* HET animals, but not WT-like controls, suggesting *Nrg1 TM* HET mice are more susceptible to the effects of THC on startle behaviour after chronic CBD. Preclinical and clinical studies on this topic have produced contradictory results, suggesting that concurrent treatment with CBD either can limit the psychoactive effects of THC (248, 258), or potentiate the behavioural effects of THC if administered concurrently or shortly before THC challenge (261, 415). There have been no studies to date investigating whether CBD modifies the effects of THC if administered chronically in advance. Clinically, this is an important question as CBD may alter endocannabinoid pathways during adolescence in a way that either blocks later effects of THC or potentiates them. Considering cannabis use is a risk factor for schizophrenia development (160), whether prior or concurrent CBD could minimise this risk by reducing the effects of THC is an important question therapeutically. As CBD is also considered for treatment of other disorders such as stroke, drug use disorders, seizure disorders, and anxiety disorders (234, 237, 238, 337, 379, 380), there is further need for understanding its long-term effects on the endocannabinoid system. In the current work, while deficits to startle habituation and startle were seen in early adult and later adult *Nrg1* mutants respectively, CBD did not potentiate or offset any other THC-induced changes to schizophrenia-relevant behaviour in either *Nrg1* mutant or control mice. This implies that any pathways altered by chronic CBD do not affect the function of acute THC outside of startle, and also implicates *Nrg1* as a modulating factor that affects the ability of THC to reduce startle, likely via alterations to the receptor systems discussed. CBD-mediated changes to receptor levels in the endocannabinoid system may increase susceptibility to some aspects of THC even in later life where *Nrg1* mutation is present, possibly due to already-present alterations in the endocannabinoid system from this

mutation. The influence of risk mutations must be factored in when considering adolescent CBD as a potential treatment for schizophrenia.

There are several mechanisms which may underlie the way prior chronic CBD can affect later THC exposure. CBD may be acting on cannabinoid pathways later used by THC, and while when concurrently administered less THC may bind where CBD is present (260), this may not be the case when administered apart. It is unknown which components of the endocannabinoid pathway are involved in the long-term effects of CBD on later THC exposure, but it may involve CBD's low affinity for CB_1 receptors, CBD's metabolic pathway, or other indirect mechanisms involving allosteric modulation of GABA receptors (416). THC binding to CB_1 receptors does however limit the release of inhibitory GABA (417). An increase in CB_1 receptors in later adulthood in the *Nrg1* mutant hippocampus post-CBD may be the mechanism by which CBD potentiates the startle-reducing effects of THC, where inhibition in some pathways is reduced by the resulting drop in GABA release caused by increased CB_1 activity. It could also be that an inhibition of glutamate release in startle-relevant regions such as the thalamus impairs startle (372), where *Nrg1* animals have prior imbalances in NMDA receptors in the thalamus at 20 weeks (179). It is possible that these alterations to glutamatergic pathways increase sensitivity to the effects of CBD on regions involved in ASR habituation, and THC exposure can exacerbate this. While this is the case, more markers and brain regions should be analysed in order to understand the connection between prior CBD and later THC exposure.

6.4 Limitations

Due to time constraints and number of animals to be tested, the current study was limited to using only one dose of CBD rather than employing a dose range. However, the dose chosen (30 mg/kg) was based on several other studies showing CBD reverses schizophrenia-relevant behaviours in rodent models of schizophrenia (10 mg/kg (276, 278), 30 & 60 mg/kg (267), 1,

50 & 100 mg/kg (*Nrg1 TM* HET mice (190)), and was in the mid-range of many of these studies. To investigate two different ages and the persistent effects of CBD in *Nrg1 TM* HET mice, it was necessary to be able to compare the results of the studies using the same dose. Time constraints also meant that only male animals could be investigated in this context; however, females *Nrg1* mutant mice do not show a strong schizophrenia-relevant phenotype and are not as sensitive to THC as male mice (184, 188) and were therefore not included in the experimental design.

The decision to continue administration of CBD during behavioural testing for the cohorts tested during adolescence may have resulted in data less comparable between studies. However, this decision was made in order to compare whether CBD had short-term effects on the development of the phenotype in late adolescence/early adulthood where no washout was present (Chapter 4), and then separately whether adolescent CBD had long-term effects that affected the phenotype in later adulthood (Chapter 5). This meant that CBD administration was recent (within 24 h) of each test carried out in young adult animals of Chapter 4. To control for direct interactions between CBD and THC in Chapter 4, CBD was administered ~20 h before THC, and considering the half-life of CBD is 280 min (4.6 h) for blood and 289 min (4.8 h) for brain, it is unlikely CBD would have directly interacted with THC (362).

Finally, the administration route (i.p. injections) used in the study may have altered the behavioural phenotype of *Nrg1* mutants in adolescence (Chapter 4 and 5 and see also (185)), and can cause stress for animals (418). Stress during adolescence is a risk factor for schizophrenia development (332), and can alter the behavioural phenotype of *Nrg1* mutants, as can adult restraint stress (169, 170, 173). This method of delivery may have potentiated some behavioural findings, while masking others, as discussed above. This limitation could be addressed using CBD mixed into rodent food; however, this technique was not yet finalised in our laboratory by the time these studies began. Therapeutically, pharmacokinetics would also

be different between these administration techniques, as mice would be consuming food in smaller amounts throughout the day and not on a single occasion. This is not how CBD would be administered in a patient capacity as CBD would likely be in a form taken once or more daily, and not in increments. Previous work in our laboratory has also used gel pellets as an administration route for long-term CBD administration, so this would be another possibility and would allow dosing across long-term experiments (335).

6.5 Future directions

This study provided an important background for future research evaluating CBD as a preventative drug and provided valuable insights into long-term changes made by CBD that could affect behavioural domains and susceptibility to acute THC. These changes invite further investigation to understand the level at which CBD and THC influence the endocannabinoid pathway and GABAergic/glutamatergic pathways, as well as focusing on different doses and timing in future chronic CBD treatment studies in order to explore the therapeutic effects of CBD in a range of protocols.

Future research could also focus on the long-term effects of CBD in other models of schizophrenia and neuropsychiatric disorders, as well as neurogenerative disorders. CBD used in a preventative setting may be applicable to other pathologies, as has been shown for Alzheimer's disease (335), and could also be employed at different doses or over different time periods to investigate preventative effects.

6.6 Overall conclusion

The *Nrg1 TM* HET mouse model of schizophrenia requires strict standardisation to display a reliable schizophrenia-relevant phenotype. CBD may not be an appropriate preventative drug treatment if used chronically during adolescence, as there may be some long-term

consequences that affect later behaviour in adulthood, and it was not effective for treating a schizophrenia-relevant phenotype. Cannabis use could have more severe consequences in individuals with an *NRG1* mutation if those had been exposed to CBD during adolescence. Chronic CBD during adolescence can change behaviour both during treatment and later on in life, likely due to changes to the endocannabinoid and GABAergic systems whose relationship to these cannabinoids and their functions currently remains unknown.

Chapter 7. References

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