Behavioural and molecular characterisation of mice haploinsufficient for *Map2k7*, a schizophrenia risk gene

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Abstract

Schizophrenia is a serious psychiatric disorder characterised by a breakdown in thought, emotion and perception, which leads to alterations of normal behaviour and feelings, a withdrawal from reality and an impression of mental defragmentation. Of the positive, negative and cognitive symptoms, the positive symptoms are perhaps the most striking. However, it is the severity of cognitive deficits that are most closely associated with a patients' functional outcome in the long-term. Despite this, the successful treatment of the cognitive deficits has been met with difficulty, partly due to a lack of suitable animal models. There is an urgent need for animal models with appropriate face, construct and predictive validity for schizophrenia so that improved drug targets can be identified, and new drugs tested.

In 2012, Winchester *et al*. discovered that sequence variations in the *Map2k7* gene were associated with increased risk for schizophrenia, and *Map2k7* mRNA was decreased in the prefrontal cortex of the post mortem brains of patients. The primary aim of this thesis is to behaviourally and molecularly characterise mice which are heterozygous for *Map2k7* (*Map2k7*$^{+/-}$ mice) as a potential animal model of relevance to schizophrenia. Sequence variants in the *Map2k7* gene are moderately common in the population and they almost double the disease risk (OR~1.9); hence, alterations of the *Map2k7* gene in mice represent an ideal basis for an animal model with good construct validity.

The *Map2k7* gene produces the MKK7 protein, a kinase within the stress-activated JNK pathway, and is involved in a diverse range of cellular processes, such as apoptosis, synaptic plasticity and regulation of the immune response. First and foremost, the components of the MKK7/JNK pathway were quantified in *Map2k7*$^{+/-}$ mice and MKK7γ was found to be significantly decreased in the prefrontal cortex compared to their wildtype (WT) littermates, a highly disrupted brain region in patients with schizophrenia.

Map2k7$^{+/-}$ mice also exhibited behavioural phenotypes relevant to schizophrenia: hyperactivity in the open field and attentional dysfunction. Minocycline showed promise in alleviating the attentional deficits and hyperactivity in the open field, but did not influence protein levels of signalling pathway components. *Map2k7*$^{+/-}$

mice did not show a decrease in sensorimotor gating as many patients do; however, they exhibited signs of altered response to amphetamine administration just prior to testing of sensorimotor gating, compared to WT mice.

Decision-making abilities were also investigated: *Map2k7*$^{+/-}$ mice showed normal learning and performance of the rodent gambling task. Additionally, all mice were able to alter their choice pattern to be more optimal when the task contingencies were subtly switched, which was the first time this has been shown in mice in the touchscreen apparatus. However, when the task demands were altered such that 'punishment' no longer featured as prominently, *Map2k7*$^{+/-}$ mice showed huge difficulty compared to their WT littermates in shifting their choice pattern to be more optimal, suggesting they have a deficit in aspects of cognitive flexibility.

Finally, *Map2k7*$^{+/-}$ mice were investigated as a gene x environment interaction model, by injecting pregnant dams with Poly I:C and examining the resultant immune response in maternal serum and embryonic brain. *Map2k7*$^{+/-}$ dams exhibited an altered immune response to Poly I:C compared to WT dams; however, future experiments will be required to confirm whether this altered cytokine response is also present in embryonic brain.

Overall, *Map2k7*$^{+/-}$ mice show utility for dissecting the cognitive deficits and some aspects of the positive symptoms of schizophrenia that could be targeted by novel compounds. This would be aimed at restoring the function of the MKK7/JNK pathway. Further molecular and behavioural characterisation will be required, particularly into the potential gene x environment interaction model. Although no mouse model can recapitulate the full symptom spectrum of a human neuropsychiatric disorder, *Map2k7*$^{+/-}$ mice exhibit an interesting accumulation of phenotypic abnormalities relevant to schizophrenia.

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List of Abbreviations

5-CCPT	5-choice continuous performance test
5-CSRTT	5-choice serial reaction time task
ADHD	Attention-deficit hyperactivity disorder
Adol	Adolescence
Amph	Amphetamine
ANOVA	Analysis of variance
AP-1	Activator protein 1
ASK1	Apoptosis signal-regulating kinase 1
ASR	Acoustic startle response
ATF2	Activating transcriptor protein 2
BCA	Bicinchoninic acid
BPRS	Brief Psychiatric Rating Scale
BSA	Bovine serum albumin
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C
CCL-	Chemokine (C-C motif) ligand
cFLIP	Cellular FLICE (FADD-like IL-1ß-converting enzyme)-inhibitory protein
cKO	Conditional knockout
CNTRICS	Cognitive Neuroscience Treatment Research to Improve Cognitive in Schizophrenia
CNV	Copy number variant
COOH	C-terminus
CPT	Continuous performance task
CSF	Cerebrospinal fluid
CSFs	Colony-stimulating factors
CST	Cell Signalling Technologies
CV	Coefficient of variation
CXCL-	Chemokine (C-X-C motif) ligand
D (or Drd)1,2,3,4	Dopamine receptors 1, 2, 3, 4
DA	Dopamine
D-amphetamine	Dextroamphetamine
DAT	Dopamine transporter
dH_2O	Distilled water
DISC1	Disrupted-in-schizophrenia 1
DLK	Dual leucine zipper kinase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DREADDs	Designer receptors exclusively activated by designer drugs
DSM	Diagnostic and Statistical Manual of Mental Disorders
E	Embryonic day
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
ENU	N-ethyl-N-nitrosourea
ERK	Extracellular signal related kinase
F1, 2	Forward primer 1, 2
F-DOPA	Fluorodopa
fITI	Fixed inter-trial interval
GABA	Gamma-Aminobutyric acid
GAD67	Glutamic acid decarboxylase-67
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GluN1, 2, 3	Glutamate ionotropic receptor NMDA type subunit 1, 2, 3
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome-wide association study
HC	Hippocampus
HRP	Horseradish peroxidase
HZ	Heterozygous
IFN-β	Interferon-beta
IGT	Iowa gambling task
IIV	Intra-individual variability
IL-	Interleukin-
Indels	Insertions or deletions
IRFs	Interferon regulatory factors
ITI	Inter-trial interval
Jnk 1, 2, 3	c-Jun N-terminal kinase gene 1,2 3
JNK1, 2, 3	c-Jun N-terminal kinase protein 1,2 3
kDa	Kilodalton
KO	Knockout
LH	Limited hold
LLOQ	Lower limit of quantification
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
LTP	Long-term potentiation
LTR	Long terminal repeat
MAM	Methylazoxymethanol acetate
Manip 1, 2	Manipulation 1, 2
Map2k4	Mitogen activated protein kinase kinase 4/7 gene
MAP2K4/7(MKK4/7)	Mitogen activated protein kinase kinase 4/7 protein
MAP3K (MKKK)	Mitogen activated protein kinase kinase kinase
MAPK	Mitogen activated protein kinase
MAPs	Microtubule associated proteins
MATRICS	Measurement and Treatment Research to Improve Cognition in Schizophrenia
MCCB	MATRICS Consensus Cognitive Battery
MEG	Magnetoencephalography
mEPSCs	Miniature excitatory postsynaptic currents

MIA	Maternal immune activation
Mino	Minocycline
Mino 4	Minocycline 4th day treatment
Mino 7	Minocycline 7th day treatment
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine
MLK	Mixed lineage kinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH2	N-terminus
NIH	National Institutes of Health
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NRG1	Neuregulin1
ns	Nonsignificant
Nurr1	Nuclear receptor related 1
OF	Open field
OOR	Out of Range
PAL	Paired associates learning
PANSS	Positive and Negative Syndrome Scale
PBS	Phosphate-buffered saline
pc-Jun	Phospho-cJun
PCP	Phencyclidine
PCR	Polymerase chain reaction
PD	Pairwise discriminiation
PET	Positron emission tomography
PFC	Prefrontal cortex
PGK-neo	Phosphoglycerine Kinase-neomycin
Poly I:C	Polyinosinic:polycytidylic acid
PPI	Prepulse inhibition
PSD-95	Postsynaptic density 95
PV	Parvalbumin
PVDF	Polyvinylidene difluoride
RDoC	Research Domain Criteria
rGT	Rodent Gambling Task
RIP1	receptor interacting protein 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
SD	Stimulus duration
SEM	Standard error of the mean
SHIRPA	SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment
SM	Strawberry milkshake

SNP	Single nucleotide polymorphism
TAB2, 3	TAK1 binding protein
TAK1	MKKK7 complex
TAOK2	Thousand-and-one amino acid kinase 2
TBS	Tris-buffered saline
tc-Jun	Total-cJun
TIR	toll/interleukin 1 resistance/receptor protein
tJNK	Total-JNK
TLR3	Toll-like receptor 3
tMKK4/7	Total MKK4/7
TNF-α	Tumor necrosis factor alpha
TO	Time out
TRAF6	TNF receptor-associated factor 6
TRIF	TIR domain-containing adaptor proteins inducing IFNβ
TRIKA2	TRAF6-regulated IKK activator 2
TTBS	Tris Buffered Saline, with Tween® 20
ULOQ	Upper limit of quantification
UTR	Untranslated region
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
vITI	Variable inter-trial interval
VRKs	Vaccinia-related kinases
vSD	Variable stimulus duration
WT	Wildtype

Declaration of Originality

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: _____

Rebecca Openshaw

Chapter 1 Introduction

1.2 General introduction to schizophrenia

Schizophrenia is a complex neuropsychiatric disorder characterised by disruption in behaviour, thoughts, emotion and perception (Tandon *et al.*, 2013). Approximately 0.7% of the worldwide population will develop schizophrenia in their lifetime, although prevalence varies significantly by geographical location (Goldner *et al.*, 2002; Saha *et al.,* 2005); for example, people in non-Asian countries are nearly four times more likely to develop schizophrenia than people in Asian countries (Goldner *et al.*, 2002). Age of symptom onset varies between patients and has been shown to be modulated by environmental factors (Stepniak *et al.*, 2014), genetic predisposition (Chow *et al.*, 2016), and sex (Aleman *et al.*, 2003). However, evidence suggests that there is not a simple correlation with age of onset of schizophrenia and any of these factors, but rather sex may be a modulator in the way that male age of onset is more influenced by environmental factors whereas female age of onset is more influenced by genetic predisposition (Hilker *et al.*, 2017). However, overall, diagnosis of schizophrenia in males occurs earlier on average compared to females (Forrest & Hay, 1971). Males also have a slightly higher prevalence, with the ratio of male: female diagnoses being 1.4:1 (McGrath *et al.*, 2008), although this observation is not clear-cut: before the age of 30, more males are diagnosed with schizophrenia than females, and after the age of 30 more females are diagnosed than males (Sham *et al.*, 1994). Females have a second "wave" of diagnosis after the age of 40 and, in general, the earlier the onset of schizophrenia the more severe and debilitating the symptoms (Forrest & Hay, 1971; Sham *et al.*, 1994).

Patients with schizophrenia are 2.6 times more likely to die prematurely compared to the general population (McGrath *et al.*, 2008). This is for a number of reasons, such as: cardiovascular or metabolic problems, suicide, or the fact that patients are more likely to engage in high-risk behaviours (McGrath *et al.*, 2008; Saha *et al.*, 2007). Furthermore, patients have only a 30% chance of sustained recovery to a life of good quality in the long-term (defined as 25 years following a psychotic episode); only 20% of patients are employed throughout Europe, and ~20% are homeless (Insel, 2010). There is a clear requirement to produce more effective medication than currently available.

1.3 Symptoms

The behavioural symptoms of schizophrenia were first described by the German psychiatrist Emil Kraepelin in 1887 (Jablensky, 2010). Symptoms usually emerge in adolescence or early adulthood, and they can be grouped into 3 main subtypes: positive, negative and cognitive symptoms (American Psychiatric Association, 2013). Positive symptoms (which "add" to normal functioning) include hallucinations, delusions, psychomotor agitation and disorganised speech. Negative symptoms (or "diminution" of normal functioning) comprise of reduced emotional expression, anhedonia, alogia and lack of motivation; cognitive symptoms consist of deficits in working memory, decision-making, speed of processing, social cognition, attention and executive control (American Psychiatric Association, 2013). Some symptoms can fall into multiple subtypes, such as deficits in prepulse inhibition, which has been described as the "interface between cognitive and positive symptoms", one reason being it is a pre-attentional (i.e. cognitive) process that is ameliorated by antipsychotics (Desbonnet *et al.*, 2009).

Although the positive symptoms are perhaps the most striking, more recently it has been realised that it is the severity of cognitive deficits which are the main determinant of long-term functional outcome for patients with schizophrenia (Lesh *et al.*, 2011; Mishara & Goldberg, 2004). However, the exact set of symptoms, severity, clinical course and response to the treatments currently available varies greatly across individuals (van Os & Kapur, 2009).

1.4 Diagnosis

Despite the evident symptoms and outcomes, the diagnosis of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) is quite difficult. First, other potential diagnoses that have overlapping symptoms must be ruled out (e.g., bipolar disorder, schizoaffective disorder, drug abuse, autism spectrum disorder), and the symptoms must have been present for at least 6 months (Tandon *et al.*, 2013). Additionally, at least two of the following symptoms are to have been existent for the duration of one month or more: delusions, hallucinations, disorganised speech, grossly disorganised or catatonic behaviour, and/or negative symptoms (e.g., affective flattening, alogia, anhedonia; Tandon *et al.*, 2013). Finally, patients must have been functioning in

normal, everyday life (e.g. work, or social situations) significantly worse than they did prior to onset of symptoms, for a prolonged period (Tandon *et al.*, 2013). These diagnostic criteria can take too long and usually lead to distressing circumstances where people are sectioned and/or hospitalised before diagnosis. Additionally, despite the significance of the cognitive symptoms in the lives of patients with schizophrenia, these are not included in diagnosis because they do not sufficiently distinguish between schizophrenia and other, similar disorders. This emphasises the need to substantially increase the neurobiological understanding of schizophrenia not just so that new treatments can be developed, but also so that reliable biological markers, or ways of diagnosing this disorder more quickly, can be produced.

In an attempt to address this issue, new initiatives such as the Research Domain Criteria (RDoC) project, established in 2009 by the National Institute of Mental Health (Cuthbert & Insel, 2013), have developed a classification system for mental disorders that puts more emphasis on symptom domains rather than relying purely on distinct signs and symptoms laid out by the DSM. The main goal of RDoC is to create a more precision-based diagnosis of patients with a psychiatric disorder. The RDoC project aims to incorporate novel information gathered by research into the genetic, behavioural, physiological and neural circuitry underpinnings of domains (or subtypes) of symptoms, rather than aiming to treat a single disorder with one drug. Any one psychiatric disorder will never be characterised by a single symptom and will always involve multiple mechanisms; it is arguably not feasible to attempt to create a drug which will alleviate all the symptoms altogether (Cuthbert & Insel, 2013). This approach may lead to a better understanding of the pathophysiology of multiple disorders by providing a more accurate framework for future research. For example, a mechanism underlying a process such as working memory is disrupted in multiple disorders and it could be hard to decide which disorder to pursue further, from a researcher's perspective. If "working memory" was investigated *per se*, it may have positive implications for all disorders it is connected to. In this way, disorders that have similar or overlapping symptoms can eventually be treated more relevantly. RDoC is currently a work in progress, but more aspects of this new framework may be incorporated into future diagnoses and research plans.

1.5 Neuropathology

In addition to behavioural symptoms, patients with schizophrenia have consistently altered brain structure, function and neurochemical regulation (Keshavan *et al.*, 2011). The brain structural changes in schizophrenia were first noted by post mortem examination of patient's brains and were later confirmed with structural MRI when the technology became available about 35 years ago (see van Os & Kapur (2009) for review). Well-established brain structural changes include: reduction of whole brain volume; reduction of prefrontal cortex (PFC) volume whilst maintaining a similar number of cells (Buchanan *et al.*, 1998); reduction of striatal volume (Keshavan *et al.*, 1998); increased volume of lateral and third ventricles (Andreasen *et al.*, 1982); white matter integrity abnormalities (Boos *et al.*, 2013; de Leeuw *et al.*, 2015; Kubicki *et al.*, 2013; Munoz Maniega *et al.*, 2008) and reduction of grey matter volume of the hippocampus, PFC, thalamus and anterior cingulate cortex (Fitzsimmons *et al.*, 2013; Shenton *et al.*, 2001). In addition to the structural differences in these areas, brain imaging studies (e.g., functional MRI, PET and MEG) of patients taking part in various cognitive tasks have shown that they have functional impairments, in particular, hypofunction in the PFC (Fusar-Poli *et al.*, 2007).

1.5.1 Prefrontal cortex dysfunction in schizophrenia

Clinical, post-mortem and neuroimaging studies show disruption in multiple areas of the brain, and have identified the PFC as a particularly key site of dysfunction in schizophrenia (Arnsten, 2011; Weinberger *et al.*, 1986). The PFC is critical for higher-order cognitive processes and emotional regulation, and can be divided in rodents and humans into several sub regions. In humans, the dorsolateral PFC mainly influences cognition, including attention, executive control and working memory, and the ventromedial PFC controls emotional and motivational regulation (Sigurdsson & Duvarci, 2016). In rodents, the medial PFC corresponds to both the dorsolateral and ventromedial subdivisions of the PFC in humans, and has analogous functions crucial for attention, working memory, short-term associative memory (Benn *et al.*, 2016), attentional set shifting and motivation (reviewed in Sigurdsson & Duvarci, 2016). The human PFC has major reciprocal connections with the mediodorsal thalamus (Pratt *et al.*, 2017) and the hippocampus. Connections from the PFC to the dorsal hippocampus are important for memory retrieval and connections from the ventral hippocampus are important

for working memory, anxiety and learned fear. The nucleus reuniens of the thalamus also has reciprocal connections to the medial PFC, which are thought to play a role in working memory (Sigurdsson & Duvarci, 2016).

Investigations of cellular pathology in the post mortem brains of patients show that there is reduced spine density on deep layer 3 pyramidal neurons of the dorsolateral PFC, reflecting a diminished number of excitatory (glutamatergic) inputs to this area and thereby causing lower activity levels (Glantz & Lewis, 2000). Also, it is well established that the calcium-binding protein parvalbumin (PV) subclass of gamma-amino butyric acid (GABA) neuron markers are reduced in patients with schizophrenia (Gonzalez-Burgos *et al.*, 2010). PV-positive GABAergic neurons provide strong inhibition onto pyramidal cells and also inhibit other GABAergic neurons, including other PV-positive neurons (Gonzalez-Burgos *et al.*, 2010). Their regulated inhibition helps create gamma oscillations that are important for normal cognitive and attentional function (Gonzalez-Burgos *et al.*, 2010). Additionally, decreased levels of mRNA (Akbarian *et al.*, 1995) and protein (Curley *et al.*, 2011) of the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}), a key enzyme involved in the synthesis of GABA, are also consistently found in the PFC of schizophrenia, especially in the axon terminals of PV-containing neurons (Curley *et al.*, 2011); hence, less GABA synthesis occurs in these regions. Patients with schizophrenia also have smaller somal volumes, decreased dendritic arbour size and density onto subtypes of pyramidal cells in the PFC (Hill *et al.*, 2006). As the formation of gamma oscillations are a result of the coordinated action between the inhibitory GABA interneurons and the excitatory pyramidal cells, it would seem reasonable that any alterations in GABA would have a knock-on effect on gamma oscillations. In fact, studies using optogenetic techniques have demonstrated that activity in PV-positive interneurons is essential for driving cortical gamma oscillations in mice (Sohal *et al.*, 2009), and overall reductions in the power and synchrony of gamma oscillations, including in the PFC during cognitive tests and at rest, have been detected in schizophrenia (reviewed in Uhlhaas & Singer, 2010).

Gamma oscillations are involved with social cognition, working memory and attention (Fries *et al.*, 2001; Williams & Boksa, 2010). They are evoked by external stimuli in sensory cortices and exploratory behaviour in the hippocampus, and they precede motor responses in the premotor areas of the cortex (Atallah & Scanziani,

2009). Oscillations synchronise and bind complex brain activity between different brain regions, allowing coordinated activity. With respect to cognition, gamma oscillations move between the PFC, thalamus and hippocampus, improving the efficiency and clarity of transmission of information between these regions (Atallah & Scanziani, 2009). Therefore, disruption of gamma oscillations will have widespread effects and can at least partially explain many of the cognitive deficits seen in schizophrenia, such as attentional deficits, working memory and social cognition (Shin *et al.*, 2011).

Another key feature in the PFC of patients with schizophrenia is that they have reduced mRNA and protein levels of the GluN1 subunit of NMDARs in the dorsolateral PFC (Weickert *et al.*, 2013). NMDARs are composed of four subunits: two obligatory GluN1 subunits, plus two out of the four GluN2 subunits and/or the two GluN3 subunits (Traynelis *et al.*, 2010). The fact that the obligatory subunit is reduced both in mRNA and protein levels suggests that NMDAR function is reduced in the PFC of schizophrenia, thus further contributing to hypofunction and therefore the cognitive deficits seen in schizophrenia (Weickert *et al.*, 2013)

1.5.2 Behavioural deficits as a result of prefrontal cortex dysfunction

Behaviourally, patients with schizophrenia show deficits in several tasks that are reliant on the PFC. These include reversal learning and attentional set shifting as evidenced by their reduced performance in the Wisconsin card sort task and/or the intradimensional/extradimensional attentional set-shifting task (Ceaser *et al.*, 2008; Elliott *et al.*, 1995; Gold *et al.*, 1997), decision-making in the Iowa gambling task (Shurman *et al.*, 2005), working memory in a spatial delayed response task (Mayer & Park, 2012), sustained attention in the 5-choice continuous performance test (5-CCPT; Suwa *et al.*, 2004), and associative memory in paired associates learning (Hutton *et al.*, 1998). Overall, patients with schizophrenia have widespread molecular, structural, physiological and functional deficits in the PFC.

1.6 Hypotheses of Schizophrenia

Many hypotheses attempting to explain the causes of schizophrenia have been formulated. These include: the NMDAR hypofunction hypothesis (reviewed in Snyder & Gao, 2013), the dopamine hypothesis (reviewed in Howes & Kapur, 2009; Meltzer & Stahl, 1976), the neurodevelopmental hypothesis (Weinberger, 1987), the serotonin hypothesis (reviewed in Eggers, 2013) and the microglia hypothesis

(reviewed in Monji *et al.*, 2009), although they are not thought to be mutually exclusive. As an overview of all the hypotheses are beyond the scope of this thesis, only the NMDAR hypofunction and dopamine hypotheses will be described as these are most relevant to the investigations of this thesis.

1.6.1 The NMDA receptor hypofunction hypothesis

The prefrontal NMDAR hypofunction hypothesis of schizophrenia posits that schizophrenia is caused by reduced function of the NMDA class of glutamate receptor (reviewed in Snyder & Gao, 2013). This hypothesis initially came about because of the psychotomimetic effect of the non-competitive NMDAR antagonist phencyclidine (PCP) (Deutsch *et al.*, 1989; Olney & Farber, 1995). Intake of PCP by healthy human subjects produces symptoms almost indistinguishable from an acute psychotic episode seen in patients with schizophrenia, and also exacerbates psychotic symptoms in patients (Luby *et al.*, 1959). Additionally, brain imaging studies have shown that PCP induces hypofrontality in human subjects when taking part in a cognitive task in the same way that occurs in schizophrenic patients (Wu *et al.*, 1991). Investigations into other NMDAR antagonists, such as MK-801 (a potent NMDAR antagonist) and ketamine (a derivative of PCP), which have similar mechanisms of action, provided further evidence for the NMDAR hypofunction hypothesis. Chronic administration of ketamine also produced the cognitive and negative symptoms of schizophrenia (Krystal *et al.*, 1994), and subanaesthetic doses of ketamine reinstated the symptoms of schizophrenia when given to patients who were not experiencing symptoms beforehand (Lahti *et al.*, 1995). Additionally, MK-801 given at low doses to healthy human subjects induce a range of symptoms associated with schizophrenia, including aspects of positive, negative and cognitive symptoms (Nakazawa *et al.*, 2012). These studies suggest that NMDARs at least partially underlie the hypofunction seen in the PFC of schizophrenia patients.

In animal models, long-term administration of NMDAR antagonists reduce PV-positive neurons as seen in schizophrenia patients (Cochran *et al.*, 2003), and this produces disinhibition of the neuronal circuits with subsequent aberrant response, and because the NMDARs are pharmacologically disrupted, the homeostasis is not re-maintained (Lisman *et al.*, 2008). It is thought that this disinhibition contributes towards disruption of the gamma oscillations and participates towards the cognitive symptoms seen in schizophrenia, as described in **Section 1.5.1**

(Gonzalez-Burgos *et al.*, 2010). Moreover, the dysfunction of GABAergic neurons is linked to repeat administration of NMDAR antagonists: the expression of GAD_{67} and PV are decreased in the cortical GABAergic neurons in mice following repeated administration of NMDAR antagonists (Nakazawa *et al.*, 2012). Similarly, chronic intermittent PCP treatment induced prefrontal hypofrontality, reduced levels of PV-positive interneurons and induced deficits in executive function (as measured by the attentional set shifting task; Dawson *et al.*, 2012), which was not reversed by clozapine or haloperidol, consistent with what occurs in human patients (Cochran *et al.*, 2003). Repeated PCP treatment also induced deficits in cognitive processing speed in the 5-choice serial reaction time task (Thomson *et al.*, 2011; see **Chapter 5** for more information on this task). As the rodent models relevant to the NMDAR hypofunction hypothesis of schizophrenia so closely match what occurs in patients with respect to molecular, cellular and behavioural changes, it is hoped that cognitive treatments may be derived from continuing to study the neurobiological mechanisms that occur to cause this.

1.6.2 The dopamine hypothesis of schizophrenia

The dopamine hypothesis has arguably been the dominant theory of the pathogenesis of schizophrenia for around 40 years (reviewed in Howes *et al.*, 2017). In a similar way to the NMDAR hypofunction hypothesis, it originated from pharmacological studies: antipsychotics were found to work by affecting the dopamine system and the clinical efficacy of antipsychotics was discovered to be positively correlated with antagonism of the dopamine D2 receptor (Seeman & Lee, 1975). Furthermore, dopaminergic agonists induce psychosis in healthy humans (Connell, 1957), and some patients display increased psychotic symptoms after acute exposure to psychostimulants at doses that do not induce psychosis in healthy subjects (Lieberman *et al.*, 1987). Direct evidence was then provided by post-mortem studies, which showed that dopamine, its receptors and metabolites, were increased in the striatum of patients with schizophrenia (Howes *et al.*, 2017). Later studies showed that there was an increase of dopamine D2 receptor activity in the subcortical structures but a decrease of dopaminergic D1 receptor activity in the PFC (Desbonnet *et al.*, 2009). Researchers formulated the dopamine hypothesis and now have a clearer explanation for how this may occur, which is described in more detail in the following paragraph (summarised by Howes *et al.*, 2017).

Under normal circumstances, the regulation of striatal dopamine release is well controlled in the dopaminergic mesocorticostriatal system (Howes *et al.*, 2017). Cortical dopamine acts on dopamine D2 receptors (which are inhibitory; Cass & Gerhardt, 1994), to regulate the activity of excitatory glutamatergic projections from the cortex to the midbrain. These projections act on the striatum, so this is one way in which striatal dopamine release is controlled to a suitable level. Additionally, cortical glutamatergic neurons projecting to NMDARs of GABAergic interneurons in the midbrain stimulate GABA release, which inhibits/limits dopaminergic output to the striatum.

In patients with schizophrenia, it has been shown that the mesocorticostriatal control of dopaminergic function is disrupted, ultimately leading to aberrant striatal dopamine synthesis and release. As there is reduced dopaminergic signalling in the PFC of patients with schizophrenia as revealed by PET (Okubo *et al.*, 1997), there is disinhibition and therefore increased glutamatergic activity to the midbrain dopaminergic neurons, which increases striatal dopamine synthesis and release. This occurs together with hypoactive NMDAR signalling from the PFC onto the midbrain GABAergic interneurons, which leads to disinhibition and therefore increased striatal dopaminergic activity (Howes *et al.*, 2017).

Neuroimaging studies support the idea that there is a yin-yang relationship between dopamine in the cortical and subcortical structures: patients with schizophrenia carrying out the Wisconsin Card Sorting Test exhibited decreased PFC blood flow accompanied by an increase in striatal F-DOPA uptake (a marker that reflects dopamine synthesis) and these two processes were tightly coupled (Meyer-Lindenberg *et al.*, 2002). It is thought that dopaminergic hypofunction in the PFC contributes towards the negative symptoms and cognitive deficits, and the hyperfunction in the subcortical regions underlies the positive symptoms (Desbonnet *et al.*, 2009). In fact, the severity of negative symptoms and cognitive deficits correlates with the degree of prefrontal metabolic hypofrontality (Hill *et al.*, 2004; Potkin *et al.*, 2002). Targeting the dopaminergic system in the PFC should treat the negative and cognitive deficits. However, a challenge arises from being able to affect the dopaminergic function here, whilst not affecting the psychotic symptoms arising from the dopaminergic hyperfunction in the subcortical structures, as these are already (albeit with side effects) treated with antipsychotics.

1.7 Treatments for schizophrenia

Antipsychotic drugs are used to alleviate positive symptoms; however, they have little or no efficacy for negative symptoms and cognitive deficits, and cause multiple severe side effects which limit their therapeutic timespan (Leucht *et al.*, 2013). Although the positive symptoms of schizophrenia are perhaps the most striking and noticeable of the symptoms of schizophrenia, the cognitive symptoms are most closely associated with a patients' functional outcome in the long-term (Green, 2006).

1.7.1 Antipsychotics as a treatment for the positive symptoms of schizophrenia

Antipsychotics can be categorised in two groups: the typical or first generation (chlorpromazine, loxapine, haloperidol), and atypical or second-generation antipsychotics (clozapine, olanzapine, sertindole, risperidone) (Insel, 2010). The typical antipsychotics were first developed in the 1950's but it was not until the mid-1970's that their main mechanism of action was found to be dopamine D2 receptor antagonism (Seeman & Lee, 1975; Seeman *et al.*, 1975). However, long-term administration of typical antipsychotics causes extrapyramidal symptoms, such as tardive dyskinesia and/or akathisia, due to chronically decreased levels of dopaminergic signalling in the nigrostriatal pathway (Tandon *et al.*, 2010). Up to 50% of patients stop taking their medication due to the extrapyramidal symptoms and other, intolerable side-effects (for example, metabolic effects and weight gain), lack of efficacy, or other reasons (Haddad *et al.*, 2014). Atypical antipsychotic drugs were then developed in an attempt to improve the side effects and efficacy. They affect dopamine D1, D4, histamine H1, serotonin 5HT2 and acetylcholine muscarinic M1 receptors, as well as the dopamine D2 receptors (Kapur & Mamo, 2003). Although the atypical antipsychotics produce fewer side effects and are safer than typical antipsychotics (Tandon *et al.*, 2010), they did not enhance functional recovery because they still did not address the cognitive deficits and negative symptoms (Hill *et al.*, 2010). A meta-analysis concluded that any improved outcomes were likely due to the improved side effect profile of atypical antipsychotics rather than their specific improvement of cognitive deficits (Crossley *et al.*, 2010). Although the understanding of the neurobiology underpinning positive symptoms has improved, since the generation of atypical antipsychotics little progress has been made in developing new therapeutic

targets for these symptoms in an attempt to eliminate side-effects completely (Buckley & Stahl, 2007; Insel, 2010).

1.7.2 Minocycline as a potential treatment for the cognitive and negative symptoms of schizophrenia

Minocycline, a broad-spectrum, second-generation, tetracycline antibiotic, has been identified as a potential treatment for the cognitive and negative symptoms of schizophrenia due to its anti-inflammatory, neurotrophic, antioxidant and anti-apoptotic properties (Monte *et al.*, 2013). These properties give minocycline a neuroprotective quality, as discovered via animal models when investigating it as a treatment for neurodegenerative conditions such as Huntington's disease, amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (Garrido-Mesa *et al.*, 2013). Additionally, minocycline can readily cross the blood brain barrier, even by multiple routes of administration (Zhang *et al.*, 2007; Zink *et al.*, 2005). It is safe for intake by humans, available for immediate clinical use, and patients can tolerate doses of therapeutic value of minocycline over long periods (6 months was tested) with little-to-no side effects and no interactions with other drugs (Domercq & Matute, 2004). This is particularly important, as discussed above, current drugs that treat the symptoms of schizophrenia are already the cause of multiple unpleasant side effects. Moreover, research is showing clinical potential for the treatment of schizophrenia with minocycline as add-on medication along with anti-psychotics, so it is imperative that interactions of minocycline with antipsychotic drugs do not occur (reviewed in Chaudhry *et al.*, 2012; Levkovitz *et al.*, 2010; Miyamoto *et al.*, 2013; Oya *et al.*, 2014; Zhang & Zhao, 2014).

1.7.2.1 Minocycline in clinical trials

A recent meta-analysis (Xiang *et al.*, 2017) incorporated the results of 8 randomised controlled clinical trials in patients with schizophrenia and overall, minocycline showed significant improvement of negative, positive and overall symptoms, as assessed by the Positive and Negative Syndrome Scale (PANSS) and the Brief Psychiatric Rating Scale (BPRS). Moreover, the number and type of adverse drug reactions reported were similar in the minocycline and placebo group, emphasising that minocycline is safe as well as efficacious. This meta-analysis did not find a significant improvement of cognitive deficits as measured

by the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) Consensus Cognitive Battery (MCCB; MATRICS is described in **Section 1.10**); however, only two of eight of the studies actually included cognitive measurements as part of the study (Levkovitz *et al.*, 2010; Liu *et al.*, 2014). Therefore, although the two studies found improvement of cognitive deficits following minocycline treatment separately (including improved working memory, cognitive shifting, attentional performance and cognitive planning tasks), combined they did not. However, this may be due to the type of statistical model applied in the meta-analysis, or the fact that different research environments cause distinct types of variation in results, so it may not be relevant to compare such a small number of studies with each other.

Another, open label study not included in the meta-analysis showed that atypical antipsychotic administration, plus 300mg/day (quite a high dose) minocycline for 4 weeks significantly improved the PANSS score, which persisted for 4 weeks after cessation of treatment (Miyaoka *et al.*, 2008). Additionally, minocycline improved reaction time of healthy volunteers, and attenuated the amphetamine-induced subjective reward deficits in a Go No-Go task (Sofuoglu *et al.*, 2011).

1.7.2.2 Minocycline effects in animal models

The effects of minocycline in pharmacological animal models in general shows good predictive validity and matches well with its effects in humans. Deficits in the Morris Water Maze and prepulse inhibition were restored by minocycline in mice that had received the NMDAR antagonist MK-801 (Levkovitz *et al.*, 2007). In another study, MK-801-induced hyperlocomotion and PPI deficits were also significantly attenuated by pre-treatment with minocycline (Zhang *et al.*, 2007). Subchronic minocycline treatment resulted in improvement of novel object recognition deficits in repeated PCP treated mice (Fujita *et al.*, 2008), and Mizoguchi *et al.* (2008) found a similar effect in mice that had been administered amphetamine for 7 days then minocycline for 7 consecutive days in the novel object recognition task. This suggests that minocycline can interact with both the glutamate and dopamine pathways to produce a therapeutic effect.

1.7.2.3 Minocycline mechanism of action

Despite minocycline having been shown to have beneficial effects in both healthy and pathological human and rodent studies, its exact mechanism of action is

incompletely characterised, although several, not mutually exclusive mechanisms have been identified. Minocycline has shown to affect various mechanisms of relevance to schizophrenia, including the inhibition of apoptosis by attenuation of caspase 1 and 3 (Chen *et al.*, 2000), inhibition of microglial activation, and reduction of the levels of specific cytokines (TNF-α, IL1β, NO) that are released from activated microglia (Filipovic & Zecevic, 2008; Lee *et al.*, 2004; Tikka & Koistinaho, 2001; Watabe *et al.*, 2012). Patients with schizophrenia have highly activated microglia in the brain, and NMDAR antagonists such as PCP and ketamine are known to induce microglial activation in the brains of rodents (Monji *et al.*, 2009), so these mechanisms are highly relevant. Minocycline also inhibits inducible nitric oxide synthase (which synthesises nitric oxide; NO); there is a whole plethora of research going into NO and its metabolites contributing towards the cause of schizophrenia (Nasyrova *et al.*, 2015). Minocycline may also protect neurons against glutamate-induced excitotoxicity, which has been implicated in the pathophysiology of several neuropsychiatric conditions (Dean *et al.*, 2012). Furthermore, the neuroprotective property of minocycline is thought to partially be due to its inhibitory effects on 5-lipoxygenase, an inflammatory enzyme associated with brain ageing (Oya *et al.*, 2014). These proposed mechanisms of action are all relevant to the neural underpinnings of schizophrenia and are linked with the MKK7/JNK pathway (see **Section 1.9** for more information on this pathway).

There is an essential requirement for more specific treatments for the negative and cognitive symptoms of schizophrenia that are not associated with debilitating side effects. Minocycline is very promising for this purpose, as the pharmacological and therapeutic profile of minocycline suggests that it could improve symptoms caused by multiple processes involved in the current hypotheses of schizophrenia, for example, the microglia, dopamine and glutamate hypotheses (Dean *et al.*, 2012), whilst being safe and not affecting the function of any other drugs that patients may be on.

1.8 Causes of Schizophrenia

Unlike some other conditions where a single, fully penetrant causal gene produces a disorder, the exact cause of schizophrenia is not attributed to a specific genetic mutation (Singh *et al.*, 2014; Sullivan *et al.*, 2012). Rather, it is caused by a

complex combination of multiple genetic and environmental influences, none which are necessary or sufficient, but instead all act as risk factors (Gottesman & Shields, 1967). These interact to bring about the complex array of brain structural, functional and chemical changes seen in schizophrenia (Keshavan *et al.*, 2008). The genetic contribution to the disorder is currently thought to be a state where large numbers of common genetic variants of small effect, as well as rare variants of larger effect, contribute towards predisposition to developing schizophrenia if they are exposed to certain environmental risk factors (Tandon *et al.*, 2008). Early twin, family and adoption studies clearly show this dual genetic/environmental contribution: monozygotic twins, which share 100% of DNA, have a concordance rate for schizophrenia of 40-60%, leaving 60-40% that could be explained by environmental factors, including those of epigenetic mechanisms (Farmer *et al.*, 1987; Kaminsky *et al.*, 2009). Dizygotic twins, on the other hand, have a concordance rate of 6-14%, which is like non-twin siblings in both the number of shared genes and the schizophrenia concordance rate (Farmer *et al.*, 1987). Additionally, several studies on adoption cases found significant effects to support this when comparing the prevalence of schizophrenia in children who were raised by parents who did not have schizophrenia but whose biological parents did (Wender *et al.*, 1974). These simple yet informative early studies demonstrate the importance of genetic inheritance in the prevalence of schizophrenia and show that environmental factors must also be involved.

1.8.1 Environmental factors

It has been acknowledged that environmental risk factors for schizophrenia include: maternal stress, infection and other neurodevelopmental threats (Buka *et al.*, 2001; Khashan *et al.*, 2008), cannabis use (Di Forti *et al.*, 2015), *Toxoplasma gondii* exposure (Arias *et al.*, 2012), advanced paternal age (Dalman & Allebeck, 2002), being born in late winter/early spring, growing up in urbanised areas, and being part of an immigrant group (Giusti-rodríguez & Sullivan, 2013; Torrey *et al.*, 1997). It is still not completely clear how the genetic and environmental factors interact with each other to cause schizophrenia, although some links have been made; for example, polymorphisms of particular genes involved in predisposing sensitivity to stress (Modinos *et al.*, 2013). Also, many risk factors for schizophrenia occur whilst the brain is undergoing development through the perinatal and early postnatal stages; it is thought that the processes

underlying the neuroinflammatory hypothesis of schizophrenia have a major part to play in this (explored further in **Chapter 7**).

1.8.2 Genetic factors

There is a major research focus on finding the genetic variants which confer increased risk for schizophrenia (Tandon *et al.*, 2008). A variety of methodologies have been developed with the aim of locating the genetic variants, including family linkage analysis, candidate gene association studies, genome wide association studies (GWAS), copy number variant (CNV) analyses, cytogenetic screens and deep re-sequencing (Winchester *et al.*, 2014). Research has been directed towards finding many common genetic variants, each contributing a small risk, as well as rare variants of large effect, that interact with one another and/or with environmental risk factors to cause schizophrenia (Mulle, 2012). In a population, schizophrenia is believed to be caused by an aggregate accumulation of many of these genetic variants that confer risk, at thousands of loci, which is known as called the polygenic inheritance model (Glessner & Hakonarson, 2009; Purcell *et al.*, 2009).

There are several ways in which genetic variants can be identified. Sequence variants consist of single nucleotide polymorphisms (SNPs), indels (INsertions or DELetions; rare, pathogenic single or small polynucleotide variants of which the importance in schizophrenia is unclear), and trinucleotide or hexanucleotide repeats. There are also structural variants, which consist of copy number variants (CNVs) and chromosomal abnormalities. CNVs and SNPs are the most common type of genetic variation that occur in the genome of patients with schizophrenia (especially SNPs), but even so, they explain less than 5% of heritability (Marshall *et al.*, 2017). This is thought to be an underestimate due to a few reasons: there are probably very many more than 108 loci associated with schizophrenia as found out by the most recent GWAS conducted (Ripke *et al.*, 2014). Perhaps most importantly, there are gene x gene interactions (epistasis) and gene x environment interactions, which are not considered in the calculation of heritability (Zuk *et al.*, 2012).

1.8.2.1 Copy number variants

Copy number variants are small chromosomal abnormalities with excessive deletions or duplications of DNA segments that occur in the genome of every

human being. Where they are located, and the gene "dosage", dictates whether they have a detrimental effect, or no effect whatsoever. CNVs associated with schizophrenia are relatively rare but have a high genotypic relative risk score, i.e., a strong effect on disease risk (Marshall *et al.*, 2017).

Eight consistent CNVs associated with schizophrenia have been identified (Marshall *et al.*, 2017), most of which have also been implicated in other psychiatric disorders, such as autism and bipolar disorder (Sullivan *et al.*, 2012). The most common CNVs are the 16p11.2 duplication, 15q13.3 deletion and 22q11.2 deletion (Marshall *et al.*, 2017). The 22q11.2 region is also strongly associated with velo-cardio-facial syndrome, patients of which have a 30% chance of developing psychosis (Williams *et al.*, 2006), suggesting psychotic symptoms may at least be partly produced by genetic alterations at this locus.

1.8.2.2 Single nucleotide polymorphisms

A single nucleotide polymorphism is an exchange of a single nucleotide at a specific locus for a different nucleotide. Just 0.1% of the human genome is different between individuals, and SNPs make up approximately 90% of this 0.1% variation (Collins *et al.*, 1998; Collins & Mansoura, 2001). More than 10 million SNPs have been identified so far, and the majority have no known effect or function; however, some are causative mutations. SNPs with causative mutations can either be located within the coding regions of a gene, where they may have effects on the structure or function of a protein and may or may not be important for normal functioning. Or, they can be located in non-coding regions of a gene, where they can have an effect on regulation of gene expression, and therefore quantity, or different splicing of isoforms of the protein made which, again, may or may not be important for normal functioning (Samson & Wong, 2015).

SNP association with a particular disorder can be identified in several ways. Microarrays can study thousands of SNPs at once, for example, GWAS. A GWAS uses a chi-squared test to identify whether the frequency of each SNP (up to ~500,000 of them at a time) differs between patients and controls. To control for multiple testing, huge sample numbers are required. The most recent schizophrenia GWAS (Ripke *et al.*, 2014) included 36,989 cases and 113,075 controls, and identified 108 loci that reached genome wide significance between patients and controls. This information is useful in order to know where to begin

looking, and for spotting trends in the associations. For example, many of the loci included genes which were part of the same well-characterised pathways (Ripke *et al.*, 2014). The most common convergence appears to occur on pathways for NMDAR signalling, synaptic plasticity, calcium signalling and immune function (Giusti-rodríguez & Sullivan, 2013; Morris & Pratt, 2014). This has led to the idea that schizophrenia may be a "pathway" disorder, with genetic variation conferring risk converging on a particular cellular pathway (or pathways), causing alterations of whole molecular systems (Horváth & Mirnics, 2015; Sullivan, 2012).

However, there are problems with using solely GWAS to identify SNPs. GWAS works by spanning the entire genome and testing for significant SNPs at roughly equal intervals (about every 50,000 bases), missing large parts of the genome. Additionally, each locus is huge; there are implicated genes at these regions, but this does not actually causally link them to a disorder. Moreover, there is very limited clinical information available for such large sample sizes, so genotype-phenotype relationships are unclear, and most of the data used in GWAS studies are from Caucasian subjects, but there are major ethnic differences in SNPs and in the prevalence of schizophrenia (Huang *et al.*, 2015). However, GWAS's are useful in providing information about where to start looking for genetic variation associated with a particular disorder.

Specific SNPs can then be genotyped in candidate gene association studies, which focus on finding associations between genetic variation of pre-selected genes of interest and disease states. Recently, Winchester *et al.* (2012) conducted a candidate gene association study and found a significant association between two SNP variants of the *Map2k7* gene and schizophrenia, as well as decreased expression levels of *Map2k7* mRNA by ~30% in the post mortem PFC of schizophrenia patients (Winchester *et al.*, 2012). *Map2k7* was not identified in the GWAS's of schizophrenia to date, most likely because it was poorly represented on the chip arrays used (Winchester *et al.*, 2012).

1.9 *Map2k7* and the JNK pathway

The *Map2k7* gene encodes an intracellular signalling kinase in the c-Jun NH_2-terminal protein kinase (JNK) pathway, which is activated by cellular stress stimuli and modulates critical cellular functions, such as cell growth and proliferation, differentiation, transcription, apoptosis and cellular migration (Rincón & Davis,

2009; Yamasaki *et al.*, 2011; Yamasaki *et al.*, 2012). The MAP2K7 (MKK7) protein specifically phosphorylates and activates JNK (facilitated by MAP2K4; MKK4; Wang *et al.*, 2007), which is one of three subgroups in the mitogen activated protein kinase (MAPK) superfamily (the other two being p38 and the extracellular signal related kinase (ERK)). Dual phosphorylation is required to activate JNK, on the Threonine-Proline-Tyrosine motif in its activation loop, and in order to keep the activation level under control, JNK is deactivated by phosphatases. MKK4 and MKK7 preferentially phosphorylate the tyrosine and threonine residues, respectfully (Wada *et al.*, 2001). Although MKK7 is specific for JNK, MKK4 is involved with activating JNK and also p38 MAPK in response to stress (Wang *et al.*, 2007). Cellular stress stimuli, such as: osmolarity changes, DNA damage, heat/cold shock, inflammatory cytokines, UV irradiation and mechanical sheer stress lead to phosphorylation of MAP kinase kinase kinases, sometimes via NMDARs (Centeno *et al.*, 2007), which then activate the MAP kinase kinases MKK7 and MKK4, which, in turn phosphorylate and activate JNK (Kyriakis & Avruch, 2001; Watanabe *et al.*, 2002). JNK then either interacts with other cellular effectors within the cytoplasm, or translocates to the nucleus to have further effects on transcription factors, such as c-Jun, AP-1, c-Myc and ATF2, microtubule associated proteins (MAPs), and various apoptotic proteins (Bogoyevitch & Kobe, 2006). This then leads to regulation of the critical, distinct cellular processes mentioned above (Yamasaki *et al.*, 2012; Yamasaki *et al.*, 2011). The main components of the signalling pathway are depicted in **Fig. 1.1.**

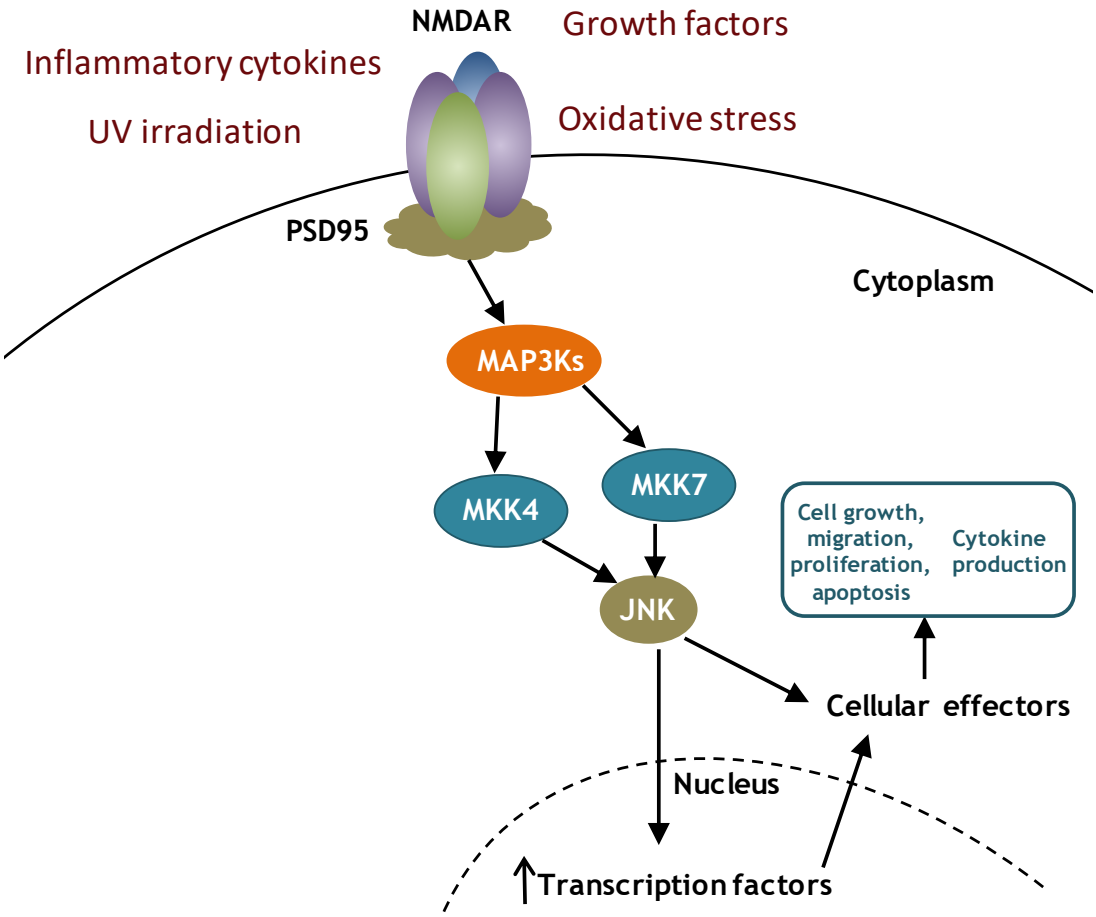


Figure 1.1. The MKK7/JNK signalling pathway. MKK7 is part of a three-tier intracellular signalling cascade that begins with the activation of MKKKs by stress factors such as inflammatory cytokines, growth factors, UV irradiation and oxidative stress, among others. NMDA receptors are one, but not all, of the ways in which the stress factors mediate their response intracellularly, activating the MKKKs, which include TAOK2, DLK, MLKs and apoptosis signal-regulating kinase 1 (ASK1). These then go on to activate MKK4 and MKK7, which together activate JNK1, 2 and 3. JNK then either translocates to the nucleus to have further effects on transcription factors and therefore gene transcription, or has direct mediation of other cellular effectors within the cytoplasm. Figure and legend composed using information from (Coffey, 2014; Morris *et al.*, 2014; Traynelis *et al.*, 2010). This is a simplified depiction of the MKK7/JNK pathway. MKK7 = mitogen activated protein kinase kinase 7; MKK4 = mitogen activated protein kinase kinase 4; MAP3Ks/MKKKs = mitogen activated protein kinase kinase kinases; PSD-95 = postsynaptic density 95; NMDAR = N-methyl-D-aspartate receptor; JNK = c-Jun N-terminal kinase.

1.9.1 Information about MKK7/JNK signalling has been provided by studying knockout mice

The importance of the role of MKK7/JNK signalling *in vivo* has been investigated using genetically altered mice for different components of the MKK7/JNK pathway (Yamasaki *et al.*, 2012). Mice completely lacking the *Map2k7* or *Map2k4* gene are embryonically lethal between E11.5 and E12.5 due to impaired liver formation and decreased JNK activation (Wada *et al.*, 2004), showing that activation of JNK by MKK4/7 is crucial for hepatoblast growth in mouse development (Watanabe *et al.*, 2002). MKK4/MKK7 double KO mice die at E9.5 prior to neural tube formation, suggesting that MKK4 and MKK7 can compensate for each other to a certain extent (Asaoka & Nishina, 2010). *Nestin-Cre Map2k7* mice lacking *Map2k7* in just the nervous system died at birth and showed severe brain defects, including enlarged ventricles, enlarged overall brain volume, defects in axonal tract formation, altered radial migration and reduced striatal volume (Yamasaki *et al.*, 2011). There was also a compensatory increase of activated MKK4 (Yamasaki *et al.*, 2011, 2017), showing that MKK4 and MKK7 work together; however, they also have distinct functions as *Map2k4 Nestin-Cre* mice lacking *Map2k4* in just the nervous system did not show a compensatory increase in MKK7, but did show a decrease in p38, which is consistent with the physiological role of MKK4 in mediating p38 MAPK activation in response to stress (Wang *et al.*, 2007). The *Map2k4* conditional KO mice died at 3 weeks and exhibited misalignment of the Purkinje cells of the cerebellum (Wang *et al.*, 2007). In both nervous system cKO MKK4 and MKK7 mice, JNK activation was reduced to 20% of the normal extent of activation in the developing brain and they also exhibited delayed neuronal migration in the cortex (Yamasaki *et al.*, 2011). It has been suggested that these differences/similarities between signalling by MKK7 or MKK4 in the JNK pathway may be due to specific regulation by extracellular stimuli, distinct tissue distribution and their different biochemical properties (Coffey, 2014; Lee *et al.*, 2012; Wang *et al.*, 2007).

JNK knockout mice have also been useful in providing information about the function of the MKK7/JNK pathway. Three *Jnk* genes (*Jnk 1, 2* and *3*) encode 10 isoforms in mammals (Wang *et al.*, 2007); JNK1 and JNK2 proteins are ubiquitously expressed, including in heart, brain, lung, liver and skeletal muscle, whereas JNK3 is only expressed in the brain and testes (Kuan *et al.*, 1999). JNK1 KO mice displayed abnormal dendritic architecture in the motor cortex, increased

apoptosis and impaired LTP (Komulainen *et al.*, 2014; Li *et al.*, 2007). JNK2 KO mice exhibited increased apoptosis and defective synaptic plasticity (Chen *et al.*, 2005; Sabapathy *et al.*, 1999). JNK3 KO mice exhibited reduced glutamate toxicity in neurons and abnormal circadian rhythms (Yang *et al.*, 1997; Yoshitane *et al.*, 2012). Additionally, JNK1/JNK2 double KO mice die at 11.5 with failure of neural tube closure and decreased apoptosis in the hindbrain but increased apoptosis in the forebrain (Kuan *et al.*, 1999). These JNK KO studies show that JNK1, 2, and 3 have both functional differences and the ability to compensate for each other to a certain extent (Yamasaki *et al.*, 2012), in a comparable way to MKK4 and MKK7.

Altogether, these KO studies show the importance of the MKK7/JNK pathway in embryogenesis and mammalian body plan organisation from very early stages, and produce effects that are related to schizophrenia pathology (reduced striatal volume, enlarged ventricles, dendritic architecture, axonal tract (i.e. white matter) changes; discussed in **Section 1.5**). In particular, the double JNK1/JNK2 KO mice showed differential apoptosis over the brain regions that clearly links with the dopaminergic hypofunction and hyperfunction of schizophrenia neuropathology in the PFC and subcortical structures, respectively (described in **Section 1.6.2**). Moreover, several of the knockout mice show deficits in long term potentiation, which is mediated by NMDARs, and there is evidence to suggest that NMDAR function is altered in schizophrenia (see **Section 1.6.1** above and reviewed in (Snyder & Gao, 2013). Therefore, the MKK7/JNK pathway has important roles in brain development, neuronal activity and controlled cell death which will likely have consequences on the brain that continue into adulthood. Additionally, cellular stress stimuli itself during development plays a role in the pathogenesis of schizophrenia (Meyer, 2013). Therefore, these highly relevant and important findings from Winchester *et al.* (2012) could pave the way to a deeper understanding of the abnormal neurobiological mechanisms involved in schizophrenia.

1.9.2 Map2k7 and schizophrenia

Animal, clinical and genetic studies show there are numerous ways in which disrupted MKK7/JNK signalling may be a factor in the pathophysiology of schizophrenia. Mutations in JNK3 cause severe intellectual disability in humans (Shoichet *et al.*, 2006; Kunde *et al.*, 2013), and JNK1, MKK7 and MKKK12 (a kinase upstream of MKK7) are all crucial for the development of the neocortex (Hirai *et*

al., 2002; Hirai *et al.*, 2011; Yamasaki *et al.*, 2011; Riches & Reynolds, 2014; Xu *et al.*, 2014). Also, NMDARs are located upstream of MKK7 and interact directly with the MKK7/JNK pathway (Mukherjee *et al.*, 1999), and JNK1 and 2 mediate aspects of synaptic plasticity via NMDARs in the mature mouse hippocampus (Chen *et al.*, 2005; Li *et al.*, 2007). Additionally, there is an orthologue version of the *Map2k7* gene present in *C. elegans: Jkk-1.* A mutation in *Jkk-1* of *C. elegans* impairs long-term potentiation, therefore implicating *Map2k7* in fundamental aspects of memory (Lakhina *et al.*, 2015). Notably, as the genes of *C. elegans* are very old phylogenetically, this shows the significance and importance of this gene in humans as it still exists and not been removed by natural selection (Asaoka & Nishina, 2010). Additionally, Winchester *et al.* (2012) identified two SNPs in the *Map2k7* gene associated with increased risk for schizophrenia. It is interesting that one of these polymorphisms was located in a region that had extremely high inter-species sequence conservation (Winchester *et al.*, 2012). This, again, highlights the physiological importance of the *Map2k7* gene and suggests fundamental processes may be compromised if there is disruption in this gene/protein and the genes/proteins it interacts with, and will probably cause disruption of functional importance. It also shows that the relevant (i.e. associated with schizophrenia) parts of the *Map2k7* gene are highly likely to be the same in mice and humans. This is especially likely considering the whole *Map2k7* gene is 99% conserved in mice and humans overall (Foltz *et al.*, 1998), and the polymorphism associated with schizophrenia represents one of the highest inter-species sequence conservation of the gene (Winchester *et al.*, 2012, with data obtained from Ensembl: www.ensembl.org). Therefore, mice heterozygous for the *Map2k7* gene would be expected to have excellent construct validity (i.e. the procedures used to create the model are related to the underlying mechanisms involved in disease aetiology; Pratt *et al.*, 2012).

Genetic abnormalities affecting several other levels of the JNK/MKK7 pathway also confer risk for psychiatric disorders such as schizophrenia (summarised in Morris & Pratt, 2014). For example, thousand-and-one amino acid kinase 2 (TAOK2) partially activates JNK1 (de Anda *et al.*, 2012) and is located on chromosome 16p11.2, a region associated with increased risk for schizophrenia (McCarthy *et al.*, 2009). Also, MKK7/JNK signalling may impact on calcium levels (Brnjic *et al.*, 2010), and the *CACNA1C* gene (which encodes an alpha-1 subunit of

a voltage-dependent calcium channel, and mediates the influx of calcium ions into the cell upon membrane polarization) has also been implicated in schizophrenia. Calcium signalling has very widespread effects *in vivo* (Giusti-rodríguez & Sullivan, 2013), including on synaptic plasticity, so it is possible that MKK7/JNK signalling can interact with calcium signalling to contribute towards the pathogenesis of schizophrenia.

Evidence is also mounting in support of an altered inflammatory response playing a role in the pathophysiology of schizophrenia (Meyer, 2013; Monji *et al.*, 2009) and stress-activated protein kinases, including MKK7, have been shown to regulate the immune response (reviewed in Rincón & Davis, 2009). Therefore, it is feasible that insults such as hypoxia and infection during a critical point in development induce cellular stress in an individual who has a schizophrenia risk polymorphism in the *Map2k7* gene, and thereby cause aberrant activation of the JNK pathway, which may result in a compensatory long-term down-regulation of *Map2k7* in the brain (Winchester *et al.*, 2012). The immune system is important for normal brain development (Bilbo & Schwarz, 2012), so abnormal regulation of the immune response throughout the developmental period could have damaging effects on brain function, structure and behaviour which only begin to show as symptoms of schizophrenia later in life.

Collectively, these findings link MKK7/JNK signalling to the pathogenesis of schizophrenia. As mentioned earlier, the genetic, pharmacological and physiological causes of schizophrenia converge on pathways for NMDAR signalling, synaptic plasticity, calcium signalling and immune function (Morris & Pratt, 2014; Giusti-Rodriguez & Sullivan, 2013). MKK7/JNK signalling falls at least partially into all four of these, making it an excellent basis for future study as a potential therapeutic target. Further investigation will be required into the cellular mechanisms and behavioural phenotypes associated with this signalling pathway when it is disrupted. Mice haploinsufficient for the *Map2k7* gene will be valuable in this objective and is what will be explored throughout this thesis.

1.10 Animal models for schizophrenia

Animal models of relevance to schizophrenia are vital for progress in drug development and have been the topic of many reviews (Ayhan *et al.*, 2009; Geyer & Moghaddam, 2002; Jones *et al.*, 2011; Marcotte *et al.*, 2001; Mouri *et al.*, 2013;

Nestler & Hyman, 2010; Pratt *et al.*, 2012; Young *et al.*, 2010, to name but a few). The idea that it is difficult to model human characteristics in non-human animals has led to criticism of models of relevance to complex mental disorders (Young *et al.*, 2010). However, animal models provide valuable neurobiological information that is not possible to obtain by studying humans. A genuinely novel therapeutic drug has not been developed for the symptoms of schizophrenia since atypical antipsychotics were clinically introduced in the 1970's, and this is thought to be primarily due to the unavailability of accurate and reliable animal models (Nestler & Hyman, 2010; Pratt *et al.*, 2012; Yee & Singer, 2013). As schizophrenia and other psychiatric disorders are behaviourally, genetically and neuropathologically heterogeneous, a common approach when generating new animal models is to focus on modelling particular aspects of symptoms domains, e.g. the cognitive symptoms. Approaching this as the RDoC initiative suggests in **Section 1.4**, rather than attempting to model the whole disorder in one animal model, will provide relevant information that can potentially span multiple disorders. That is, assuming that the domains share similar underlying mechanisms across species and disorders (Young *et al.*, 2010). The research domains that RDoC have identified include negative valence (fear, anxiety), positive valence (motivation, reward learning, initial and sustained responsiveness to reward), cognitive systems (attention, working memory, cognitive control, declarative memory), social processes (social communication, perception and understanding of self and others), and, finally, arousal/modulatory systems (sleep/wake cycle, circadian rhythms) (Cuthbert & Insel, 2013).

Initiatives such as the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) and the Cognitive Neuroscience Treatment Research to Improve Cognitive in Schizophrenia (CNTRICS) also aim to facilitate boundaries between clinical and basic research, and they focus on the cognitive deficits of schizophrenia as there are no efficacious treatments for those currently. MATRICS have developed a standardised battery of tests to examine the functioning of each of the cognitive constructs in patients so that the efficacy of novel treatments can be assessed (Nuechterlein *et al.*, 2008). Based on the battery of tests laid out by MATRICS, the CNTRICS panel selected analogous behavioural tasks for evaluating these cognitive domains in animal models (Carter & Barch, 2007). Therefore, by following the guidance of CNTRICS, researchers investigating the phenotype of

potential animal models will be re-assured that they are using accurate tasks that have been reliably shown to be highly translatable to human patients.

1.10.1 Validity of animal models

For an animal model to be effective it must be carefully designed to fulfil its specified function, be reliable and satisfy certain criteria (Young *et al.*, 2010). The behavioural symptoms of schizophrenia are primarily unique to humans. However, endophenotypes (behavioural, neuroanatomical, cognitive or neuropsychological markers that are heritable and that expose the link between genetic and clinical expression; Uhlhaas & Singer, 2010) reflecting specific symptoms in humans that can be observed in rodent models are becoming more established (Braff, 2015).

Ideally, an animal model should have good face, construct and predictive validity and the behavioural tests used to examine it would be relevant, precise, objective and perfectly translatable to human patients (Young *et al.*, 2010). Face validity is defined as when the observed phenotype of the model resembles that seen in patients, which is not as vital as construct or predictive validity and if this is the only criteria an animal model fulfils, would not be considered a reliable model. Construct validity describes a model created using mechanisms based on actual, known underlying mechanisms involved in disease aetiology, which is perhaps the most important of the three criteria and is hard to achieve, yet is still not useful if this is the only criteria a model satisfies. A model has predictive validity if predictions made using the model (for example, drug efficacy) reflect what occurs in patients (Pratt *et al.*, 2012; Sharma *et al.*, 2016; Young *et al.*, 2010). All the aforementioned validity criteria are important and ideally an animal model will possess all of them. As described in this introduction, mice heterozygous for the *Map2k7* gene have excellent construct validity, and their face and predictive validity will be investigated throughout this thesis by utilising translational behavioural tasks and molecular techniques.

There are currently multiple subtypes of animal models for schizophrenia, broadly grouped into pharmacological, genetic and developmental models; a brief overview and examples of each type are given in the following sections (Jones *et al.*, 2011).

1.10.2 Pharmacological animal models of schizophrenia

Pharmacological models are created by acute or sub chronic administration of psychotomimetic compounds to animals in order to recapitulate symptoms of schizophrenia. As mentioned in **Section 1.6**, ketamine is a derivative of PCP, and they are both primarily NMDAR antagonists. PCP and ketamine induce remarkably similar experiences in people who abuse it to those who have schizophrenia, mirroring the negative, positive and cognitive deficits (Adler *et al.*, 1999). This occurs to such an extent that chronic PCP abusers have previously been mis-diagnosed as having schizophrenia (Morris *et al.*, 2005). Acute or subchronic PCP administration in rodents produces hyperlocomotion, neuropathological changes and impairment in: PPI, novel object recognition, reversal learning, attentional set-shifting and attentional function that are relevant to schizophrenia (Jones *et al.*, 2011; Neill *et al.*, 2010; Pratt *et al.*, 2012; Thomson *et al.*, 2011). These studies provide evidence towards the NMDAR hypofunction hypothesis of schizophrenia, and, in a similar way, administration of amphetamine in rodents provide evidence towards the dopamine hypothesis of schizophrenia (Van Den Buuse, 2010). Although offering good face validity, pharmacological animal models of schizophrenia have come under some criticism because of the fact that schizophrenia is not thought to be caused by selective dysfunction of a single neurotransmitter system (Nestler & Hyman, 2010). However, combinational administration of drugs in animal models have been attempted in the context of drug abuse. Ketamine plus amphetamine administration in mice showed additive effects on locomotor activity but differential effects on GAD_{67} expression (Lai *et al.*, 2013); therefore, it would be useful to pursue these combinatorial pharmacological models further, with a focus on schizophrenia.

1.10.3 Developmental animal models

Developmental animal models of schizophrenia typically involve administration of drugs or manipulation of the environment during critical periods of development and then studying the effect(s) this has on the offspring. Exposure to Polyinosinic:polycytidylic acid (Poly I:C), a synthetic analogue of double-stranded RNA that acts as a viral mimetic, during the gestational period is one example of this. It causes behavioural phenotypes in offspring relevant to schizophrenia, such as deficits in: PPI (described in **Section 4.4.1**), latent inhibition, selective attention, sociability and social novelty, and hyper-exploration in a novel

environment (Moran *et al.*, 2016). Poly I:C exposure during gestation also produces an enhanced immune response in maternal serum and foetal brain that are relevant to those which contribute to schizophrenia in offspring (Reisinger *et al.*, 2015).

Another well-studied neurodevelopmental rodent model is the gestational methylazoxymethanol acetate (MAM) model. MAM is an agent naturally occurring in cycad plant seeds and it methylates DNA, producing anti-mitotic and anti-proliferative effects during development (Matsumoto & Higa, 1966). Administration of MAM to gestating rats produces long-term histological, neurophysiological and behavioural deficits in the offspring (Lodge, 2013). Another example of developmental animal models are neonatal lesion or disconnection studies of parts of the hippocampus, which produce a collection of behavioural and cellular alterations that mimic several aspects of schizophrenia (Lipska, 2004). However, these models do not show particularly good construct validity.

1.10.4 Genetic animal models of schizophrenia

One approach when creating a relevant animal model is by genetic manipulation of specific gene(s) associated with schizophrenia. Genetic manipulation can be in the form of point mutations using N-ethyl-N-nitrosourea (ENU) mutagenesis, structural changes such as inducing chromosome abnormalities, CNV alterations and haploinsufficiency models (Tomoda *et al.*, 2016). Although it must be acknowledged that animal models relevant to schizophrenia created by genetic manipulation may show phenotypes also related to other conditions such as depression and bipolar disorder, they have significant value as they have the potential to have excellent construct validity. Having a genetic model that is relevant to the pathogenesis of not just schizophrenia but related disorders also, would conform well with the RDoC initiative. This may be valuable because schizophrenia shares some of its risk genes with disorders such as bipolar disorder and autism (Carroll & Owen, 2009; Goes *et al.*, 2016). Very specific genetic alterations can be designed to mimic what occurs in humans, and is present from birth, which is relevant to what is likely to naturally occur. On the other hand, genetic manipulation that can be induced whenever desired using optogenetic techniques and designer receptors exclusively activated by designer drugs (DREADDs) are showing promise for precisely deconstructing the pathophysiological mechanisms of schizophrenia-relevant neural circuits (Cho &

Sohal, 2014) and have been shown to impair PPI, cognitive flexibility and attention (Koike *et al.*, 2015; Nguyen *et al.*, 2014; Parnaudeau *et al.*, 2015).

22q11.2 deletion, 15q13.3 microdeletion and 16p11.2 duplication (see **Section 1.8.2.1**) mouse models have also been developed. Mice with various deletions in the 22q11.2 region are perhaps the best studied of these and show alterations in hippocampal synaptic plasticity, reduced synchrony between the hippocampus and PFC, dysregulation of presynaptic calcium, enhanced glutamate release, age-dependent decrease in parvalbumin positive cell density, and increased LTP (Earls *et al.*, 2011; Piskorowski *et al.*, 2016; Sigurdsson *et al.*, 2010). Behaviourally, they exhibit hyperactivity in the open field, and deficits in working memory, social memory, spatial memory, PPI and fear conditioning (Earls *et al.*, 2011; Piskorowski *et al.*, 2016; Stark *et al.*, 2008). The 22q11.2 deletion is one of the largest known genetic risk factors for schizophrenia (Karayiorgou & Gogos, 2004), so these animal models represent excellent face and construct validity.

Disrupted-in-schizophrenia 1 (DISC1) is a gene initially identified in a Scottish pedigree in which loss of DISC1 function is associated with hugely increased risk for schizophrenia and other psychiatric disorders (such as depression and bipolar disorder; Millar *et al.*, 2007). Various genetic mouse models of DISC1 have shown behavioural, cellular, circuitry and molecular changes relevant to schizophrenia. Behavioural changes include working memory deficits, increased impulsivity, reduced PPI, reduced sociability and social novelty, hyperlocomotion, enhanced dopamine function, deficits in interneuron development and enlarged ventricles (Brandon & Sawa, 2011; Koike *et al.*, 2006; Kuroda *et al.*, 2011; Lee *et al.*, 2013; Lipina *et al.*, 2010). These animal models have proven useful in advancing the knowledge of the underlying neurobiology of schizophrenia and are expected to continue providing the means to test novel treatments and diagnostic possibilities (Tomoda *et al.*, 2016).

1.10.5 Gene x environment interaction models

The advantage of genetic models is that it is easy to simultaneously "superimpose" a different type of model onto them to create a gene x environment interaction model. Genetic manipulations have been combined with developmental models (**Section 1.10.3**), including induction of stress, maternal infection, isolation

rearing and drug abuse, allowing for a more hypothesis-driven and accurate animal model (Ayhan *et al.,* 2009).

It is well established that prenatal exposure to infection that involves immune activation is associated with increased risk of developing schizophrenia in offspring (Cannon *et al.*, 1996). Exposure of a genetically altered gestating dam to infection or direct immune activation such as human influenza, lipopolysaccharide (LPS) or Poly I:C and subsequent investigation of changes in offspring are an interesting way to study gene x environment interactions in schizophrenia. Exposure to Poly I:C is perhaps the most frequently studied. In non-genetically altered animals, Poly I:C administration to a gestating dam causes (in offspring) behavioural phenotypes (PPI, latent inhibition, selective attention, sociability and social novelty deficits, and hyper-exploration in a novel environment), including an enhanced immune response in maternal serum and foetal brain that are relevant to those which occur in schizophrenia (reviewed in Moran *et al.*, 2016; Reisinger *et al.*, 2015). Both Neuregulin-1 and DISC1 animal models have been subject to the Poly I:C immune challenge procedure, and produced differential (O'Leary *et al.*, 2014) and synergistic (Tatiana *et al.*, 2013) effects on schizophrenia-related phenotypes, respectively. **Chapter 7** will examine the potential of *Map2k7* heterozygous mice as a gene x environment model with the administration of Poly I:C.

1.11 Assessing schizophrenia-related behavioural phenotypes in mice

When investigating complex disorder such as schizophrenia, it is common to utilise a battery approach and examine mice in a broad range of tasks, in order to get a full representation of impairments. As with human tasks, no single rodent behavioural task is uniquely relevant to schizophrenia, or adequately captures the full spectrum of its symptoms (Powell & Miyakawa, 2006). There are many rodent tasks of relevance to schizophrenia, which will be briefly discussed.

1.11.1 SHIRPA assessment

SHIRPA (an acronym of **S**mithKline Beecham, **H**arwell, **I**mperial College, **R**oyal London Hospital **p**henotype **a**ssessment) tests are usually carried out in the first instance on any potential novel animal model. It is a battery of short tests that

detect overt differences in animals to produce a general phenotypic screen (Rogers *et al.*, 1997). *Map2k7*$^{+/-}$ mice have previously undergone SHIRPA tests (R. Thompson, PhD thesis, 2013) and appeared completely normal in every aspect assessed. **Table 1.1** gives a summary of results of the tests relevant to the current thesis. To summarise, *Map2k7*$^{+/-}$ mice can move normally, are not hindered by tremor or by exhibiting convulsions, can see properly, and are normal in their balance, strength and motor coordination, compared to their WT littermates (R. Thompson, PhD Thesis, 2013). These non-significant results are important because it means that any differences between WT and *Map2k7*$^{+/-}$ mice in subsequent behavioural tests are likely to be due to the impact of the genetic manipulation upon behaviour, rather than a confound, such as impaired vision.

Test	WT score	HZ score	Significance
Gait	0 ± 0	0 ± 0	p >0.05
Tremor	0 ± 0	0 ± 0	p >0.05
Bizarre behaviours	0 ± 0	0 ± 0	p >0.05
Convulsions	0 ± 0	0 ± 0	p >0.05
Visual placing (eyesight)	1 ± 0	1 ± 0	p >0.05
Hanging wire (s) (grip strength)	54.9 ± 3.3	52 ± 3.7	p >0.05
Rotarod (s) (balance, motor coordination)	111.5 ± 14.7	121.2 ± 12.6	p >0.05

Table 1.1. Summary of primary phenotype screen of Map2k7+/- mice (HZ) and their WT littermates, carried out by R. Thompson (PhD thesis, 2013). Data is presented as the mean ± SEM, WT n=7, *Map2k7*$^{+/-}$ n=9. Data analysed by Student's t-test.

1.11.2 Maze and arena-based tasks

Behavioural tasks carried out in a maze or arena are usually relatively brief tasks and require little to no training. Maze-based tasks can give a variety of information, such as the Morris water maze for working and spatial memory, elevated plus or zero maze for anxiety, 8-arm radial maze for working memory, and the Y-maze for working memory and reversal learning. Arena-based tasks such as the open field or three-chamber arena are used for assessing many behaviours; for example, locomotor activity, anxiety, sociability and social novelty, novel object recognition and light/dark preference, to name but a few. The tasks can also be carried out following pharmacological intervention. Many of these maze and arena-based tasks can be used effectively in conjunction with animal tracking

software (such as Ethovision®) whilst performing the tasks so that distance moved, velocity moved, interactions with objects or other animals, number of entries and time spent in pre-defined areas can be recorded automatically, which avoids researcher bias (reviewed in Powell & Miyakawa, 2006).

1.11.3 Operant-based tasks

Bussey *et al*. (2012) described the following desirable traits for a rodent behavioural assay. It should be: automated to diminish experimenter interaction/bias, minimise stress to the subject, be translational to human tasks, and utilise similar neural circuitry. All in all, the assay itself must present with good face, construct and predictive validity. It must also be able to measure multiple cognitive domains within the same equipment to minimise environmental factors. Therefore, Bussey *et al*. created the touchscreen operant-based method in order to meet these criteria, and designed the tasks to match the MATRICS tasks for phenotypic screens in humans as closely as possible, even sometimes utilising the same stimuli. Robbins (2002) previously developed the 9-hole operant box which, again, minimises experimenter bias and allows a battery of tasks to be carried out in the exact same environment on the same subjects. These operant-based methods are also relatively high throughput, meaning that many animals can be tested within one day, which is useful as extensive, daily training is usually required.

Throughout this thesis, I will use both the 5-Choice serial reaction time task for attention in 9-hole operant chamber and the rodent gambling task for decision-making using the touchscreen, and will describe the tasks in further detail in the relevant chapters.

1.12 Thesis aims

Following their finding that variations in the *Map2k7* gene are associated with schizophrenia, Winchester *et al*. conducted some preliminary behavioural analysis on mice haploinsufficient for *Map2k7* (*Map2k7*$^{+/-}$ mice) and found deficits potentially relevant to those in patients with schizophrenia. *Map2k7*$^{+/-}$ mice showed signs of exhibiting increased perseveration and a reduction in correct responses compared to WT littermates during a T-maze task to probe working memory, which involves the PFC (Winchester *et al*., 2012). As this genetic

manipulation is based on findings from human patients, this suggests the *Map2k7*$^{+/-}$ mice could have good face validity and model the cognitive deficits of schizophrenia; hence, this thesis will primarily focus on the cognitive systems and, to a lesser extent, the positive valence domains from those identified by RDoC (outlined in **Section 1.10**).

As described throughout the thesis Introduction, there are many ways in which *Map2k7* is linked with the pathogenesis of schizophrenia and many features that suggest mice heterozygous for the *Map2k7* gene have the potential to possess the main qualities of a good animal model. For example, *Map2k7*$^{+/-}$ mice have appropriate construct validity and the predictive and face validity are what I will be investigating throughout this thesis, following on and contributing to the findings by Winchester and colleagues (2012).

Chapter 2 describes the materials and methods that are relevant to all subsequent experimental chapters and how the *Map2k7*$^{+/-}$ mice were created, by J. Penninger. The experimental chapters will aim to answer the following questions:

Chapter 3

- Do *Map2k7*$^{+/-}$ mice show decreased MKK7 protein in the PFC?
- Do *Map2k7*$^{+/-}$ mice show altered amounts of other proteins in the MKK7/JNK pathway in the PFC?
- Do *Map2k7*$^{+/-}$ mice show altered pathway components following one week's treatment with minocycline?

Chapter 4

- Do *Map2k7*$^{+/-}$ mice show phenotypes grossly related to the positive symptoms of schizophrenia?
- Do they show an altered behavioural response to an NMDAR antagonist?
- Does minocycline alleviate any positive-related phenotypes observed?

Chapter 5

- Do *Map2k7*$^{+/-}$ mice show attentional deficits?
- Do they show an altered behavioural response to an NMDAR antagonist?
- Does minocycline alleviate any deficits observed?

Chapter 6

- Can mice learn the rodent gambling task in the touchscreen?
- Do *Map2k7*$^{+/-}$ mice exhibit a deficit in decision-making?
- Are *Map2k7*$^{+/-}$ mice flexible in their decision-making abilities?

Chapter 7

- Will *Map2k7*$^{+/-}$ mice be suitable as a gene x environment risk factor model of schizophrenia?
- Do *Map2k7*$^{+/-}$ mice (gestating adult and/or embryo) exhibit an altered cytokine response to maternal immune activation?

Finally, Chapter 8 will bring together and discuss the findings of the thesis, including how it fits in with the relevant literature.

Chapter 2 Materials and Methods

Materials and methods which are applicable to all chapters are included here; more detailed "Materials and Methods" will be described within each chapter.

2.1 *Map2k7*$^{+/-}$ mice

Mice heterozygous for a functional *Map2k7* gene (*Map2k7*$^{+/-}$; HZ) and wildtype (WT) littermate controls are the mice used throughout this thesis. They were originally a gift from Professor J. Penninger (IMBA, Institute for Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria) and were produced by replacement of a portion of exon 9 with a PGK-Neo cassette as described in Sasaki *et al*. (2001) (see **Fig. 2.1**). They were backcrossed for at least 5 generations onto a C57Bl6/J mouse strain to achieve >98% genetic background similarity (the rest is 129SvEvBrd strain). Homozygous disruption of *Map2k7* results in embryonic lethality, thus only WT and *Map2k7*$^{+/-}$ mice are available to study. WT and HZ mice were fertile together and were always mated in-house with WT x HZ breeding pairs, giving ~ 50% WT mice and ~ 50% *Map2k7*$^{+/-}$ pups; WT littermates were always used as controls. Half of breeding pairs consisted of WT females with HZ males, and half consisted of WT males with HZ females. Mice were weaned and genotyped (utilising the genotyping protocol outlined in **Section 2.2**) at 3 weeks of age. All mice were kept in a humidity and temperature-controlled room (21°C, 45–65% humidity). Animal weight, age, light cycle and testing times vary for each experiment so these details are given in the "**Materials and Methods**" section of the corresponding chapter. It was not possible to calculate sample sizes for each experiment using power analyses because no previous data had been obtained using *Map2k7*$^{+/-}$ mice for the experiments in this thesis. Therefore, all sample sizes chosen were based on wildtype mouse data obtained by our lab for the corresponding experiment. All behavioural testing and procedures were carried out in accordance with the Animals (Scientific Procedures) Act, 1986.

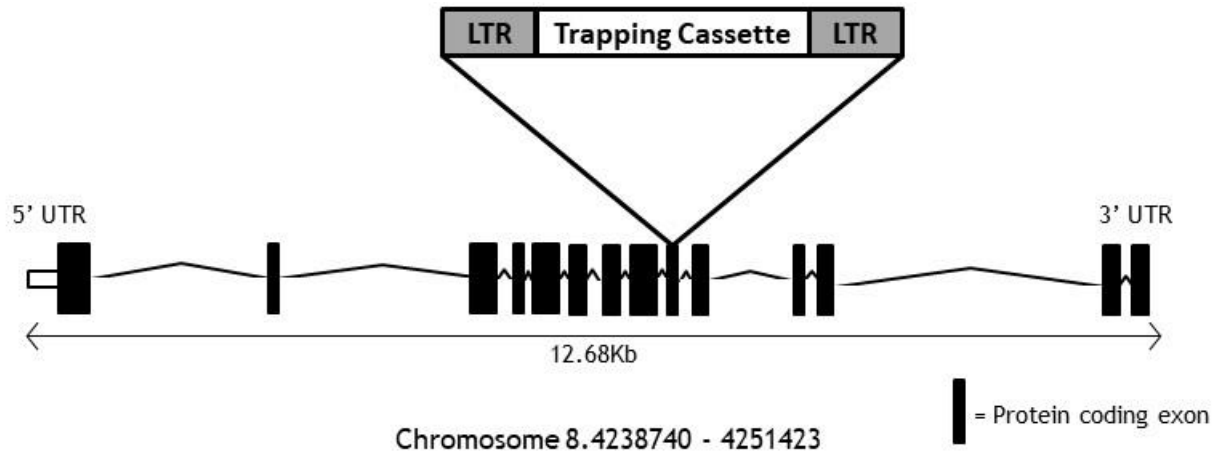


Figure 2.1. Disruption of *Map2k7*$^{+/-}$ mice was induced by insertion of a cassette into exon 9. Not to scale. UTR = untranslated region; LTR = long terminal repeat.

2.2 Genotyping

Genotyping followed a standard protocol of DNA extraction, polymerase chain reaction (PCR) to amplify gene areas of interest, then gel electrophoresis and examining bands under UV light to identify the genotype. If problems occurred during the genotyping protocol (for example, no bands on the gel), DNA quantity and quality was then measured (**Section 2.2.4**) and PCR repeated with adjustments as necessary.

2.2.1 DNA Extraction

Ear clippings were obtained from mice using a 2mm diameter ear punch at weaning (aged 3 weeks old), placed in a 1.5ml Eppendorf tube and kept at -20°C until digestion.

38µl 0.5M EDTA at pH8 (Invitrogen, #15575020), 7µl Proteinase K (Invitrogen, #25530015) and 155µl of cell lysis solution (Buffer ATL; QIAGEN, #19076) were added to each sample in the Eppendorf and vortexed to ensure the ear clipping was submerged in the fluid. The ear clippings were then digested overnight at 50°C with gentle shaking.

The following day, tubes were heated to 99°C for 10 minutes, then 470µl 100% ethanol added and vortexed. Samples were centrifuged at 13,000rpm for 5 minutes, supernatant discarded, 1ml 70% ethanol added to each tube and centrifuged again at 13,000rpm for 2 minutes. The supernatant was then

discarded, and DNA was air-dried for 30 minutes at 37°C. Finally, 100µl of nuclease-free water was added to each sample and incubated in an Eppendorf Thermomixer at 50°C for 20 minutes, shaking at 1000rpm for 10 seconds per minute. DNA extracts were kept at -20°C until the PCR stage.

2.2.2 Polymerase Chain Reaction

PCR was used to amplify regions of mouse genomic DNA extracted from *Map2k7*$^{+/-}$ mice to determine their genotype using Top Taq DNA Polymerase (QIAGEN, #200203). PCR primers specific for *Map2k7* were designed using OligoPerfect™ Designer (Invitrogen, UK). Proposed forward and reverse primers were 'blasted' using Nucleotide Blast (NCBI) to ensure primers were specific to the gene of interest. See **Fig. 2.2** for primer locations: WT mice were distinguished from *Map2k7*$^{+/-}$ mice by primers designed to target regions either side of the trapping cassette insertion site (exon 9), plus a primer designed to anneal to the trapping cassette in order to identify *Map2k7*$^{+/-}$ mice. Primer sequences are displayed in **Table 2.1**.

All equipment and PCR tubes (0.5ml) were placed in a UV hood and sterilised of DNA for 30 minutes using the UV light. A master mix was then made for the samples, plus three control tubes, one each for nuclease-free water, a sample from a known wildtype mouse and a sample from a known *Map2k7*$^{+/-}$ mouse. Master mix components are outlined in **Table 2.2**. If using primers for the first time, each lyophilised primer was resuspended in nuclease-free water to 100µM. The primer mix was then made by adding 10µl 100µM primer F1, 10µl 100µM primer F2, 10µl 100µM primer R1 and 70µl nuclease free water, then added to the master mix as directed in **Table 2.2**. 18µl of master mix was added to each 0.5ml PCR tube, then 2µl of each DNA sample diluted 10X, (or controls) added to the individual tubes. The tubes were then placed in a Veriti® Thermal Cycler (ThermoFisher Scientific) and the PCR was run according to the conditions outlined in **Table 2.3**.

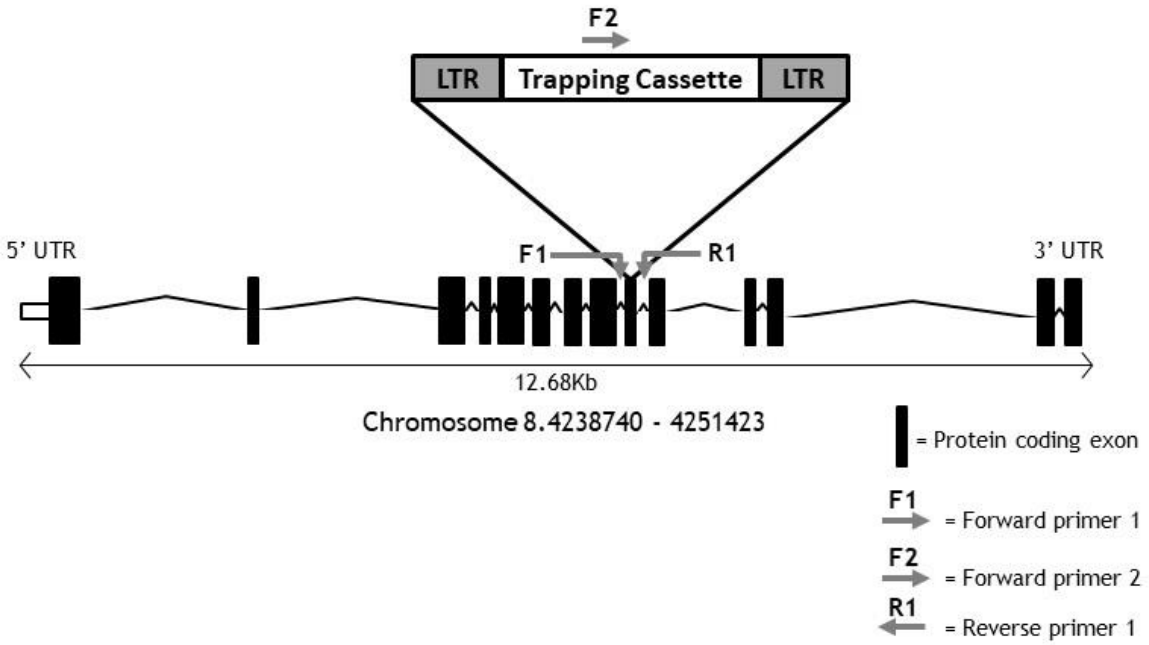


Figure 2.2. Primer locations for genotyping of *Map2k7*$^{+/-}$ mice. Forward primer 1 (F1; WT primer) is located in the intronic region between exon 8 and 9; forward primer 2 (F2; Mutant primer) is located in the trapping cassette inserted into exon 9 of *Map2k7*$^{+/-}$ mice only, and reverse primer 1 is common to both F1 and F2, located in the intronic region between exons 9 and 10.

Primer name	Sequence
Forward 1 (WT)	5' - GCTCTGTGACTTTGGCA
Reverse primer 1 (common)	5' - GCCCCAACTAACCAGTGAGA
Forward 2 (mutant)	5' - GGATGTGGAATGTGTGCGAG

Table 2.1. List and sequences of *Map2k7* primers. Three primers were used which create one product for WT mice and two products for HZ mice. These products were then run on a gel and examined. WT mice presented with one band and HZ mice presented with two bands. Primer locations are shown in **Figure 2.2** and band locations shown in **Figure 2.3**.

Component	Volume per Reaction
Nuclease free H_2O	12.5µl
10X PCR Buffer for Top Taq DNA Polymerase	2µl
2mM each dNTP	0.4µl
Primer Mix (see **Section 2.2.2**)	1µl
Coral Load	2µl
Top Taq DNA Polymerase	0.1µl
Total Reaction Volume (inc. DNA)	**20µl**

Table 2.2. Components of the master mix for PCR reaction. The components are added together to make a master mix. 18µl is added to each PCR tube, then 2µl DNA sample (diluted x 10 with nuclease-free water), to finish with a total reaction volume of 20µl.

Step	Temperature	Duration	Cycles
Polymerase activation	94°C	3 minutes	1X
Denature	94°C	30 seconds	33X
Annealing	61°C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	3 minutes	1x

Table 2.3. PCR cycling parameters for the amplification of *Map2k7* genomic DNA.

2.2.3 Gel Electrophoresis

PCR products were then separated by gel electrophoresis. 3.5% (w/v) agarose gels with Gelstar™ nucleic acid gel stain (Lonza) were prepared and 5µl PCR product was loaded per well. Gels were run for 3.5 - 4 hours at 34V and imaged on a UV light box. A representative gel is shown in **Fig. 2.3**.

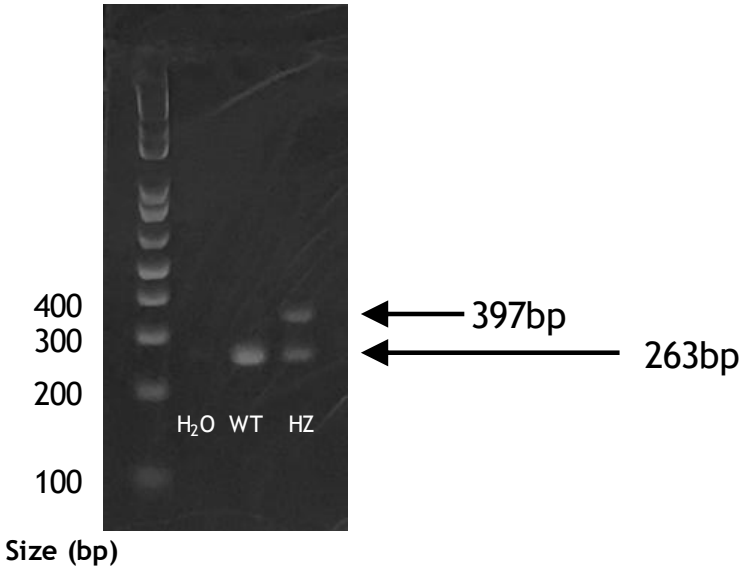


Figure 2.3. Representative gel to identify *Map2k7*$^{+/-}$ mice and their WT littermates. WT mice have a single band at 263bp, and *Map2k7*$^{+/-}$ (HZ) mice present with two bands at 263bp and 397bp. The water control (H_2O) allows for identification of contamination and should present with no bands. Bp = base pairs.

2.2.4 DNA quantification

If any problems were encountered with genotyping results, the quantity and quality of DNA samples were determined using a NanoDrop spectrophotometer (ND-1000; ThermoFisher Scientific). Prior to measurements, the spectrophotometer was blanked to sterile water and then the samples were calibrated to the buffer in which the samples were in (in this case, nuclease-free water). One microliter of each sample was measured and nucleic acid concentration was displayed by the NanoDrop in ng/ml along with 260/280 and 230/260 ratios. A 260/280 reading of between 1.6 and 2.6 is an acceptable standard of purity for DNA samples. The 230/260 measurement gives a secondary indication of sample purity: ideal readings should be ~2.0, and values significantly lower than this could indicate contamination with carbohydrates or phenols.

2.3 Statistical analysis

The details of specific statistical analyses are given in each chapter. Minitab® software was always used for analysis. For all repeated measures designs, mice were assigned a number and nested within either genotype, sex, or both as appropriate to ensure Minitab® knew that each mouse being tested within a repeated measure is the same mouse. Pairwise interactions were made as appropriate using Tukey's method. All data points (unless otherwise specified) are shown/described as the mean ± SEM.

2.4 Graphs and Figures

All data which was expressed as a percentage, or other data which could have a value of zero is shown as a bar chart. Other data which could not have a value of zero is presented as a line graph or box plot as appropriate. All difference scores are represented as a bar chart. All bar and line graphs were made using GraphPad Prism 7 and box plots were made using BoxPlotR, an application available at http://shiny.chemgrid.org/boxplotr/ and described in Nature Methods Editorial "Kick the bar chart habit" 2014, p113.

Chapter 3 MKK7 signalling pathway protein quantification in *Map2k7*$^{+/-}$ mice

3.1 Introduction

This thesis will primarily focus on behaviourally characterising mice that are heterozygous for the *Map2k7* gene in order to assess their suitability for being a model relevant to schizophrenia. It is important to verify the effect that *Map2k7* heterogeneity has on protein levels in the brain because this will establish whether there are likely to be behavioural differences related to schizophrenia (and therefore be more likely to show face validity) and will also confirm that the mice have an element of construct validity for schizophrenia. This chapter will focus on the PFC as it is a highly relevant brain area: *Map2k7* mRNA is decreased in the PFC of patients with schizophrenia (Winchester *et al.*, 2012) and the PFC has extensive molecular, structural and functional disruption in patients (described in **Section 1.5.1**).

The entire known MKK7/JNK pathway is outlined in **Fig. 1.1** and is described in detail in **Section 1.9**. However, this chapter will focus on those proteins downstream of MKK7 (outlined in **Fig. 3.1**).

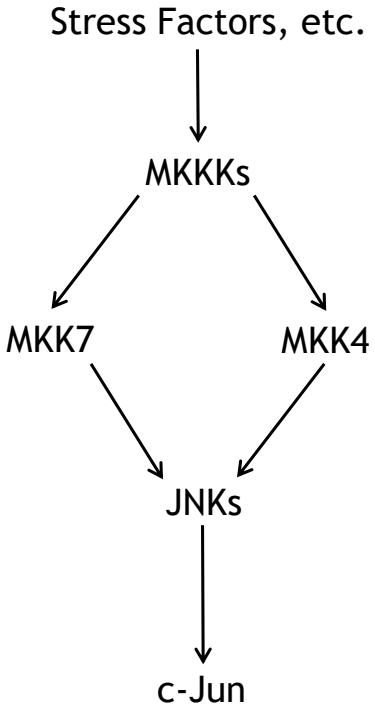


Figure 3.1. A brief overview of the MKK7/JNK signalling pathway components to be quantified by Western blotting.

MKK7 is activated in response to a variety of cellular stress signals, such as inflammatory cytokines, UV irradiation, heat/cold shock, DNA damage and osmolarity changes. These signals lead to phosphorylation of MAP kinase kinase kinases (MKKKs), for example, dual leucine zipper kinases (DLKs), mixed lineage protein kinases (MLKs), but there are many more (Johnson & Lapadat, 2002), which then activate the MAP kinase kinases MKK4 and MKK7. These, in turn, phosphorylate and activate JNK (Holland *et al.*, 1997; Kyriakis & Avruch, 2001; Watanabe *et al.*, 2002; Yao *et al.*, 1997). JNK then either translocates to the nucleus and has further effects on transcription factors, or it activates other targets within the cytoplasm (Plotnikov *et al.*, 2011). One of the most well-known targets of JNK is c-Jun (Hibi *et al.*,1993), which gave it its name and is a component of the activator protein 1 (AP-1) transcription complex (Johnson & Lapadat, 2002; Plotnikov *et al.*, 2011). The phosphorylation of c-Jun represents a central mechanism by which JNK mediates its various cellular processes, which include upregulation of genes involved in cell differentiation and proliferation, induction of an inflammatory response, and apoptosis; therefore, c-Jun activity is often observed as an indication of MKK7/JNK pathway activity levels (Bode & Dong, 2010). The *Map2k7* gene is comprised of 14 exons that are alternatively spliced to yield a group which contains two different COOH- termini (which give isoforms 1 and 2), and three different NH2-termini (alpha, beta, gamma). The individual functions of the isoforms are unclear (Asaoka & Nishina, 2010).

3.2 Aims

The aims of this chapter are to establish if/how the MKK7/JNK pathway is altered in *Map2k7*$^{+/-}$ mice by using Western Blotting to determine protein levels of each of the downstream pathway components: total JNK (46 and 54 kDa), total c-Jun (48 kDa), phospho c-Jun (49 kDa) and also total MKK4 (44 kDa), and total MKK7 (39 kDa (α isoform) and 56 kDa (γ isoform)).

We have shown in a separate cohort of mice that MKK7γ is decreased in the PFC and hippocampus (Openshaw *et al.*, 2017), and that minocycline showed signs of alleviating attentional deficits seen in *Map2k7*$^{+/-}$ mice (described in **Chapter 5**). The rationale for investigating minocycline further with respect to *Map2k7*$^{+/-}$ mice is outlined in **Section 1.7.2.3**. Minocycline is a tetracycline antibiotic that has shown promise as a potential add-on treatment along with antipsychotics for the

treatment of negative and cognitive aspects of schizophrenia (more detail about minocycline in **Section 1.7.2** and **1.7.2.1;** Chaudhry *et al.*, 2012; Jhamnani *et al.*, 2013; Levkovitz *et al.*, 2010; Liu *et al.*, 2014). To investigate how minocycline could alleviate attentional deficits in *Map2k7*$^{+/-}$ mice, this chapter will also aim to establish whether minocycline appears to produce its therapeutic effect by altering the protein levels of the components of the MKK7/JNK pathway.

3.3 Materials and Methods

3.3.1 Mice and drug administration

23 *Map2k7*$^{+/-}$ (12 female, 11 male) and 17 WT mice (6 female, 11 male) were used to supply tissue. Females weighed 21.94 ± 0.64 g and males weighed 25.10 ± 0.64 g on average and all mice were 11.06 ± 0.02 weeks old at the time of dissection. The week immediately prior to dissection, half of these mice received minocycline in a similar way to McKim *et al.* (2016): 0.5 mg/ml; protected from light (Sigma-Aldrich M9511, St. Louis, USA) in their standard drinking water for 7 days, whilst the other half received standard drinking water without minocycline (see **Table 3.1**). The dose and duration of the minocycline administration were chosen to reflect, as closely as possible, the treatment protocols that are associated with symptomatic improvement in patients with schizophrenia. Administration of minocycline to mice in drinking water at 0.5 mg/ml produces a brain concentration of around 2μM (Smith *et al.*, 2003), which is equivalent to the CSF concentrations achieved in humans during standard antibacterial dosing regimens (Agwuh & MacGowan, 2006; Macdonald *et al.*, 1973). Fresh water or minocycline solution were prepared every second day and provided at room temperature. Consumption of water and minocycline treated water was monitored daily for each cage. On average, the treated group received 99.14 ± 17.72 mg/kg/day of minocycline and, per day, mice drank 5.18 ± 0.49ml water or 4.84 ± 0.43ml minocycline treated water, which are both within the normal daily water intake range for mice (Bachmanov *et al.*, 2002).

	Water	**Minocycline**
Female WT	3	3
Female HZ	6	6
Male WT	4	7
Male HZ	6	5

Table 3.1 Numbers of mice treated with minocycline or water.

3.3.2 Protein extraction

Exactly one week after the beginning of the minocycline treatment, mice were killed by cervical dislocation. The brain was carefully removed, which was placed on a ceramic tile on ice, thus keeping it at ~ 4°C. Using a sharp scalpel, the PFC of the brain (coronal section ~1.5mm from the front of the brain without the

olfactory bulb) was removed and placed in a 1.5ml Eppendorf on ice and then frozen at -80°C until required. Total protein was then extracted from the PFC tissue as follows: approximately 20mg tissue was homogenised in RIPA buffer (10mM Tris-HCL pH 7.4, 150mM NaCl, 1mM EDTA pH 8, 0.5 % w/v NP-40, 0.1 % w/v SDS, 0.1 % w/v sodium deoxycholate), 1% w/v protease inhibitor cocktail (Sigma, P8340) and 1M phosphatase inhibitor (Na_3VO_4). Cellular extracts were then centrifuged at 10,000g for 10 minutes at 4°C and supernatant collected and frozen at -20°C until required.

3.3.3 Bradford protein assay

Protein concentrations of each individual sample were determined by using a Bradford protein assay. Bovine serum albumin (BSA) (Sigma-Aldrich, UK) was diluted in distilled water (dH_2O) to make six standards of concentrations 0, 2.5, 5, 10, 15 and 20 µg/ml. If frozen, samples were defrosted on ice and then diluted in dH_2O to ensure the protein concentration would fall on the linear portion of the standard curve. Protein assay dye reagent concentrate (Bio-Rad, #500-0006) was diluted 1:4 in dH_2O, then 200µl was added to each sample/standard and mixed thoroughly. 200µl of each standard and sample mixture were assayed in duplicate into a flat-bottomed 96-well plate and the optical density read at 595nm on a plate reader (Multiskan Spectrum, Thermo Fisher) using SkanIt™ Software 2.4.4. The duplicate readings were averaged, and the blank readings were subtracted from all other standards and samples in order to give corrected optical density. The sample readings were then interpolated from the standard curve and multiplied by the dilution factor to give the concentration of each sample in mg/ml.

3.3.4 Western blotting

Protein samples were normalised to equal levels by diluting in dH_2O according to the total protein that was quantified using the Bradford assay. The normalised samples were then denatured by heating to 80°C for 10 minutes in sample buffer (NuPAGE, Novex, NP0007) and sample reducing agent (NuPAGE, Novex, NP0004), which created a mixture that was 65% normalised protein, 10% reducing agent and 25% sample buffer, before being subjected to SDS-PAGE in 10% Bis-Tris gel (NuPAGE, Novex, NP0302BOX) and transferred to Invitrolon PVDF membrane (Novex, LC2005). Membranes were placed in TTBS buffer (20 mM Tris pH 7.6, 150

mM NaCl, 0.05% Tween-20), supplemented with 3% skimmed milk and blocked for 30 minutes at room temperature. Membranes were then incubated with the appropriate primary antibody (see **Table 3.1**) overnight at 4°C with constant agitation. The next day, they were washed 3 × 10 minutes in TTBS and then incubated with anti-rabbit secondary antibody (1:10,000; Millipore, 12-348) for 90 minutes at room temperature with constant agitation. Blots were then washed 1 x 10 minutes in TTBS, then 2 × 10 minutes in TBS (20 mM Tris-HCL pH 7.6, 150 mM NaCl). Membrane-bound secondary antibodies were detected using Chemiluminescent HRP Substrate (Immobilon, Millipore, WBKLS0100), and digital images of Western blots were captured by PXi4 (Syngene) using the appropriate exposure time as outlined in **Table 3.1**. Blots were then re-probed with housekeeping protein GAPDH-HRP antibody (1:20,000, Genetex, GTX627408) in TTBS with 1% milk for two hours at room temperature with constant agitation and then washed 1 × 10 minutes in TTBS, followed by 2 x 10 minutes in TBS. GAPDH specific bands were detected using ECL reagent (Signalfire, Cell Signalling Technologies, 68835), and digital images of Western blots were captured by PXi4 (Syngene) using an exposure time of ~3 minutes. Digital images of blots were quantified using ImageJ software (W. Rasband, NIH), then antigen values normalised to the appropriate GAPDH values. Normalised values for each antigen were then expressed as a percentage of control (WT mice that received water) from the same blot, to allow the multiple blots to be compared reliably with each other. This was calculated using the formula:

$$\% \text{ of control} = \left(\frac{\text{GAPDH-normalised value for the antigen of interest}}{\text{WT Water GAPDH-normalised values from that blot, averaged}} \right) * 100$$

Antigen	Company	Dilution	Diluent	Time	ECL	Company	Exposure time (using PXi4)
tMKK7	Genetex, GTX103563	1:10000	Signalboost, Millipore, KP31812	Overnight, 4°C	Immobilon	Millipore, WBKLS0100	~10 minutes
tMKK4	Bethyl, A302-658A	1:5000	Signalboost, Millipore, KP31812	Overnight, 4°C	Immobilon	Millipore, WBKLS0100	~15 minutes
tJNK	CST, #9258	1:10000	TTBS with 1% milk	Overnight, 4°C	Immobilon	Millipore, WBKLS0100	~10 minutes
tc-Jun	CST, #9165	1:1000	Signalboost, Millipore, KP31812	Overnight, 4°C	Immobilon	Millipore, WBKLS0100	~15 Minutes
pc-Jun	CST, #9166	1:1000	TTBS with 5% BSA	Overnight, 4°C	Immobilon	Millipore, WBKLS0101	~10 minutes
GAPDH-HRP	Genetex, GTX627408	1:20000	TTBS with 1% milk	2 hours, Room Temp.	Signalfire	CST, 68835	~3 minutes

Table 3.2 Primary antibodies used for Western blotting. CST = Cell Signalling Technologies, BSA = bovine serum albumin, ECL = enhanced chemiluminescence

3.3.5 Statistical Analysis

Data from each antigen expressed as a % of control were analysed separately by a three-way ANOVA with sex, treatment (water or minocycline) and genotype (WT or *Map2k7*$^{+/-}$*)* as between subjects factors with Tukey's post hoc analysis where appropriate.

For tMKK7γ, there were 4 extreme outliers on one blot, so these were removed from analysis. There was also an extreme outlier for c-Jun and two extreme outliers for tJNK, so these were also removed. All extreme outliers in the current experiment were identified from Minitab® 17 software as a Large Standardised Residual. Each outlier was first removed, and the data reanalysed to determine whether it was an influential observation that had a disproportionate impact on the ANOVA model. If it had no effect, it was kept; if the model changed significantly, the outlier was investigated further. First, a check was made for data input errors. Next, the origin of the data was investigated. The outliers from these Western blots may have occurred because the control (WT Water) for the particular blot they came from was unusually faint, so when expressing as a % of control the data was skewed. The outliers did not exist when looking at protein levels normalised to GAPDH for that blot, therefore, it was necessary to remove them from % of control analyses.

3.4 Results

3.4.1 Map2k7$^{+/-}$ *mice have decreased MKK7 protein in the PFC*

Western blotting shows 56 kDa MKK7 (γ isoform; effect of genotype: $F_{(1,28)}$=40.97; p<0.0001), but not 38 kDa MKK7 (α isoform; $F_{(1,32)}$=0.01; p=0.905), to be significantly decreased in the PFC of *Map2k7*$^{+/-}$ mice compared to their wildtype littermates (**Fig. 3.2**). For both MKK7α and γ, there were no significant effects of treatment, sex or any interactions between them (p>0.05).

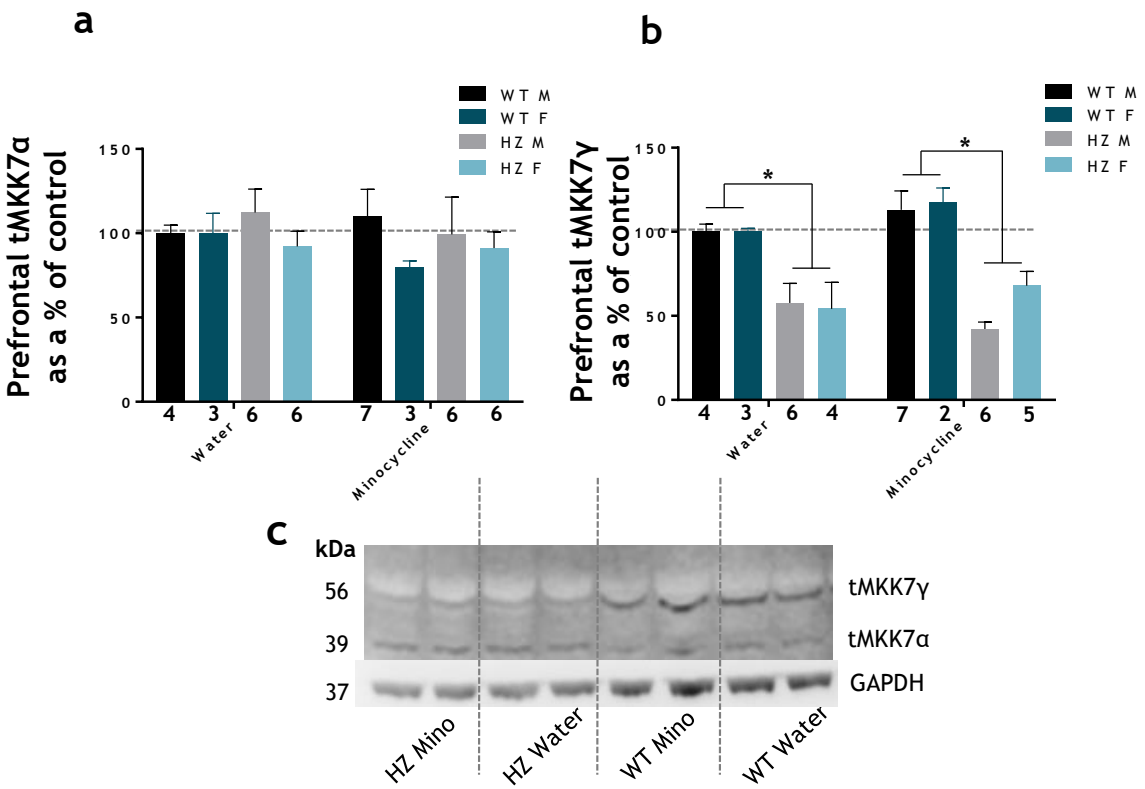


Figure 3.2. MKK7 protein levels in the PFC as determined by Western blotting. a) Total MKK7α protein levels in *Map2k7*$^{+/-}$ PFC were similar to WT levels. **b**) *Map2k7*$^{+/-}$ mice have reduced total MKK7γ protein in the PFC compared to WT littermates. **c**) representative Western blot. MKK7γ and α levels were normalised to GAPDH levels from the same blot and then expressed as a percentage of control (WT Water). Minocycline did not significantly alter protein levels of tMKK7α or tMKK7γ. Numbers under each bar represent the *n* number of that group. Four extreme outliers from a single Western blot were removed from MKK7γ analysis. Data were analysed by a 3-way ANOVA with genotype, sex and treatment as between subjects factors. 2- and 3-way pairwise comparisons were made between all factors using Tukey's method. *p<0.05 (ANOVA).

3.4.2 Map2k7$^{+/-}$ *mice have similar levels to WTs for all other proteins measured in the PFC*

Protein levels in the PFC were similar in *Map2k7*$^{+/-}$ mice compared to their wildtype littermates for tMKK4, tJNK, tc-Jun and pc-Jun. tMKK4, tJNK and tc-Jun had no significant effects of treatment, sex or any interactions between them (**Fig. 3.3**; $p>0.05$). For pc-Jun, there was an overall significant genotype x treatment interaction ($p=0.015$; $F_{(1,20)}=7.05$); however, Tukey's post hoc analysis did not reveal where this statistical significance arose.

3.4.3 Minocycline does not alter protein levels of MKK7/JNK pathway components

Following seven days of minocycline treatment, the levels of all PFC proteins measured (tMKK7α, tMKK7γ, tMKK4, tJNK, tc-Jun and pc-Jun) were not altered compared to mice that received water (**Fig 3.2** and **3.3**; $p>0.05$ for all).

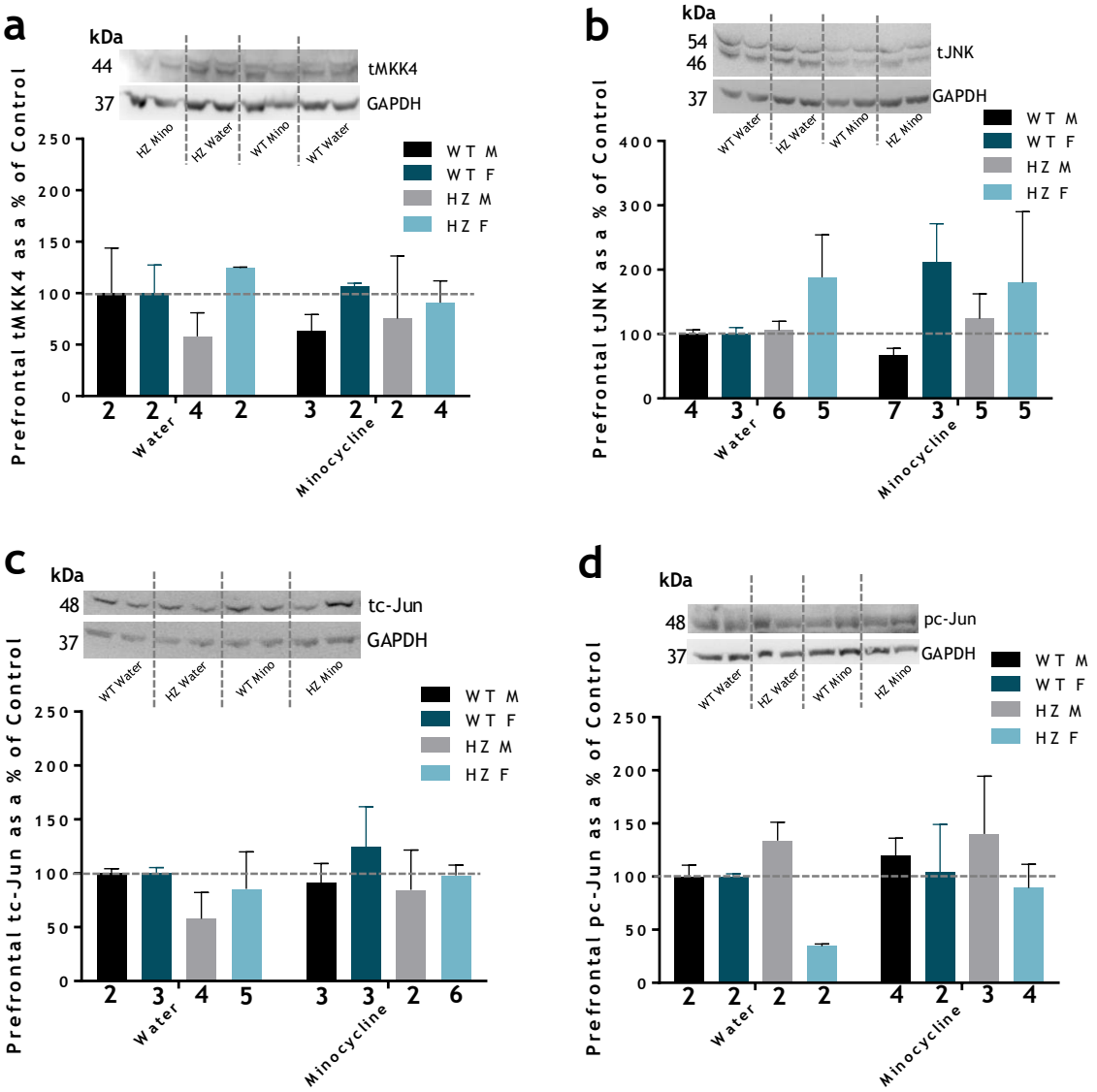


Figure 3.3. PFC levels of various proteins from components of the MKK7/JNK pathway as determined by Western blotting. Representative Western blots shown above the corresponding graph. tMKK4 (**a**), tJNK (**b**), tc-Jun (**c**) and pc-Jun (**d**) all showed similar protein levels in the PFC in *Map2k7*$^{+/-}$ mice compared with WT littermates. There were no significant effects of minocycline on the levels of any of the proteins measured. All protein levels were normalised to GAPDH levels from the same blot and then expressed as a percentage of control (WT Water). Numbers under each bar represent the n number of that group. Two extreme outliers were removed from tJNK analysis and one extreme outlier was removed from tc-Jun analysis. Data were analysed by a 3-way ANOVA with genotype, sex and treatment as between subjects factors. 2- and 3-way pairwise comparisons were made between all factors using Tukey's method.

3.5 Discussion

Western blotting revealed that MKK7γ was significantly decreased in the PFC of *Map2k7*$^{+/-}$ mice compared to their wildtype littermates. On the other hand, protein levels of MKK7α, JNK, MKK4, tcJun and pc-Jun were not altered in the PFC (**Fig. 3.4**). Minocycline had no significant effect on protein levels in the PFC, including that of the decreased MKK7γ, suggesting that it does not produce its therapeutic effects by restoring MKK7γ protein levels in the MKK7/JNK pathway in the PFC. These results show that *Map2k7* heterogeneity may have been compensated for by MKK7α, but not MKK7γ, and that downstream pathway components do not have altered protein levels as a consequence of reduced MKK7γ as measured by Western blotting in the current experiment.

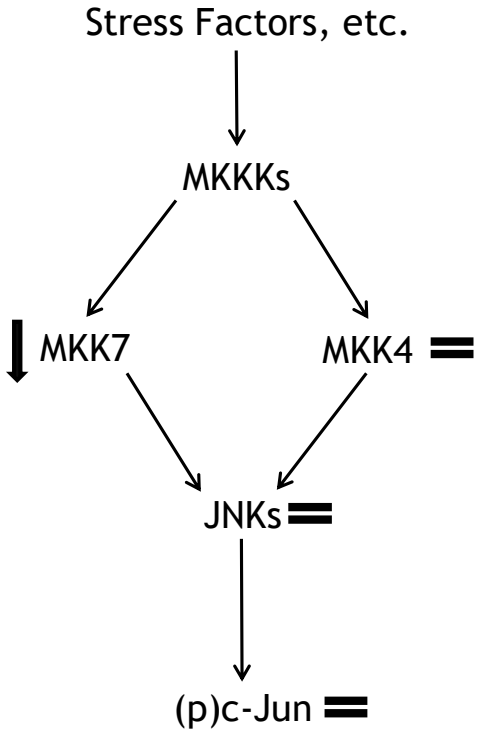


Figure 3.4. Simplified overview of MKK7/JNK signalling pathway components and how their levels are altered in *Map2k7*$^{+/-}$ mice compared to WT littermates, as determined by Western blotting. The levels of tMKK7γ are significantly reduced compared to WTs, however the levels of tMKK7α, tMKK4, tJNK, tc-Jun and pc-Jun remain similar in WT and *Map2k7*$^{+/-}$ mice.

3.5.1 The γ isoform of MKK7 is decreased in the PFC of Map2k7$^{+/-}$ *mice*

Western blotting confirmed that MKK7γ is decreased in the PFC of *Map2k7*$^{+/-}$ mice; however, MKK7α and the other downstream proteins in **Fig 3.4** remain similar to WT levels. It is not clear what the physiological relevance of the different isoforms

of MKK7 are (Asaoka & Nishina, 2010; Wang *et al.*, 2007); however, using a coupled protein kinase assay *in vitro,* Tournier *et al.* (1999) showed that, under basal conditions, MKK7α has a lower level of activity for JNK than MKK7β and γ (which have similar activities for JNK), suggesting that the different isoforms of MKK7 may have different functions (Haeusgen *et al.*, 2011). Also, MKK7γ is the only isoform of MKK7 that can bind Filamin A, which plays an important role in the organisation of actin filaments in the cytoskeleton and forms very strong signalling complexes together with MKK4 (Nakagawa *et al.*, 2010). Activation of JNK is significantly stronger when activated by MKK4 plus MKK7 than either separately (Asaoka & Nishina, 2010), so this suggests that MKK7γ can have a powerful effect on JNK signalling, perhaps more so that the other isoforms.

We have replicated these results of MKK7γ but not MKK7α being decreased in the PFC of *Map2k7*$^{+/-}$ mice compared to WTs in a separate cohort of mice, and which also occurred in the hippocampus (Openshaw *et al.*, 2017), demonstrating that this is a robust finding relevant to different brain areas. The specific importance of MKK7γ in schizophrenia is emphasised by the fact that one of the two SNPs increased in a cohort of patients with schizophrenia vs. healthy controls is located immediately upstream of an alternatively spliced exon only present in the MKK7γ isoform (Winchester *et al.*, 2012). Moreover, the location of this SNP is in a region that is associated with particularly high levels of inter-species sequence conservation, and highlights the significance of this part of the gene in the MKK7γ isoform (Winchester *et al.*, 2012).

Decreased MKK7γ in the PFC and HC are likely to have a disruptive effect on behaviour because hippocampal-prefrontal interactions occur in various cognitive and behavioural functions, and disruption of the PFC is consistently implicated in psychiatric disease (reviewed in Sigurdsson & Duvarci (2016) and discussed further in **Section 1.5.1**). The potential functional implications are explored behaviourally in the next chapters of this thesis. However, the reason why only the γ isoform of MKK7 is decreased is not clear, and is surprising considering the disrupted portion of the gene in *Map2k7*$^{+/-}$ mice is not within the first four exons (Sasaki *et al.*, 2001), which is where alternative splicing occurs to create either the α, β or γ isoforms (Tournier *et al.*, 1999). It is most likely that compensatory mechanisms have occurred (discussed further below); however, additional experiments will be required to shed more light on this.

3.5.2 The potential impact of decreased MKK7γ from early development

As *Map2k7*$^{+/-}$ mice have had *Map2k7* heterogeneity in all tissues from the beginning of conception, it is likely this will have an impact on development of the brain as MKK7 is required for various aspects of embryogenesis, including brain development and mammalian body plan organisation (reviewed in Asaoka & Nishina, 2010). The role of MKK7 in development is striking, as *Map2k7* full knockout (KO) mice die between embryonic day (E) 11.5 and 13.5 (Wada *et al.*, 2004). Conditional *Map2k7* KO mice in which *Map2k7* was specifically knocked out in the nervous system showed enlarged ventricles, a pathological hallmark of schizophrenia, and showed that MKK7 plays a major role in neuronal migration and axon elongation (Yamasaki *et al.*, 2011). This would certainly have an impact on the extent and quality of connectivity, structure and function of the brain once fully developed. However, these mice died immediately after birth, preventing any further molecular and/or behavioural investigation, but still provided information that is likely to also be relevant for the development of the brain in mice that are heterozygous for *Map2k7*.

Analysis of knockout mice for other members of the MKK7/JNK pathway have also been examined: *Map2k4* KO mice die at E10.5 and *Map2k4 Map2k7* double KO mice die at E9.5, earlier than either KO separately (Asaoka & Nishina, 2010), showing that MKK4 and MKK7 have non-redundant functions *in vivo* but they can compensate for each other to some extent. Additionally, KO mice for the different isoforms of JNK have been investigated (reviewed in Yamasaki *et al.*, 2012): *Jnk1, 2* and *3* KOs (separately) can survive postnatally but *Jnk1* KOs show dysregulation of neuronal migration and dendritic architecture, and *Jnk2* and *Jnk3* KOs show resistance to neuronal stress, demonstrating their necessity in stress-induced neuronal cell death in the adult brain. Finally, *Jnk1 Jnk2* double KO mice die during development due to defective neural tube morphogenesis and reduced apoptosis in the hindbrain (Kuan *et al.*, 1999; Sabapathy *et al.*, 1999). Altogether, these KO studies show the importance of the MKK7/JNK pathway from very early stages of brain development; produce effects that are related to schizophrenia pathology, and also may have consequences on the brain that continue into adulthood (see **Section 1.9.1** and **1.9.2** for further discussion on this). Although only MKK7γ was decreased at adulthood in *Map2k7*$^{+/-}$ mice in the current experiment, it is possible that other proteins in the pathway are altered more

subtly than can be detected by Western blotting, are only altered within certain cell types or parts of the cell, and/or other proteins were indeed altered but were not measured here. It is also feasible that pathological alterations had already occurred throughout development but the brain levels of other molecules in the pathway have adapted back to normal levels by adulthood. This is supported in a study by Yamasaki *et al*. (2017), who found relatively subtle changes in components of the MKK7/JNK pathway, even with complete deletion of MKK7 in the brain, as well as changes in pJNK levels throughout embryonic development. It would be interesting to examine the levels of protein of MKK7/JNK pathway components during earlier stages of development, and to investigate protein levels utilising a more sensitive technique, such as ELISA.

The vast majority of cellular signalling pathways in the CNS, including the MKK7/JNK pathway, are extremely tightly controlled (Winchester *et al.*, 2012). Therefore, mice heterozygous for *Map2k7* with accompanying decreased protein expression throughout the course of development may show adjustment in other components of the pathway (or from other interacting proteins) in an attempt to compensate for lack of MKK7 (Pratt *et al.*, 2012). *Map2k4* and *Map2k7* genes cannot compensate for each other in vivo (Asaoka & Nishina, 2010); however, other components of the MKK7/JNK pathway in the brain have been shown to compensate for each other, such as JNK1 increasing for lack of *Jnk2* (Chen *et al.*, 2005) and JNK2 increasing for lack of *Jnk3* (Brecht *et al.*, 2005). On the other hand, MKK7 has been shown to compensate for over-activation of itself: Winchester *et al*. (2012) showed that increased MKK7 activation by sorbitol in embryonic mouse cortical neurons initially increased phosphorylated MKK7, then dramatically reduced MKK7 expression 24 hours afterwards. In the current study, there are no signs of other downstream proteins in the pathway increasing or decreasing for lack of MKK7γ, but it may be that the α isoform has indeed compensated for lack of itself, as its protein level appears normal. Again, it will be important to investigate protein levels at distinct stages of embryonic development. In addition, the study by Winchester *et al*. (2012) highlights the need to examine gene expression levels as well as protein levels in order to get a more accurate picture of any compensatory mechanisms that may be occurring.

3.5.3 Minocycline does not alter protein levels of any component of the MKK7/JNK pathway

Minocycline or standard drinking water was administered to *Map2k7*$^{+/-}$ and WT mice for one week in order to investigate whether minocycline alters the protein levels of the components of the MKK7/JNK pathway. It was of particular interest to establish whether minocycline restored any protein alterations in *Map2k7*$^{+/-}$ mice back to WT levels. However, minocycline had no significant effect on protein levels in the PFC, including that of the decreased MKK7γ in *Map2k7*$^{+/-}$ mice.

Minocycline has previously been shown to activate MKK7 in cultured neuronal cells (spoken communication with Prof. Brian Morris, unpublished data), and inhibit JNK1/2 in microglia following lipopolysaccharide stimulation (Nikodemova *et al.*, 2006), so it is proven possible for minocycline to interact with the MKK7/JNK pathway. However, these results also suggest that minocycline acts in a cell- and/or stimulus-specific manner which may not have been strong enough to be identified via Western blotting, particularly if other cell types are present in which minocycline does not mediate its effect as this may mask any significant differences. Additionally, minocycline can act via alternative pathways; for example, by affecting a non-MKK7/JNK pathway (such as p38 MAPK) downstream of NMDA receptors (Chaves *et al.*, 2009). In a case such as this, it is unlikely that an alteration in protein levels in the MKK7/JNK pathway would be detected.

3.5.4 Future Directions

This chapter has provided useful information, such as showing that MKK7γ is decreased in the PFC of *Map2k7*$^{+/-}$ mice; however, there are further experiments that would be necessary to carry out in order to further validate any findings and to provide more detailed information regarding how this pathway is disrupted in *Map2k7*$^{+/-}$ mice. As briefly mentioned above, it will be beneficial to look at MKK7 in other brain areas, and to use a more sensitive method of protein quantification, such as ELISA. It would be interesting to look at the proteins at an earlier stage of development; in individual cell types, such as microglia, astrocytes and neurons; in separate cellular compartments (JNK signalling is known to have different effects depending on the cellular component in which the signalling takes place; Coffey, 2014), and to look at other proteins that interact with the MKK7/JNK pathway. This could include upstream proteins such as the MKKK DLK, which is an

important MKKK with respect to MKK7 activation (Haeusgen *et al.*, 2011). DLK-mediated activation of MKK7 has been shown to position JNK signalling modules in neurites in order to control microtubule bundling in embryonic hippocampal neurons (Feltrin *et al.*, 2012), and DLK KO mice produce major disruption of neuronal migration and axon elongation , similar to what is seen in *Map2k7* conditional KO mice. (Yamasaki *et al.*, 2012). Furthermore, DLK is almost exclusively expressed in neural tissues in the developing mouse embryo, including central, peripheral and autonomic nervous systems; the only exception outside of the nervous system is that it is also temporally expressed in the liver at E11, which, interestingly, is the same time at which *Map2k7* KO mice die due to impaired liver formation. Finally, it is of interest to investigate any changes in the levels of inhibitory molecules that interact with the MKK7/JNK pathway, such as phosphatases, vaccinia-related kinases (VRKs), cFLIP and ITCH (Haeusgen *et al.*, 2011).

3.5.5 Conclusion

To conclude, *Map2k7*$^{+/-}$ mice have decreased MKK7γ in the PFC compared to WT mice, showing good construct validity. Although the protein levels in some of the downstream components of the pathway are unaffected as measured by Western blotting, it remains possible that behavioural effects will be seen as *Map2k7*$^{+/-}$ mice have had decreased MKK7 throughout the whole of development and the MKK7/JNK pathway is vital for the developing brain. Finally, minocycline did not affect the protein level of downstream MKK7/JNK signalling pathway; however, it may exert any therapeutic effects reported in **Chapters 4** and **5** via other means. The following chapters will consider the face validity of *Map2k7*$^{+/-}$ mice by characterising their behavioural phenotype, as well as investigate them as a suitable gene x environment interaction risk model with relevance to schizophrenia.

Chapter 4 Positive symptom-related tasks

4.1 Introduction

The positive symptoms of schizophrenia, such as hallucinations and delusions, are likely to be uniquely human because of other animals' inability to express and/or experience them. Although not necessarily producing the same behaviours as those seen in humans, positive symptoms can be explored in rodent models by using behavioural studies which utilise similar areas of the brain and neurotransmitter systems to those which are dysfunctional in patients with schizophrenia that cause positive symptoms (van den Buuse, 2010). Examining locomotor activity, sensorimotor gating and sensitivity to psychotomimetic drugs are the main behavioural measures used to identify phenotypes relevant to the positive symptoms (Van Den Buuse 2010; Powell & Miyakawa 2006; Pratt *et al.*, 2012).

4.1.1 Sensorimotor gating

Sensorimotor gating is a physiological process that filters out irrelevant, incoming sensory stimuli as it is transmitted to motor output systems. This is thought to occur so that potentially disruptive reactions (such as the startle reflex) can be attenuated until conscious perception of the startling stimulus is completed (Graham, 1975). Sensorimotor gating abilities are reliably examined by measuring the extent of prepulse inhibition (PPI) of the startle response, a cross-species paradigm with excellent face validity (depicted in **Fig. 4.1;** Graham, 1975; Swerdlow & Geyer, 1998).

In the majority of mammals, including humans and mice, the startle response is in the form of sudden movement, or "jumping", and can be measured (in rodents) using an accelerometer to quantify the whole-body startle amplitude. In humans, the startle response is usually measured by eye blink response (e.g. Braff *et al.*, 1992). PPI is a normal, automatic, pre-conscious inhibition of the startle response when the startling stimulus is preceded closely (~100ms) by a much weaker, non-startling "prepulse" (Graham, 1975), and its measurement is robust and consistent over time in healthy adult humans (Swerdlow *et al.*, 2017). Patients with schizophrenia reliably present with reduced PPI, to the extent where it is considered to be an endophenotype (Braff *et al.*, 2007; Swerdlow *et al.*, 2008;

Swerdlow *et al.*, 2014). Additionally, the degree of reduced PPI in patients with schizophrenia has been shown to correlate with extent of psychotic symptoms and is also reversed with antipsychotics, in a way in which treatment positively correlates with drug clinical potency (Swerdlow & Geyer 1998). However, this is controversial; for example, Ludewig & Vollenweider (2002) did not find any correlation between psychotic symptoms and degree of reduced PPI in patients.

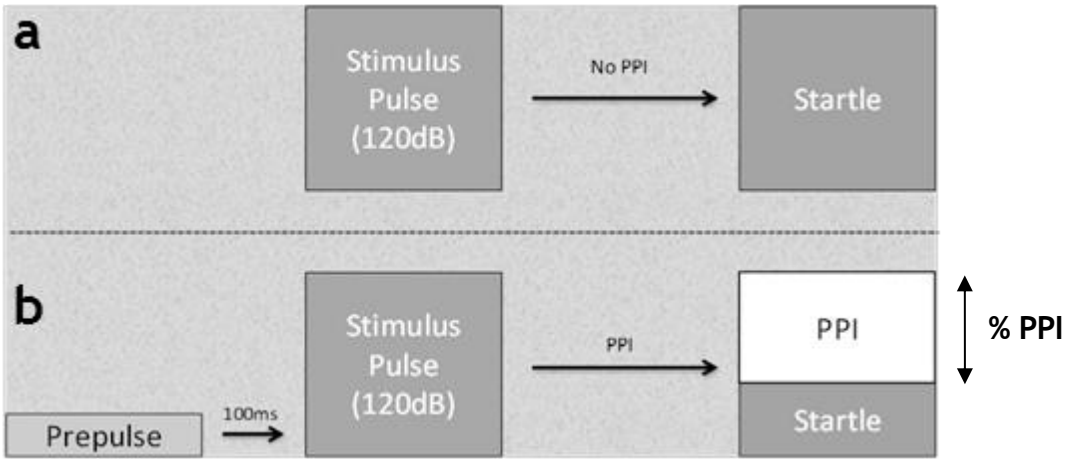


Figure 4.1. Schematic depiction of PPI. a) Normal startle response to a 120dB startling stimulus above background noise. **b**) When a prepulse precedes the startling stimulus by ~100ms, the startle response is attenuated. The percentage of full startle that is attenuated is the extent of prepulse inhibition.

As the measurement of normal PPI is a consistent phenomenon in all species, deficits in PPI have emerged as a phenotype used for identifying genetic mouse models of potential relevance to schizophrenia (Pratt *et al.*, 2012). Additionally, PPI represents an excellent way to examine the sensitivity of models to psychotomimetics. Many studies have demonstrated the psychotomimetic effects of dopaminergic agonists, such as amphetamine, on PPI (e.g. Varty *et al.*, 2001). Indeed, amphetamine administration in rodents have shown that dopamine is a regulator of PPI and it is suggested that increased mesolimbic dopamine (DA) activity, a dopaminergic reward pathway connecting the ventral tegmental area to the nucleus accumbens, mediates amphetamine-induced disruption of PPI (Swerdlow *et al.*, 1990).

4.1.2 Locomotor hyperactivity

Baseline locomotor hyperactivity has also been shown to reflect aberrant mesolimbic dopaminergic activity in rodents, and is believed to relate to psychotic

episodes and the positive symptoms seen in schizophrenia (Jones *et al.*, 2011; Pratt *et al.*, 2012). The underlying neural mechanisms for hyperactivity in rodents and the positive symptoms of schizophrenia in humans are similar; however, the behavioural effects are different aside from showing aspects of psychomotor agitation (van den Buuse, 2010). Hence, although its face validity is questionable, this observation is not without value (Pratt *et al.*, 2012). Many genetic animal models with relevance to schizophrenia show hyperactivity in some form (either in response to a novel environment or generally across behavioural tests), including dopamine transporter KO, NRG1 heterozygous, NRG3 KO, Drd1 KO, ErbB4 conditional KO (parvalbumin-positive neurons only), and NMDAR glycine binding site mutant mice (Powell & Miyakawa, 2006; Wen *et al.*, 2010; Hayes *et al.*, 2016; and summarised in Pratt *et al.*, 2012).

Additionally, it is widely recognised that mild psychotic-like experiences frequently occur in a muted form during childhood or adolescence in patients with schizophrenia before they are formally diagnosed. This period, from the onset of deviation from normal behaviour to diagnosis, is referred to as the schizophrenia "initial prodrome" (Yung & McGorry, 1996). It is of significant interest to study this period because it has the most potential to provide information that could lead to early intervention and the identification of biological markers. Animal models of the schizophrenia prodromal state also display hyperactivity (Tenn *et al.*, 2005) and so part of this chapter will aim to discover whether hyperactivity is apparent in *Map2k7*$^{+/-}$ mice during early adolescence, as well as during adulthood.

4.2 Aims

This chapter will investigate *Map2k7*$^{+/-}$ mice in tasks that are thought to be sensitive to dopaminergic dysfunction and are strongly implicated with the positive symptoms of schizophrenia. Locomotor activity in the open field at adolescence and adulthood, and following minocycline treatment at adulthood will be examined. Additionally, PPI of the acoustic startle response under the influence of an acute dose of amphetamine will be carried out in order to investigate the integrity of dopaminergic systems in *Map2k7*$^{+/-}$ mice.

4.3 Materials and Methods

4.3.1 Mice

40 Mice were used in the following PPI and open field experiments: 17 WT (11M, 6F), 23 *Map2k7*$^{+/-}$ (HZ; 11M, 12F), group housed with between 9-12 mice per cage on a 12:12 reverse light/dark cycle (lights off at 7am). They weighed an average of 21.87 ± 0.46g and underwent PPI at 7.51 ± 0.026 weeks old. For the open field, these same 40 mice were tested initially, then 24 additional mice were tested when the first group of mice reached adulthood (15 WT (9M, 6F), 9 HZ (8F, 1M)). At adolescence, the 64 mice were 5.58 ± 0.03 weeks old and weighed 18.10 ± 0.30g. At adulthood, they were 10.11 ± 0.04 weeks old, weighed 23.28 ± 0.43g, and were separated 3 days prior to open field testing at adulthood so that they were housed in smaller groups of 2, 3 or 4. This was so that minocycline administration could be monitored more accurately for each mouse. One mouse in the additional group of mice died during the open field experimental period (a HZ female); all data from that mouse were excluded from open field analyses.

4.3.2 PPI apparatus

Four identical startle chambers (**Fig. 4.2;** SR-LAB, San Diego Instruments, San Diego, CA) were used to measure startle reactivity. Each chamber consisted of a sound attenuated cabinet (inside height: 28.7cm, inside width: 28.7cm, inside depth: 30cm) that was lit and ventilated, with a Plexiglass cylinder (3.7cm inner diameter, 12.7cm long) situated on top of a removable stand in which a piezoelectric accelerometer was attached underneath. A speaker was mounted 24cm above the cylinder and played acoustic stimuli in the form of white noise. During a session, the animal was placed within the cylinder with both ends blocked off by Plexiglass attachments. Motion was detected by the accelerometer and recorded as analog signals which were then stored by a computer. The delivery of acoustic stimuli and recording of responses were controlled by SR-LAB software. All four chambers were calibrated to each other so that they had similar baseline values to movement, delivered startle stimuli simultaneously, and at the same decibel level.

A continuous background noise level of 65dB was maintained throughout all sessions to provide a consistent acoustic environment and to mask any external noises that may have passed through the sound attenuated cabinet. All stimuli

were delivered as xdB above background noise. All chambers were cleaned with soap and warm water and dried in between each mouse, and each mouse was tested at the same time of day in the same chamber for all sessions. Care was taken to ensure that no chamber was being used for all mice of one particular group; for example, all WT/*Map2k7*$^{+/-}$ or all male/female. The first session consisted of the startle curve, which aims to verify that hearing is intact in the mice. The second and third sessions were PPI test sessions with amphetamine (counterbalanced): the drug administration procedure is outlined in **Section 4.3.5.**

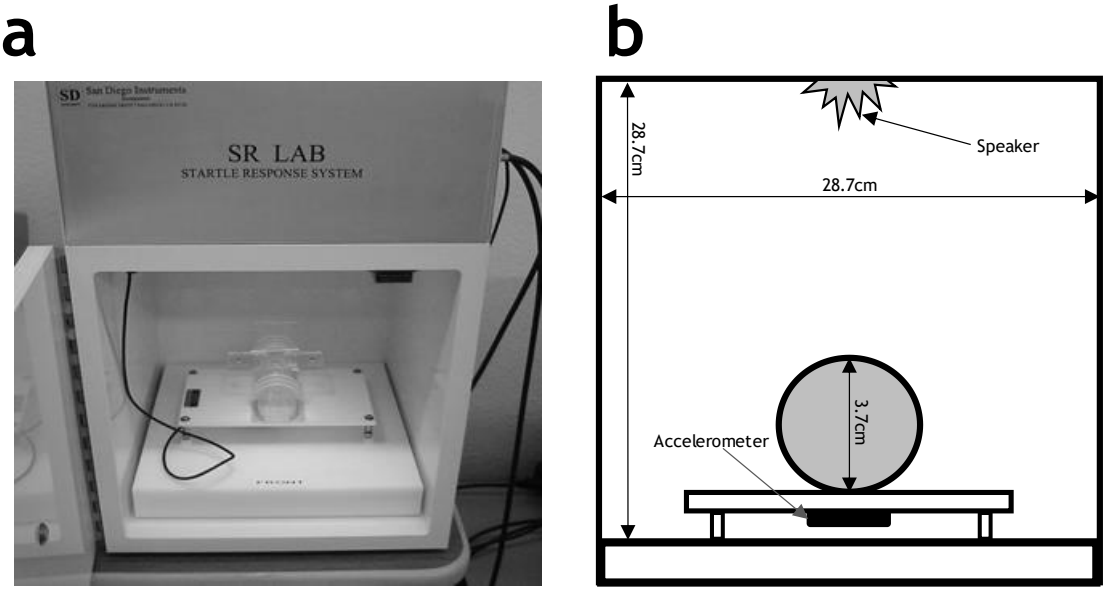


Figure 4.2. a) The startle response system. b) A schematic diagram of the view looking in the front of the startle response system. The mouse is placed in the centre of the Plexiglas cylinder and blocked in by two Plexiglas attachments at either end of the cylinder (which is 12.7cm long). Startle stimuli in the form of white noise is played through the speaker on the roof of the soundproof cabinet. Movements from the mouse are recorded by the accelerometer placed on the stand below the cylinder containing the mouse.

4.3.3 Startle curve

The startle curve session began with a 5-minute acclimation period where background white noise (65dB) was played continuously. 5 x 40ms, 120dB startling stimuli were played following the acclimation period to partially habituate the animals to the startling stimulus. Following this, the test session began, which consisted of 6 repetitions of each of the following 40ms-long stimuli: 65, 69, 73,

77, 85, 90, 100, 110 and 120 dB above background. The stimuli were presented in a random order with inter-trial intervals averaging ~15 seconds, but were either (randomly) 12, 13, 14, 15, 16 or 17 seconds long. The startle response was recorded throughout the duration of each stimulus and the peak amplitude of each response were analysed.

4.3.4 PPI test session

The PPI session began with a 5-minute acclimation period where background white noise (65dB) was played continuously. 5 x 40ms, 120dB startling stimuli were played following the acclimation period to partially habituate the animals to the startling stimulus. Following this, the PPI test session began, which consisted of 10 repetitions of each of the following prepulse trials: a 20 ms prepulse of either 4, 6 or 8 dB above background, followed by a 100ms inter-pulse interval, then a 40ms startling stimulus at 120dB above background. Randomly interspersed between prepulse trials were 10 x 120dB startling stimuli alone and 10 x "no stimulus" trials in which movements were recorded but no stimulus was delivered. These trials were presented in a random order with inter-trial intervals averaging ~15 seconds, but were either (randomly) 12, 13, 14, 15, 16 or 17 seconds long. Movements of the animal were recorded for 40ms from the beginning of the 120dB startling stimulus, or, in the case of "no stimulus" trials, from the end of the inter-trial interval for 10 ms. The peak amplitude of each response was used in the formulas in **Section 4.3.6** for analysis. The session finished with 5 x "120dB startle only" trials to give an indication of overall habituation to the startle response the mice exhibited when compared with the first 5 x "120dB startle only" trials.

4.3.5 PPI drug administration

The effect of an acute dose of D-amphetamine on % PPI was assessed. The amphetamine dose was chosen based on previous publications in the literature which suggested that 5mg/kg causes disruption of PPI in mice on a C57Bl/6 background (Martin *et al.*, 2008). Amphetamine administration was counterbalanced such that 5mg/kg D-amphetamine (Sigma Aldrich, A-5880) or 2ml/kg saline were injected interperitoneally on the first day, 5 minutes prior to PPI testing. Mice were then tested normally without drug on the following day and then D-amphetamine or saline were administered to the other half of the group on the third day, 5 minutes prior to PPI testing.

4.3.6 PPI statistical analysis

All statistical analyses were carried out using Minitab® 17 software. Results were considered significant if $p<0.05$. All error bars are expressed as ± standard error of the mean (SEM). Bar and line graphs were created using GraphPad Prism 7. Box plots were created using BoxPlotR, an application available at http://shiny.chemgrid.org/boxplotr/ and described in Nature Methods Editorial "Kick the bar chart habit" 2014, p113.

Peak amplitudes of startle response in the 'Startle Curve' session were averaged for each stimulus intensity and analysed by a 3-way ANOVA with genotype and sex as between subjects factors, stimulus intensity as a within subjects factor and each mouse nested within genotype and sex.

For the 'PPI test' session, the peak amplitude of response to each trial were used in the analyses, and were averaged for each prepulse stimulus type (4, 6 or 8dB) for each mouse over the course of a session. Responses to 120dB startle only trials at the beginning and end were not included in the calculation of % PPI, which was determined by the formula:

$$\%\ \text{PPI} = \left(\frac{\text{mean of 120dB startle only} - \text{mean of 4, 8 or 16 dB PPI startle}}{\text{mean of 120dB startle only}}\right) * 100$$

% PPI was calculated, then analysed by a 4-way ANOVA with genotype and sex as between subjects factors, prepulse stimulus intensity and treatment as within subjects factors and each mouse nested within genotype and sex.

Short term habituation to the startling stimulus is inevitable; however, it is important to verify that all mice exhibited a similar amount of habituation. Habituation to the startling stimulus was calculated by the formula:

$$\%\ \text{Habituation} = \left(\frac{\text{mean ASR to initial 120dB bursts} - \text{mean ASR to final 120dB bursts}}{\text{mean ASR to initial 120dB bursts}}\right) * 100$$

% habituation and the peak amplitude of response to "no stimulus" trials were analysed by a 3-way ANOVA with genotype and sex as between subjects factors, treatment as a within subjects factor and each mouse nested within genotype and sex.

2-, 3- and 4-way pairwise interactions were made in all analyses as appropriate using Tukey's method.

4.3.7 PPI Data anomalies

There was no effect of genotype for "120dB startle only" (p=0.965), however, WTs were significantly more startled with "no stim" than HZs (p=0.006; $F_{(1,72)}$=8.10). Minitab® 17 flagged "no stim" data from two mice (one WT female and one HZ female) as anomalies, which was driving this effect. All extreme outliers in the current experiment were identified from Minitab® 17 software as a Large Standardised Residual. Each outlier was first removed and the data reanalysed to determine whether it was an influential observation that had a disproportionate impact on the ANOVA model. If it had no effect, it was kept; if the model changed significantly, the outlier was investigated further. First, a check was made for data input errors. Next, the origin of the data was investigated. These two mice appeared to be constantly moving to the same extent regardless of stimulus type. This was biasing the data: the significant effect was ameliorated when they were removed, so these mice were removed from all PPI analyses.

4.3.8 Open field (OF) apparatus

Four black opaque Perspex XT open field arenas (40 x 40 x 40cm), semi-permeable to infrared light, were used in a small, dimly lit room. The apparatus was lit from below by infrared LED lighting. An infrared-sensitive digital camera (Sony) and computer-based video tracking software (EthoVision® XT, Noldus Information Technology, Leesburg, VA) were used to monitor and record activity levels.

4.3.9 OF procedure

Mice were tested in the open field firstly at adolescence, then at adulthood and finally, at adulthood following 7 days' minocycline treatment (or standard drinking water) and were always tested in the same arena for all three sessions. Mice were placed into transfer cages half an hour prior to testing.

One mouse was placed in the centre of each arena and allowed to explore freely for 45 minutes (15 minutes habituation directly followed by 30 minutes test). Care was taken to ensure that the same box was not being used for all mice of one particular group; for example, all WT/*Map2k7*$^{+/-}$ or all male/female. Arenas were cleaned in between each animal using disinfectant to avoid possible effects on behaviour from odour cues left by previous mice.

4.3.10 OF drug administration

Following testing at adulthood half of these mice received minocycline in a similar way to McKim *et al.* (2016): 0.5 mg/ml; protected from light (Sigma-Aldrich M9511, St. Louis, USA) in their standard drinking water for 7 days, whilst the other half received standard drinking water without minocycline (see **Table 4.1**). The dose and duration of the minocycline administration were chosen to reflect, as closely as possible, the treatment protocols that are associated with symptomatic improvement in patients with schizophrenia. Administration of minocycline to mice in drinking water at 0.5 mg/ml produces a brain concentration of around 2μM (Smith *et al.*, 2003), which is equivalent to the CSF concentrations achieved in humans during standard antibacterial dosing regimens (Agwuh & MacGowan, 2006; Macdonald *et al.*, 1973). Fresh water or minocycline solution was prepared every second day and provided at room temperature. Consumption of water and minocycline treated water was monitored daily for each cage. On average, the treated group received 99.14 ± 17.72 mg/kg/day of minocycline and, per day, mice drank 5.18 ± 0.49ml normal drinking water or 4.84 ± 0.43ml minocycline treated water, which are both within the normal daily water intake range for mice (Bachmanov *et al.*, 2002).

	Water	**Minocycline**
Female WT	6	6
Female HZ	9	10
Male WT	8	12
Male HZ	6	6

Table 4.1 Numbers of mice treated with minocycline or water.

4.3.11 OF statistical analysis

All statistical analyses were carried out using Minitab® 17 software. Results were considered significant if $p<0.05$. All error bars are expressed as ± standard error of the mean (SEM). Bar and line graphs were created using GraphPad Prism 7. Box plots were created using BoxPlotR, an application available at http://shiny.chemgrid.org/boxplotr/ and described in Nature Methods Editorial "Kick the bar chart habit" 2014, p113.

Total distance travelled (cm), mean velocity (cm/s), and duration spent in an immobile (s), mobile (s) and highly mobile (s) state were recorded by EthoVision®

for each 5-minute time bin. Duration spent in the different mobility states were calculated by Ethovision® using the pixel area of the mouse on the screen, on a frame by frame basis. The changed area (in number of pixels) for the current frame (the frame rate was set at 25 frames per second) was divided by the sum of the current area and the previous area. If a mouse was in a highly mobile state, the percentage of change in body area was defined as more than 10% from one frame to the next; if a mouse was in a mobile state, the percentage of change in body area was defined as between 2 and 10%, and if the mouse was in an immobile state, the percentage of change in body area was defined as less than 2% from one frame to the next.

For the age comparison, data were analysed by a three-way ANOVA with genotype and sex as between subjects factors, age (adolescence or adulthood) as a within subjects factor and each individual mouse nested within genotype and sex. For the treatment comparison, data were analysed by a three-way ANOVA with genotype, sex and treatment (minocycline or water) as between subjects factors, and each mouse nested within genotype, sex and treatment. Tukey's post hoc test was carried out where appropriate. As there were so many irrelevant significant effects, results for main effects and interactions are only shown and discussed when a significant effect was observed.

There were many effects of sex throughout the open field analyses. Therefore, the graphs are presented with sex separately for visual clarity. However, all p-values stated will be from the overall analyses that included sex as a factor.

4.4 Results

The acoustic startle response curve was first established for all mice. They then underwent the first PPI session two days later with half the mice receiving saline or amphetamine. Two days following that, the PPI session was repeated with the other half of mice receiving either saline or amphetamine.

4.4.1 The acoustic startle response curve showed WT and Map2k7$^{+/-}$ *mice had a similar startle response at 120dB, the stimulus intensity that was used to measure PPI*

The startle curve is shown in **Fig. 4.3.** Overall, *Map2k7*$^{+/-}$ mice have a greater startle response to stimuli than WT mice (effect of genotype: $p=0.038$, $F_{(1,280)}=4.36$) and males have a greater startle response than females (effect of sex: $p<0.0001$, $F_{(1,280)}=27.78$); however, there were no post hoc differences between WT vs. *Map2k7*$^{+/-}$ mice or male vs. female at any of the stimuli separately. As expected, as stimulus intensity increases from 65-120dB, so does the startle response to all mice overall (effect of stimulus intensity: $p<0.0001$, $F_{(8,280)}=76.04$). Post hoc analyses revealed that from 65-90dB, mice respond to each stimulus intensity to a similar extent ($p>0.05$). The startle stimulus intensity at which mice significantly begin startling at is 100dB. At 120dB, the stimulus intensity we used to measure PPI, there was no significant difference in startle between genotypes (0.759, ns), but males were significantly more startled than females (effect of sex: $p=0.002$, $F_{(1,35)}=11.79$). There were no other significant interactions/effects. This indicates that any significant genotype effects on the PPI test session are not due to *Map2k7*$^{+/-}$ mice being unable to hear properly and that male and female %PPI should be examined separately as they are so different from each other.

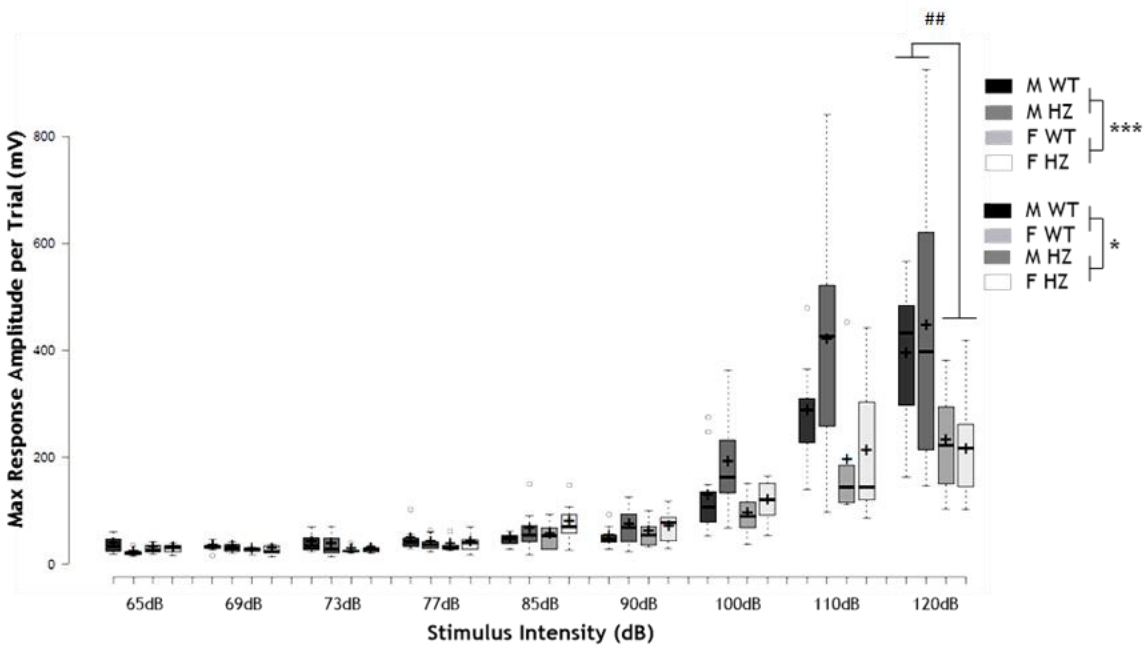


Figure 4.3. The startle curve of mice generated by measuring movement immediately following a range of stimuli with differing intensities (65 – 120dB above background noise). Overall, *Map2k7*$^{+/-}$ mice have a greater startle response than WT mice and male mice have a greater startle response than female mice. Mice begin startling at 100dB. At 120dB, there was no significant difference between WT and *Map2k7*$^{+/-}$ mice but the significant difference between males and females remained. Significant differences between groups are indicated on the graph key on the RHS. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample mean. Data analysed by a 3-way ANOVA with genotype and sex as between subjects factors, stimulus intensity as a within subjects factor and each mouse nested within genotype and sex. 2- and 3-way pairwise interactions were made as appropriate using Tukey's method. Lines joining groups show the significance between those groups: *p<0.05; ***p<0.001 (ANOVA); ##p<0.01 (Tukey's). *Map2k7*$^{+/-}$: n=23 (11M, 12F), WT: n= 17 (11M, 6F).

4.4.2 WT and Map2k7$^{+/-}$ *mice had a similar, relatively low response to the "no stimulus" condition, and exhibited a similar level of habituation over the course of the PPI sessions*

"No stimulus" trials were randomly interspersed between PPI trials, and each session started and finished with 5 x 120dB stimuli, to assess the level of baseline reactivity when there is no stimulus, and extent of habituation, respectively. Once the two mice that were moving to a high extent regardless of stimulus type were removed from analysis (see **Section 4.3.7** for more information), there were no significant differences between any measurements (genotype, sex or treatment) for both "no stimulus" trials and % habituation (**Fig. 4.4;** $p>0.05$ for all). This indicates that any disruptive effects on PPI in the test session are not simply due to differences in baseline movement, or differences in changes in movement over the course of the session, which could be potentially induced by hyperactivity owing to genotype, sex, or amphetamine administration.

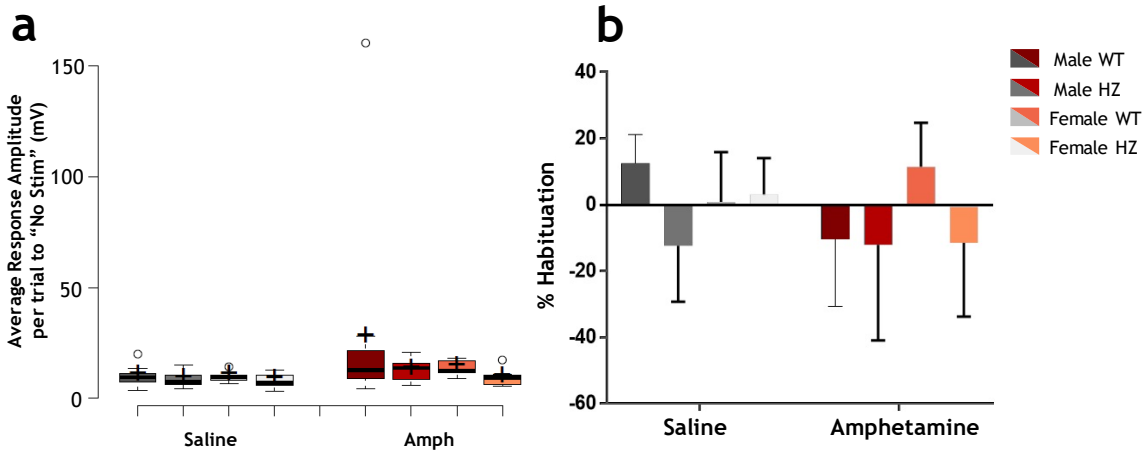


Figure 4.4. The % habituation and response of mice to the "no stimulus" control trials. There were no significant differences between any variables measured (genotype, sex, amphetamine administration) for response amplitude to **a)** the "no stimulus" trials, or for **b)** the % habituation exhibited across the two PPI sessions. Saline (2ml/kg) or equivalent volumes of D-amphetamine (5mg/kg) was administered 5 minutes prior to PPI testing. The bar chart represents the mean, with error bars showing the standard error of the mean; boxplots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample mean. Data for **a)** and **b)** were analysed separately by a 3-way ANOVA with genotype and sex as between subjects factors, treatment (amphetamine or saline) as a within subjects factor and each mouse nested within genotype and sex. 2- and 3-way pairwise interactions were made as appropriate using Tukey's method. *Map2k7*$^{+/-}$: n=23 (11M, 12F), WT: n= 17 (11M, 6F).

4.4.3 % PPI increases as prepulse intensity increases but there were no significant effects of genotype or drug

As prepulse intensity increases from 4 to 8 to 16dB, % PPI significantly increases (**Fig. 4.5**; effect of prepulse intensity: $p<0.001$; $F_{(2,170)}=101.37$). Post-hoc analyses showed there were no effects of sex, genotype or injection at each of the prepulse intensities separately, and no overall significant effects of sex or genotype ($p>0.05$ for all terms).

4.4.4 Amphetamine reduces % PPI in WT and male mice, but not Map2k7$^{+/-}$ *and female mice*

Overall, injection of 5mg/kg amphetamine immediately prior to PPI testing resulted in a decrease in % PPI (**Fig. 4.5;** significant effect of injection: $p=0.009$; $F_{(1,170)}=6.90$). Post hoc analyses revealed that this decrease in % PPI by amphetamine was significant within WT but not *Map2k7*$^{+/-}$ mice, suggesting a decreased sensitivity of *Map2k7*$^{+/-}$ mice to amphetamine. However, the genotype x injection interaction term was not significant ($p=0.120$, $F_{(1,170)}=2.44$), but there was a sex x genotype x injection interaction where male WT % PPI was significantly reduced by amphetamine whereas female WT % PPI was not ($p=0.003$; $F_{(1,170)}=9.28$). Therefore, amphetamine effects appear to be more obvious in male and WT mice.

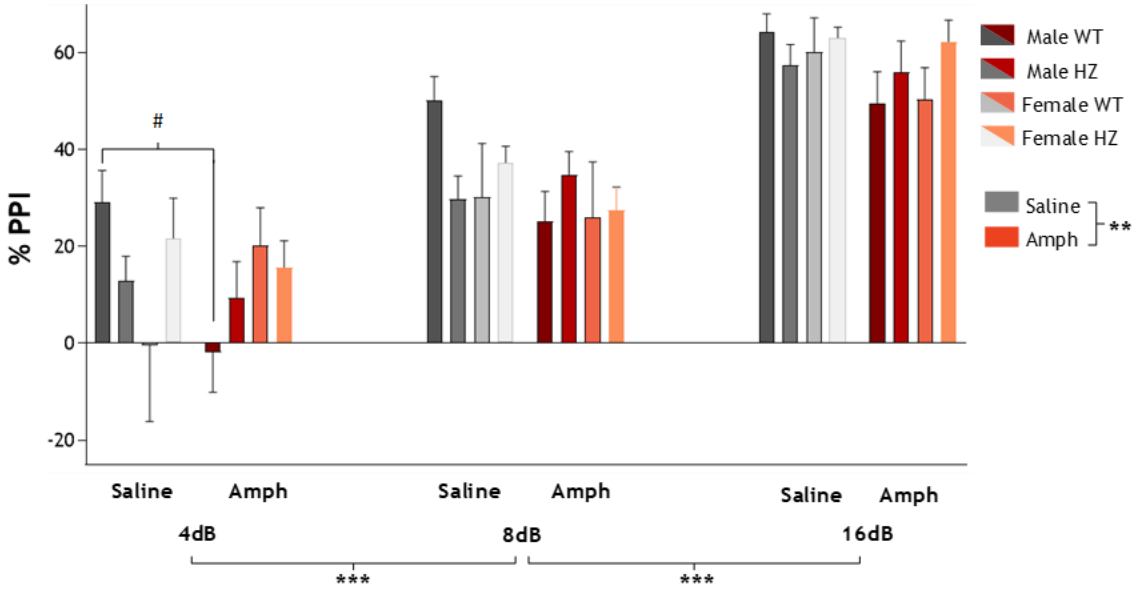


Figure 4.5. % PPI of mice to prepulses of 4, 8 and 16 dB above background. % PPI significantly increases along with prepulse intensity. Amphetamine administration decreased % PPI for WT mice but not *Map2k7*$^{+/-}$ mice and post hoc analyses revealed that this decrease in % PPI in WT mice occurred only for male mice (not indicated on the graph). Post hoc analyses indicated that the decrease in %PPI by amphetamine for male WT mice was significantly different for the 4dB prepulse intensity (indicated on the graph), even though the genotype x sex x injection x prepulse intensity interaction was not significant (p=0.132). Saline (2ml/kg) or equivalent volumes of D-amphetamine (5mg/kg) was administered 5 minutes prior to PPI testing. Bar chart represents the mean, with error bars showing the standard error of the mean. Data were analysed separately by a 4-way ANOVA with genotype and sex as between subjects factors, treatment (amphetamine or saline) and prepulse (4, 8 or 16 dB) as a within subjects factor and each mouse nested within genotype and sex. 2-, 3- and 4-way pairwise interactions were made as appropriate using Tukey's method. Lines linking groups represent significant differences between those groups: **p<0.01; ***p<0.001 (ANOVA); #p<0.05 (Tukey's). *Map2k7*$^{+/-}$: n=23 (11M, 12F), WT: n= 17 (11M, 6F).

4.4.5 Open field at adolescence, adulthood and with minocycline treatment

The locomotor activity of mice was monitored individually for 45 minutes in a 40cm x 40cm open field arena at adolescence, adulthood and then at adulthood following minocycline treatment (or normal water) for one week. The first 15 minutes within the open field was considered the "Habituation Phase", to allow activity levels of mice to stabilise before analysis, and the final 30 minutes termed the "Test Phase". Mice were not removed or disturbed throughout the 45 minutes. There were effects of sex in most of the performance parameters, so the data from males and females are presented on separate graphs for clarity; however,

all p-values stated will be from the overall analysis that included sex as a factor. P-values from the analysis for each variable, plus their relevant interactions, are presented in **Table A1** and **A2** in **Appendix 1**.

4.4.6 Map2k7$^{+/-}$ *mice move further and faster than WT mice at adulthood but not at adolescence*

Overall, mice moved further and faster at adulthood than at adolescence (**Fig. 4.6;** distance: $p<0.001$, $F_{(1,688)}=97.98$); velocity: $p<0.001$, $F_{(1,688)}=94.80$); however, post hoc analyses showed that this occurred only within female mice (age x sex interaction; distance: $p<0.001$, $F_{(1,688)}=41.85$; velocity: $p<0.001$, $F_{(1,688)}=42.41$). In fact, looking further into the post hoc analyses, it was revealed that *Map2k7*$^{+/-}$ male mice moved further and faster than WT male mice at adolescence but not at adulthood, whereas *Map2k7*$^{+/-}$ female mice moved further and faster than WT female mice at adulthood but not at adolescence (genotype x sex x age interaction; distance: $p<0.001$, $F_{(1,688)}=22.50$); velocity: $p<0.001$, $F_{(1,688)}=22.29$). Nevertheless, overall, *Map2k7*$^{+/-}$ mice moved further and faster than WT mice at adulthood but not at adolescence (genotype x age interaction, distance: $p=0.026$, $F_{(1,688)}=5.00$; velocity: $p=0.01$, $F_{(1,688)}=4.88$).

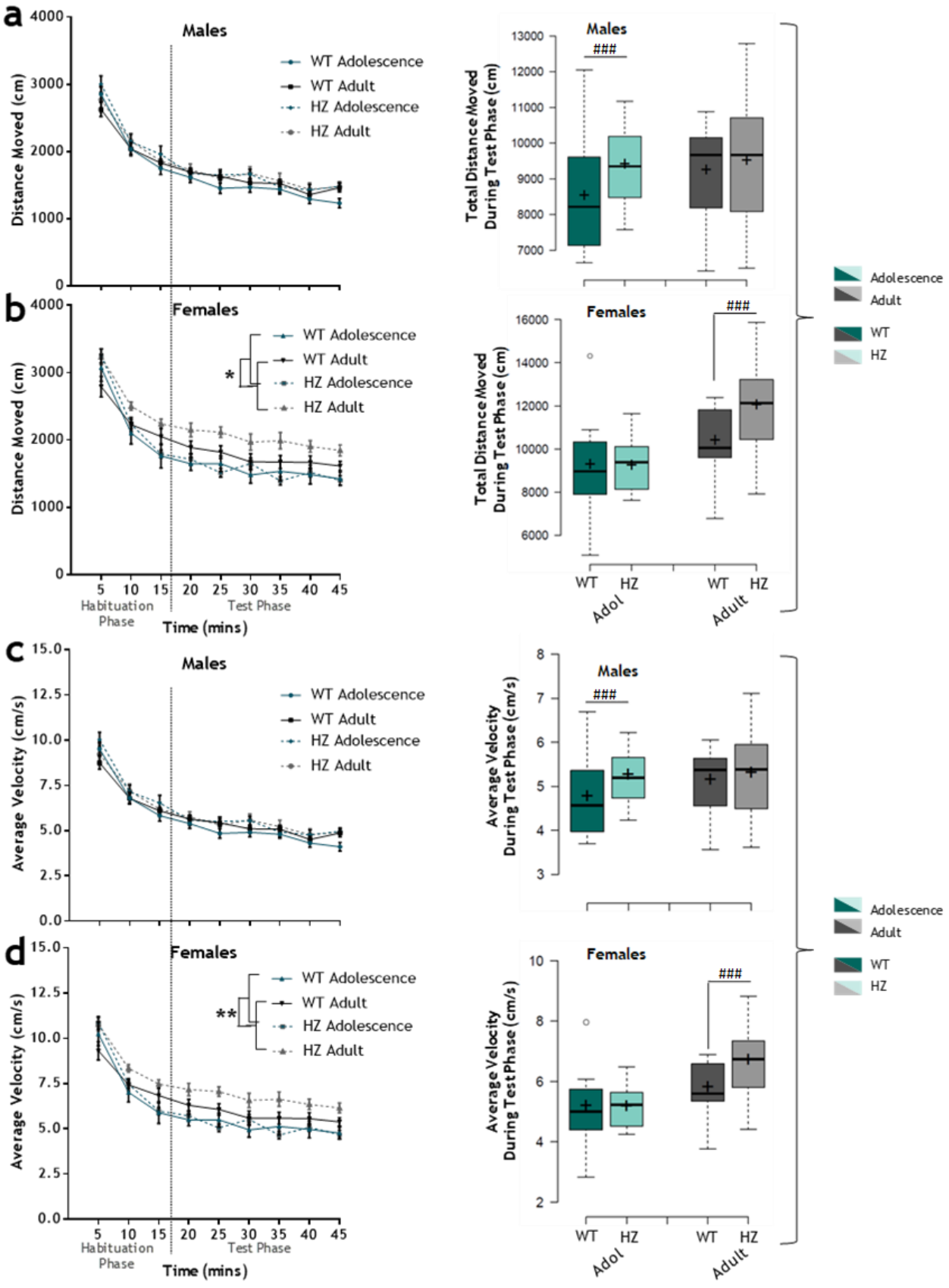
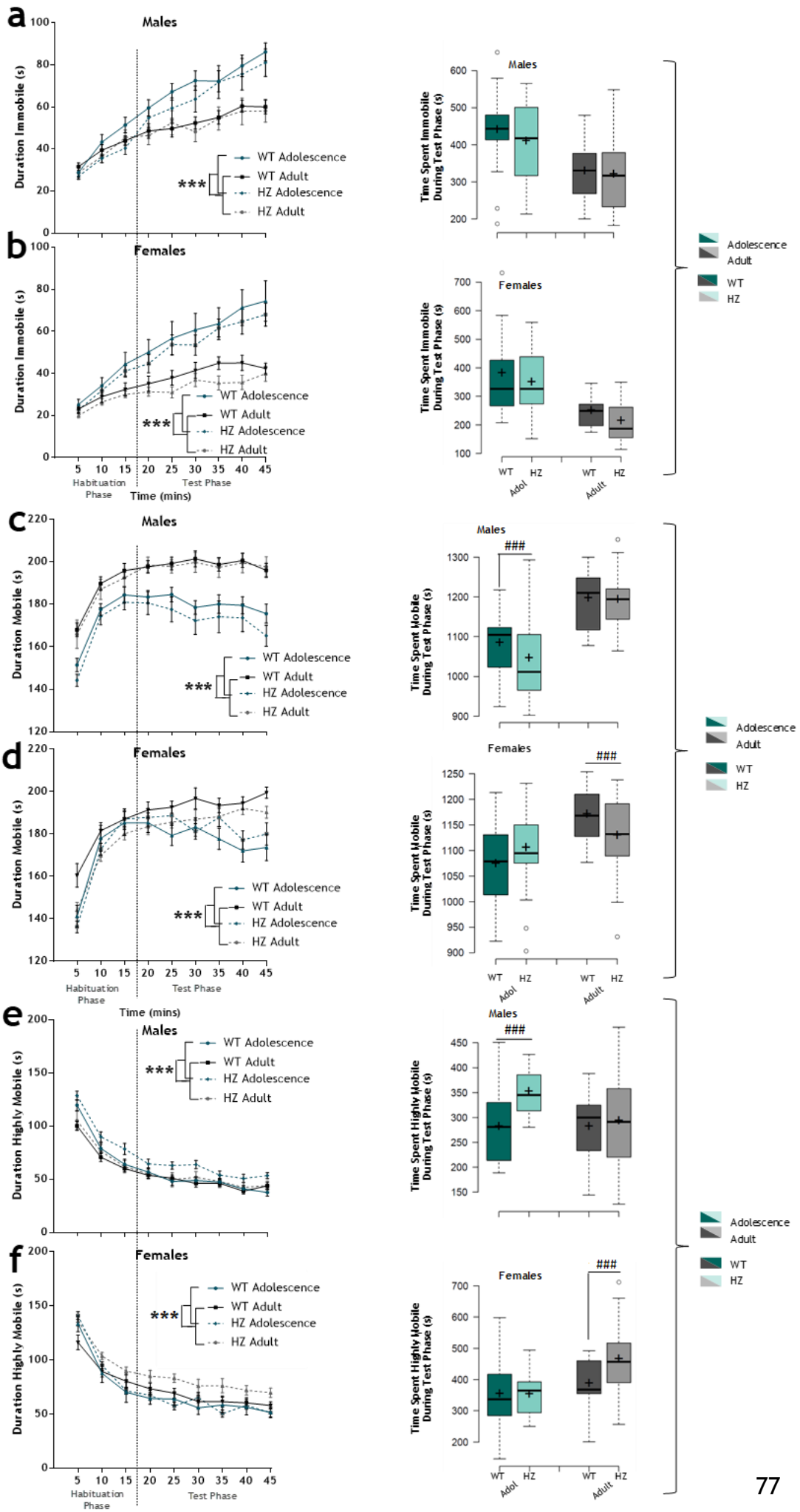


Figure 4.6. Distance moved and average velocity by WT and HZ mice. *Map2k7*$^{+/-}$ males at adolescence move **a)** further and **c)** faster than WT males at adolescence. *Map2k7*$^{+/-}$ females at adulthood move **b)** further and **d)** faster than WT females at adulthood, and all female mice at adulthood move further and faster than all female mice at adolescence. Line graphs: data points represent the mean and error bars the

standard error of the mean. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Significant sex x age interactions are indicated on the corresponding graph key. Significant genotype x sex x age interactions are indicated on the box plot on the right-hand side. *p<0.05; **p<0.01 (ANOVA); ###p<0.001 (Tukey's). *Map2k7*$^{+/-}$: n=31 (12M, 19F), WT: n= 32 (20M, 12F).

4.4.7 Mice show higher levels of mobility at adulthood compared to adolescence

As would be expected, time spent in each of the mobility levels reflect what occurs with distance moved and velocity (**Fig. 4.7**). Taken together, mice spent significantly more time immobile and less mobile and highly mobile at adolescence compared to adulthood (overall effect of age, immobile: $p<0.001$, $F_{(1,688)}=377.18$; mobile: $p<0.001$, $F_{(1,688)}=252.16$; highly mobile: $p<0.001$, $F_{(1,688)}=13.09$). WT mice spent longer in a mobile state ($p=0.032$, $F_{(1,688)}=4.59$) and *Map2k7*$^{+/-}$ mice spent longer in a highly mobile state ($p<0.001$, $F_{(1,688)}=42.74$) compared to WT mice overall. In line with distance moved and velocity, male *Map2k7*$^{+/-}$ mice spent less time in a mobile state than male WT mice at adolescence and spent a similar amount of time in a mobile state at adulthood, whereas female *Map2k7*$^{+/-}$ mice spent a similar amount of time in a mobile state to female WT mice at adolescence but less time in a mobile state than WT mice at adulthood (genotype x sex x age interaction: $p<0.001$, $F_{(1,688)}=20.54$). Additionally, male *Map2k7*$^{+/-}$ mice spent more time highly mobile than male WT mice at adolescence but not at adulthood, whereas female *Map2k7*$^{+/-}$ mice spent more time highly mobile than female WT mice at adulthood but not at adolescence (genotype x sex x age interaction: $p<0.001$, $F_{(1,688)}=32.20$). Overall, *Map2k7*$^{+/-}$ mice spent less time immobile and more time highly mobile at both adolescence and adulthood (see **Table A1** from **Appendix 1**).


a
Males
Duration Immobile (s)
WT Adolescence
WT Adult
HZ Adolescence
HZ Adult
***
b
Females
Duration Immobile (s)
***
Habituation Phase
Test Phase
Time (mins)
Time Spent Immobile During Test Phase (s)
Males
Females
Adol
Adult
WT
HZ
Adolescence
c
Males
Duration Mobile (s)
d
Females
Duration Mobile (s)
Time Spent Mobile During Test Phase (s)
###
e
Males
Duration Highly Mobile (s)
Time Spent Highly Mobile During Test Phase (s)
f
Females
Duration Highly Mobile (s)

< Figure 4.7. Mobility levels at adulthood and adolescence. All mice spend significantly more time immobile (**a** & **b**), and less time mobile (**c** & **d**) and highly mobile (**e** & **f**) at adolescence compared to adulthood. There were no significant genotype differences for duration immobile (**a** & **b**). However, male *Map2k7*$^{+/-}$ mice spend less time in a mobile state than male WT mice at adolescence and spend a similar amount of time in a mobile state at adulthood **(c)**, whereas female *Map2k7*$^{+/-}$ mice spend a similar amount of time in a mobile state to female WT mice at adolescence but less time in a mobile state than WT mice at adulthood **(d)**. Male *Map2k7*$^{+/-}$ mice spent more time highly mobile than male WT mice at adolescence but not at adulthood **(e)**, whereas female *Map2k7*$^{+/-}$ mice spent more time highly mobile than female WT mice at adulthood but not at adolescence **(f)**. Line graphs: data points represent the mean and error bars the standard error of the mean. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Significant sex x age interactions are indicated on the corresponding graph key. Significant genotype x sex x age interactions are indicated on the box plot on the right-hand side. Lines linking groups represent significant differences between those groups: ***p<0.001 (ANOVA); ###p<0.001 (Tukey's). *Map2k7*$^{+/-}$: n=31 (12M, 19F), WT: n= 32 (20M, 12F).

4.4.8 Minocycline decreased distance moved and velocity for female mice but not males

Following minocycline treatment, there was an overall significant decrease of distance moved and velocity (**Fig. 4.8**; effect of treatment, distance moved: $p=0.001$, $F_{(1,314)}=12.21$; effect of treatment, velocity: $p<0.001$, $F_{(1,314)}=12.19$). However, post hoc analyses revealed that minocycline did not significantly affect distance moved and velocity of male mice, whereas the distance moved and velocity of female mice were decreased (sex x treatment interaction, distance moved: $p<0.001$, $F_{(1,314)}=45.04$; velocity: $p<0.001$, $F_{(1,314)}=45.03$). This decrease occurred for both WT and *Map2k7*$^{+/-}$ female mice, so the genotype significance was still present in female mice with minocycline, and indeed overall (effect of genotype, distance moved: $p<0.001$, $F_{(1,314)}=16.84$; velocity: $p<0.001$, $F_{(1,314)}=16.97$). On the other hand, male mice slightly (although not significantly) moved further and faster following minocycline treatment, but only to a point where the distance moved and velocity of male mice following minocycline was more like that of females following minocycline treatment (genotype x sex x treatment interaction, distance moved: $p<0.001$, $F_{(1,314)}=13.66$; velocity: $p<0.001$, $F_{(1,314)}=13.42$).

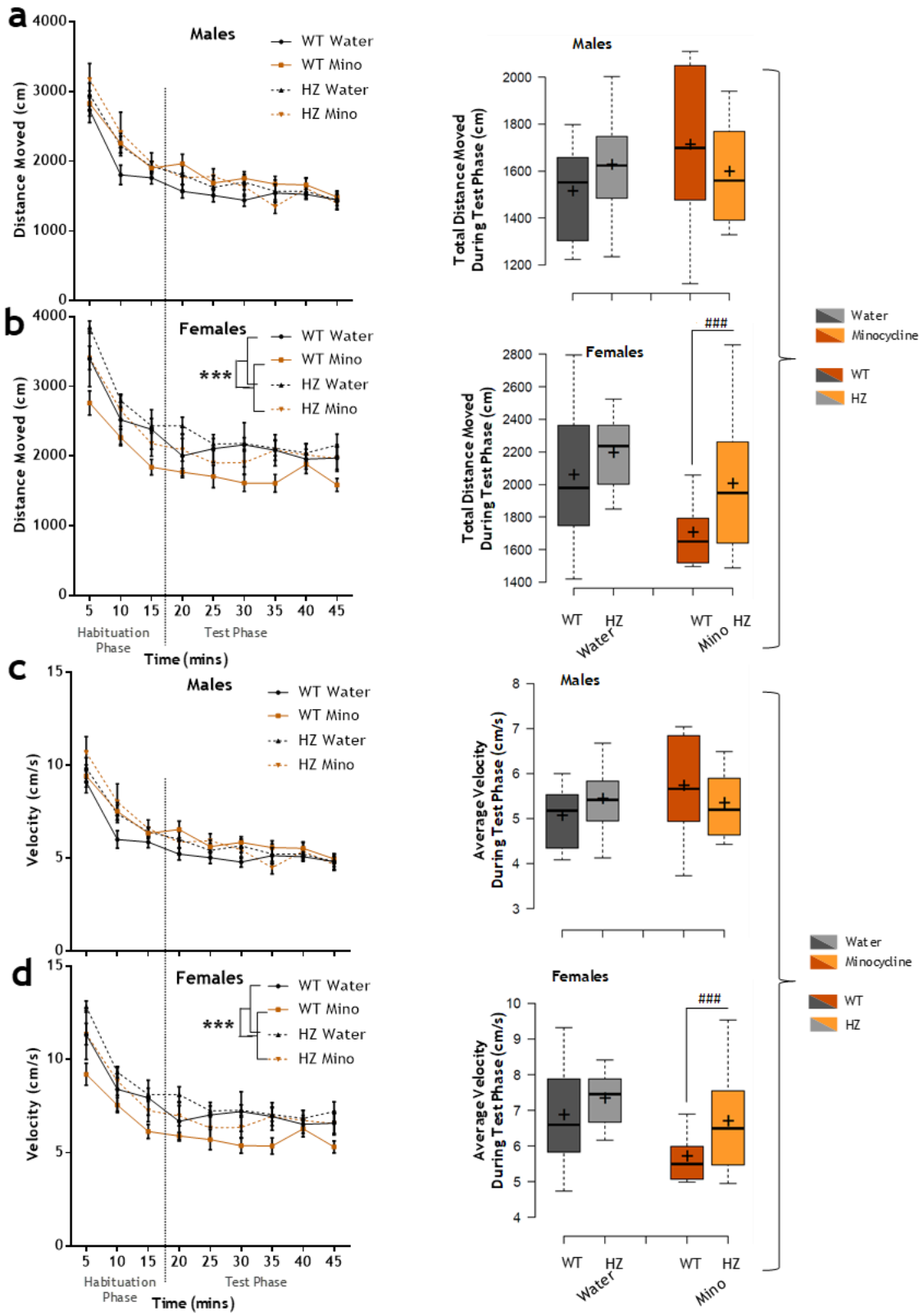


Figure 4.8. Distance moved and velocity of mice in the open field at adulthood either with normal water or treated with minocycline for one week. Male mice distance moved (**a**) and velocity (**c**) were unaffected by minocycline treatment, whereas female mice covered less distance **(c)** and with decreased velocity **(d)** following minocycline treatment compared to mice that received standard drinking water. Minocycline has the same effects on female mice regardless of genotype.

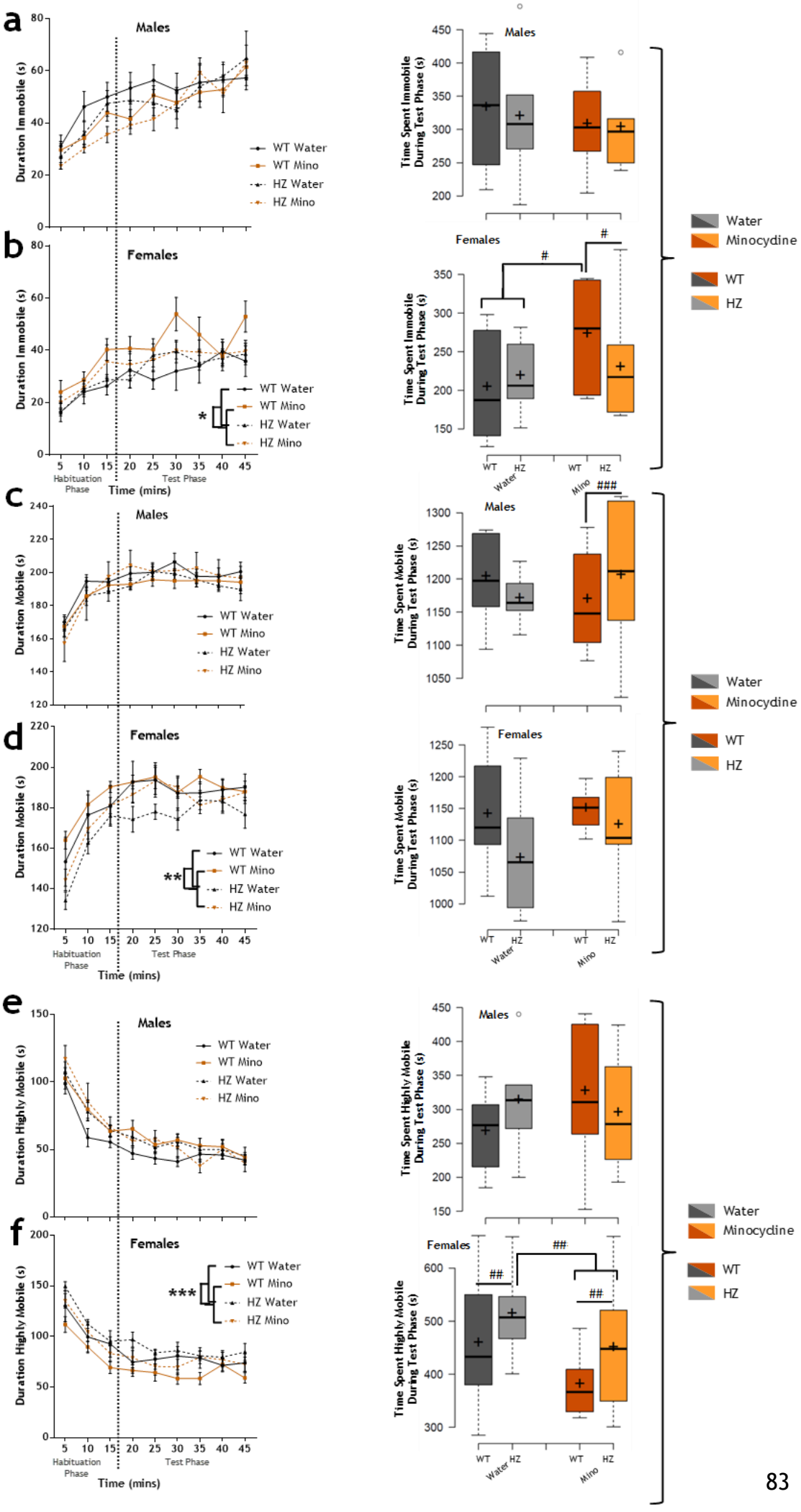
0.5mg/ml minocycline was administered via drinking water for one week. Line graphs: data points represent the mean and error bars the standard error of the mean. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Significant sex x treatment interactions are indicated on the corresponding graph key. Significant genotype x sex x treatment interactions are indicated on the box plot on the right-hand side. Lines linking groups represent significant differences between those groups: ***p<0.001 (ANOVA); ###p<0.001 (Tukey's). *Map2k7*$^{+/-}$: n=31 (12M, 19F), WT: n= 32 (20M, 12F).

4.4.9 Minocycline decreased mobility levels for female mice but not males

In a comparable way to the adolescent-adult analysis, and as would be expected, mobility levels reflect what occurs with distance moved and velocity. Within the mice that received standard drinking water, *Map2k7*$^{+/-}$ mice moved faster than WT mice overall: they spent less time in an immobile, less time in a mobile and more time in a highly mobile state (**Fig. 4.9**; genotype x treatment interaction, immobile: $p=0.037$, $F_{(1,314)}=4.39$; mobile: $p<0.001$, $F_{(1,314)}=25.11$; highly mobile: $p=0.034$, $F_{(1,314)}=4.53$).

Minocycline had an overall significant effect on time spent in a mobile and highly mobile state but not immobile (no effect of treatment, immobile: $p=0.102$). Overall, mice spent more time in a mobile state and less time in a highly mobile state following minocycline treatment, compared to controls who received standard drinking water (effect of treatment, mobile: $p=0.006$, $F_{(1,314)}=7.81$; highly mobile: $p=0.001$, $F_{(1,314)}=11.37$). However, post hoc analyses revealed that minocycline had a significant effect on the duration of time spent in an immobile and highly mobile state for female mice but not male mice for these variables. Furthermore, this difference among female mice following minocycline treatment occurred regardless of genotype. Minocycline increased the time spent immobile and decreased the time spent highly mobile for female mice (genotype x sex x treatment interaction, immobile: $p=0.004$, $F_{(1,314)}=8.30$; highly mobile: $p=0.002$, $F_{(1,314)}=9.47$). This interaction was not significant for duration spent in a mobile state ($p=0.245$).

Taken together, these results show that minocycline could decrease activity levels (by decreasing distance moved, velocity and duration in a highly mobile state, and increasing duration in an immobile and mobile state) for all female mice, regardless of genotype, but did not have the same impact on male mice.


a
Males
Duration Immobile (s)
WT Water
WT Mino
HZ Water
HZ Mino
b
Females
Duration Immobile (s)
*
Habituation Phase
Test Phase
Time (mins)
Time Spent Immobile During Test Phase (s)
Males
Females
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Water
Minocydine
WT
HZ
c
Males
Duration Mobile (s)
Time Spent Mobile During Test Phase (s)
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d
Females
**
e
Males
Duration Highly Mobile (s)
Time Spent Highly Mobile During Test Phase (s)
f
Females
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< Figure 4.9. Mobility levels following treatment with minocycline or standard drinking water for one week. Compared to standard drinking water, minocycline increased the amount of time spent in an immobile state for female mice **(b)** but not for male mice **(a)**, and increased the time spent in a mobile state for female mice **(d)** but not male mice **(c)**. Minocycline also decreased the amount of time spent in a highly mobile state for female mice **(f)** but not male mice **(e)**, compared to controls who received standard drinking water. Minocycline had these effects in female mice to the same extent in WT and *Map2k7*$^{+/-}$ mice. 0.5mg/ml minocycline was administered via drinking water for one week. Line graphs: data points represent the mean and error bars the standard error of the mean. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Significant sex x treatment interactions are indicated on the corresponding graph key. Significant genotype x sex x treatment interactions are indicated on the box plot on the right-hand side. Lines linking groups represent significant differences between those groups: *p<0.05; **p<0.01; ***p<0.001 (ANOVA); #p<0.05; ##p<0.01; ###p<0.001 (Tukey's). *Map2k7*$^{+/-}$: n=31 (12M, 19F), WT: n= 32 (20M, 12F).

4.5 Discussion

Map2k7$^{+/-}$ mice show some characteristics that could be interpreted as relevant to the positive symptoms of schizophrenia. They did not exhibit a deficit in PPI, but were hyperactive in the open field, which presented in male *Map2k7*$^{+/-}$ mice at a younger age than it did in females. Administration of amphetamine prior to PPI testing revealed a modest decreased sensitivity to its effects in *Map2k7*$^{+/-}$ mice, and treatment of minocycline in the open field decreased locomotor activity levels of all female mice, regardless of genotype, but did not have a significant effect on males.

4.5.1 Map2k7$^{+/-}$ *mice did not show a deficit in PPI*

For all mice, as prepulse intensity increased from 4 to 8 to 16dB, %PPI also increased, which is expected and is what occurred in previous PPI experiments in C57Bl/6 mice (Martin *et al.*, 2008; Yee *et al.*, 2004; van den Buuse, 2010). WT and *Map2k7*$^{+/-}$ mice were similar in the amount of PPI they exhibited. As *Map2k7*$^{+/-}$ and WT mice had a similar startle response at 120dB and a similar level of habituation, these potential confounding factors can be ruled out, and so the neural circuitry involved in PPI appears to be intact or compensated for in *Map2k7*$^{+/-}$ mice. Hence, *Map2k7* does not appear to be key to the robust deficits seen in %PPI observed in patients with schizophrenia.

The basic circuitry involved in PPI is relatively well established. There are three main pathways involved: the primary startle, PPI mediation and PPI modulation networks (Rohleder *et al.*, 2016). Brain areas involved include (among others) the caudal pontine reticular nucleus, substantia nigra, nucleus accumbens, inferior and superior colliculi, medial PFC, mediodorsal thalamus, pedunculopontine and laterodorsal tegmental nuclei (reviewed in Swerdlow *et al.*, 2001). As would be expected from the wide distribution of brain areas, genetically modified mice, lesion and pharmacological studies have shown that multiple neurotransmitter systems are involved, including dopaminergic, serotoninergic, glutamatergic and cholinergic systems (Geyer *et al.*, 2002; Maclaren *et al.*, 2014; van den Buuse, 2010; Geyer *et al.*, 2001). As *Map2k7*$^{+/-}$ mice do not have disruption in PPI, they may have these circuitries fully intact. However, these brain systems are known to interact closely, and to up- or down-regulate themselves to compensate for alterations in each other, particularly if, as is the case in *Map2k7*$^{+/-}$ mice, the

alteration has been present from the beginning of development (van den Buuse, 2010; Pratt *et al.*, 2012). It is highly possible that molecular alterations have occurred in *Map2k7*$^{+/-}$ mice, such as receptor expression or neurotransmitter level changes, which would present behaviourally as having normal PPI (Pratt *et al.*, 2012; van den Buuse, 2010). The administration of amphetamine prior to PPI testing may shed more light on this and is discussed below.

4.5.2 Map2k7$^{+/-}$ *mice showed decreased sensitivity to amphetamine-induced deficits in PPI*

Administration of an acute dose of amphetamine prior to PPI testing was then carried out to investigate whether there may be underlying alterations in the neurotransmitter systems involved; for example, neurotransmitter release, upregulation of specific receptors or other compensatory mechanisms, due to *Map2k7* deficiency. The amphetamine drug challenge significantly decreased %PPI in WT mice overall as a group, which is an effect previous studies have shown in healthy rats (Mansbach *et al.*, 1988), mice (Martin *et al.*, 2008) and humans (Hutchison & Swift, 1999). However, post hoc analyses showed that this significance was only true for male and WT mice; *Map2k7*$^{+/-}$ and WT female mice did not show a significant decrease in PPI, suggesting that they may have an attenuated response to amphetamine.

The effect of amphetamine on behaviour in rodents has been observed and recorded since the early 1970's (Scheel-Krüger, 1971). At the moderate dose of 5mg/kg in this experiment, amphetamine is known to cause increase in available dopamine in the synaptic cleft by decreasing reuptake of dopamine back into the presynaptic bouton, facilitating release of dopamine into the synaptic cleft from vesicles, and by interacting with the dopamine transporter, causing it to work in reverse (German *et al.*, 2015; Seiden *et al.*, 1993). Amphetamine also increases noradrenaline in the synaptic cleft in a similar way to dopamine (Seiden *et al.*, 1993) and increases levels of serotonin and other neurotransmitters, but dopamine is the main neurotransmitter system it affects (Fleckenstein *et al.*, 2007). Ralph *et al.* (1999) conducted PPI on mice that had each member of the dopamine D2-like receptor family knocked out, separately. Each KO mouse line (D2, D3 and D4) showed normal PPI; however, when challenged with amphetamine, D2R KO mice did not show amphetamine-induced decrease in PPI, whereas D3R and D4R KO mice did, suggesting that the amphetamine-induced decrease in PPI is mediated

by D2 and not D3 or D4 receptors. This suggests that *Map2k7*$^{+/-}$ mice may have alterations in the activity/performance of D2 receptors.

Dr. N. Dawson (Lancaster University, UK) conducted 2-deoxyglucose imaging on *Map2k7*$^{+/-}$ mice and found that, following an acute, 5mg/kg dose of amphetamine, *Map2k7*$^{+/-}$ mice (but not their WT littermates) exhibited a complete loss of response to amphetamine in multiple regions of the PFC and hippocampus and the nucleus reunions of the thalamus, and that the response to amphetamine was significantly attenuated in several other thalamic nuclei (written communication, Dr. N. Dawson). This implies that *Map2k7*$^{+/-}$ mice have developed a modest resistance to the effects of amphetamine, perhaps by decreasing receptors and/or synthesis of neurotransmitters (as amphetamine works by manipulating endogenous neurotransmitters as opposed to replacing them) so that amphetamine does not have a significant effect because the system is saturated. This may have come about if, for example, the *Map2k7*$^{+/-}$ mice had elevated levels of dopaminergic (and possibly other) signalling from the beginning of development and therefore developed differently to compensate. Both the thalamus and PFC are involved in the mediation of PPI (Rohleder *et al.*, 2016), so it is feasible that amphetamine would have attenuated the response relevant during PPI testing. Based on the Ralph *et al.* (1999) results described above, if dopamine receptors are decreased in *Map2k7*$^{+/-}$ mice as a compensatory effect, it is possible that dopamine D2 receptors are more decreased than the others. These underlying differences at a molecular and systems level may have been masked by compensatory effects from other neurotransmitter systems without the influence of amphetamine and would therefore produce apparently normal PPI in *Map2k7*$^{+/-}$ mice. It will be interesting to investigate this molecularly in the future to establish whether this is the case. One way this could be achieved is by measuring dopamine receptor density in *Map2k7*$^{+/-}$ vs. WT mouse brain sections using specific radio-ligands, or by quantifying the dopamine receptor availability using micro-PET (e.g. Dalley *et al.*, 2007).

4.5.3 Sex differences in PPI and startle

There were many sex differences when examining extent of PPI and startle, the most substantial being that males had a significantly larger overall startle response than females. **Fig. 4.3** shows that males' startle response was almost double that of females at 120dB, the volume used in PPI testing. The PPI of male

WT mice was significantly decreased by amphetamine whereas female WT PPI was not. As the calculation of %PPI relies on the startle response to 120dB without prepulse, this would make the calculation of female %PPI less accurate than if they had a larger startle response. This may explain why the female WT mice do not exhibit reduced %PPI in response to amphetamine, which is a well-established finding (in humans: Hutchison & Swift, 1999; and C57Bl/6 mice: Ralph *et al.*, 2001), as opposed to it being a real effect of sex in the current experiment. For the future, it would be necessary to carry out a power analysis to help decide whether to include more animals for increased power in this experiment.

There are several reasons that could explain the amount of sex differences in this experiment. There are profound differences in the neurochemistry of rodent male and female brains, including that of dopaminergic neurotransmission, arguably the most relevant to the current experiment. For example, in rats, regulation of the dopamine transporter is significantly more tightly controlled in females than males (Walker *et al.*, 2005). Neurochemical differences occur for a few main reasons, such as due to fluctuations in circulating hormones as a result of the oestrus cycle in females, and sex chromosome-related gene expression differences during development (Calipari *et al.*, 2017). Specifically, genes of the X-chromosome play a role in development of the dopaminergic system, leading to basal and hormone-related differences in this system in male and female offspring (Calipari *et al.*, 2017). The oestrus cycles of female mice in the current experiment were not monitored or controlled; it will be important in the future to consider doing this.

On the other hand, with respect to PPI testing, the effects of sex are likely to be at least partially due to a methodological issue: female mice are smaller and weigh less, so may have less of an influence on the accelerometer used to measure startle responses. In future, more consideration could be given to keep the weight variation in mice as little as possible, and perhaps testing males and females separately in experiments where this will be important, as well as adjusting the group sizes accordingly.

4.5.4 Map2k7$^{+/-}$ *mice exhibited hyperactivity in the open field*

By bringing together the information from distance moved/velocity, and duration immobile, mobile and highly mobile, it is possible to get an indication of locomotor activity levels of mice overall. Animals that moved further, with faster velocity

and had a larger duration highly mobile would be attributed to more hyperactive animals, and those that moved a shorter distance, with slower velocity and larger duration immobile would be attributed to more hypoactive animals. Duration mobile is merely a buffer in between the immobile and highly mobile parameters: any changes to duration spent immobile or highly mobile will have derived from duration spent mobile. Therefore, this parameter could be treated as "normal movement" and does not carry much meaning as a separate parameter.

Overall, mice were more hyperactive at adulthood, but post hoc analysis showed that *Map2k7*$^{+/-}$ male mice were significantly more hyperactive than WT males at adolescence but not at adulthood, and *Map2k7*$^{+/-}$ female mice were significantly more hyperactive than WT females at adulthood but not at adolescence. It is interesting that males show hyperactivity at an earlier stage than females, especially considering schizophrenia in male humans has an earlier onset on average, compared to females (Forrest & Hay, 1971): before the age of 30, more males are diagnosed with schizophrenia than females, and after the age of 30 more females are diagnosed than males (Sham *et al.*, 1994). Females have a second "wave" of diagnosis after the age of 40 and, in general, the earlier the onset of schizophrenia the more severe and debilitating the symptoms (Forrest & Hay, 1971; Sham *et al.*, 1994). However, these observations do not fully fit with the data seen here. Although it is interesting that the male *Map2k7*$^{+/-}$ mice showed hyperactivity earlier than female *Map2k7*$^{+/-}$ mice compared to their WT littermates, the hyperactivity observed in male *Map2k7*$^{+/-}$ mice did not continue into adulthood, and especially did not become more severe. However, this does suggest a disruption of the relevant circuitry at a critical developmental period, which will be an important focus for future research. There is also evidence to suggest that mice are generally more hyperactive during adolescence because of increased novelty-seeking and risk-taking behaviour (Laviola *et al.*, 2003), which may have occurred to a larger extent in male *Map2k7*$^{+/-}$ mice.

There are many ways in which we can correlate the age of mice to humans (Dutta & Sengupta, 2016). With respect to brain development and behavioural phenotypes, 5.58 weeks old (as tested in the current experiment) corresponds to periods of human adolescence (Semple *et al.*, 2013). In mice and humans, this is a period where locomotor and explorative activity are increased, and when the brain circuitry connections are continuing to be refined and matured (Semple *et*

al., 2013). On the other hand, 10.11 weeks old (as tested in the current experiment) corresponds to early adulthood, a time more relevant to the age at which females begin to develop the symptoms of schizophrenia (Semple *et al.*, 2013). It is unclear why male *Map2k7*$^{+/-}$ mice do not have a hyperactive phenotype by the time they reach adulthood; however, it may be that as their brain matures, the brain circuitry connections adapt to the disruption within the brain. Overall, with males and females combined, *Map2k7*$^{+/-}$ mice are more hyperactive than WT littermates at adulthood. In fact, hyperactivity in the open field was also observed in another, separate cohort of *Map2k7*$^{+/-}$ mice in the open field at 24.6 ± 0.3 weeks old (data not shown). Additionally, *Map2k7*$^{+/-}$ mice of two more, separate, cohorts of adult mice (50:50 male:female) exhibited significant hyperactivity compared to WT littermates whilst carrying out more sophisticated behavioural tasks: one in the operant-based 5-choice serial reaction time task (**Chapter 5**) and one in the pairwise discrimination/reversal (PD) and paired associates learning (PAL), both carried out in the touchscreen (data not shown; general hyperactivity of *Map2k7*$^{+/-}$ mice is discussed in more detail in **Discussion Section 8.3.1**).

Hyperactivity in rodents has been shown to be mediated by aberrant mesolimbic dopaminergic activity (Pratt *et al.*, 2012). Pijnenburg *et al.* (1975) administered the dopamine antagonist (and typical antipsychotic) haloperidol directly onto the nucleus accumbens which counteracted the hyperactivity induced by amphetamine, whereas adrenergic antagonists into the nucleus accumbens did not, and neither did haloperidol directly onto the caudate nucleus. As the nucleus accumbens is part of the neurocircuitry involved in PPI, these results also suggest that this system may be partially disrupted in *Map2k7*$^{+/-}$ mice, and in a different way in males and females, which was discussed in **Section 4.5.3** above.

4.5.5 Minocycline treatment decreased locomotor activity levels for all female mice

Minocycline was then administered in the drinking water of half the mice (the other half received standard drinking water) for one week in an attempt to alleviate the overall hyperactive phenotype. Minocycline is described in **Section 1.7.2.** Minocycline, overall, decreased activity levels of all mice; however, post hoc analyses showed that minocycline treatment did not have a significant effect on male mice but decreased activity levels of all female mice to the same extent, regardless of their genotype, closer to a level more like that of male mice. The

fact that male *Map2k7*$^{+/-}$ mice were not hyperactive compared to male WT mice at adulthood (when the minocycline was administered), and that genotype difference did occur within female mice at adulthood, suggests that minocycline may act to decrease activity levels, but only if they are elevated in the first place. It may be that minocycline can act to stabilise the mesolimbic dopaminergic system in some way. Zhang *et al*. (2007) gave 40mg/kg minocycline intraperitoneally to mice before testing in the open field with or without the NMDA receptor antagonist MK-801 and found that minocycline attenuated the hyperlocomotor effect of MK-801, in a comparable way to the effect minocycline had on *Map2k7*$^{+/-}$ mice in the current study. The authors also considered potential mechanisms and discovered that the extracellular increase in prefrontal and striatal dopamine induced by MK-801 was significantly attenuated by pre-treatment with minocycline. Moreover, minocycline did not decrease extracellular dopamine levels of mice that did not receive MK-801 (Zhang *et al.*, 2007), further demonstrating that minocycline has the ability to regulate, or normalise, extracellular dopamine levels in the brain when necessary.

4.5.6 Conclusion

Dysfunction in the mesolimbic pathway, a dopaminergic reward pathway connecting the ventral tegmental area to the nucleus accumbens, is strongly associated with the positive symptoms of schizophrenia, and both behavioural tests carried out in this chapter rely on the mesolimbic system (Van Den Buuse, 2010; Pratt *et al.*, 2012). In this chapter, although *Map2k7*$^{+/-}$ mice did not show a deficit in PPI, they were hyperactive in the open field, which presented earlier in male than female *Map2k7*$^{+/-}$ mice, and amphetamine administration shows signs of producing an attenuated effect in *Map2k7*$^{+/-}$ mice on PPI (and 2-DG imaging, carried out by N. Dawson, Lancaster, UK), compared to WT mice. These results suggest that dopaminergic (and possibly other neurotransmitter systems) may be disrupted in *Map2k7*$^{+/-}$ mice; these potential compensatory effects will need to be examined further.

This chapter also showed that minocycline acted to decrease activity levels of more hyperactive, female mice in the open field and highlights the need for further investigation into the mechanism of minocycline, as it appears to influence mice in this chapter and in the cognitive 5-CSRTT (**Chapter 5**), regardless of genotype. Minocycline may, therefore, work by balancing / regulating dopamine

levels, either directly or indirectly, and appears to have a smaller effect on less hyperactive mice, i.e. WT mice or adult male mice in the current experiment.

Therefore, although further molecular investigation will be necessary, these results suggest that *Map2k7*$^{+/-}$ mice show some characteristics relevant to the positive symptoms of schizophrenia, in a different way and time period in male and female mice.

Chapter 5 The 5-Choice Serial Reaction Time Task

This chapter has been published in part:

Openshaw RL, Thomson DM, Penninger JM, Pratt JA and Morris BJ (2017) Mice haploinsufficient for Map2k7, a gene involved in neurodevelopment and risk for schizophrenia, show impaired attention, a vigilance decrement deficit and unstable cognitive processing in an attentional task: impact of minocycline. P*sychopharmacology (Berl.)* **234(2):** 293-305.

5.1 Introduction

Current treatments for patients with schizophrenia can alleviate psychotic symptoms to some extent, but are limited in their effectiveness for negative and cognitive dysfunction, which are the symptoms carrying the strongest predictor for long-term functional outcome of patients (Keefe *et al.*, 2007; Mishara & Goldberg, 2004). The multiple cognitive domains in which patients with schizophrenia show deficits include: speed of processing, attention, decision-making, pre-attentional sensory gating, working memory, visual and verbal learning and memory, and social cognition (Green *et al.*, 2004). Attention is arguably the most important of these, as the ability to maintain proper attention inevitably has a direct effect on performance in other cognitive domains. Moreover, it is considered one of the core impairments in schizophrenia (Fukumoto *et al.*, 2014) and it has been suggested that attentional deficits may actually precede the onset of psychotic symptoms (Cornblatt & Keilp, 1994; Erlenmeyer-Kimling & Cornblatt, 1992; reviewed in Lewis, 2004).

Attention is complex; many subtypes have been described, including focussed, sustained, visuo-spatial, divided, and effortful, controlled attention (Chudasama & Robbins, 2003). Attentional deficits in humans can be assessed via multiple different tasks, however MATRICS selected the continuous performance test (CPT) as the optimal way of measuring attention because of its strong test-retest reliability and lack of ceiling effect (Nuechterlein *et al.*, 2008). The CPT was originally developed by Beck *et al.* in 1956 to probe sustained attention. It was

then developed further and adapted to probe other aspects of attention, such as visuo-spatial attention (Wilkinson, 1963; Carli *et al.*, 1983). Nowadays, various versions exist but the basic concept of CPTs is that the subject is rapidly presented with various stimuli from which they have to identify and correctly respond to "target" stimuli as quickly as possible; duration of the task is intended to be quite long (usually at least 100 trials), so it is sufficient to assess sustained attention (Riccio *et al.*, 2002). The main measurements usually used to determine if a subject has an attentional deficit are reaction times and accuracy. Deficits have been identified and widely reported in patients with schizophrenia through various versions of the CPT (Turner *et al.*, 2015), where patients show slower reaction times and decreased accuracy, but also increased omissions and worse performance over time (sustained attention), and more variability in responses (Elvevåg *et al.*, 2000; Mulet *et al.*, 2007). Patients do not tend to show a difference in false alarms or motivational state (Cornblatt *et al.*, 1989).

In order to reliably assess attentional function in rodents, a task that is highly translatable to those used in humans is required. Based on the tasks probing specific cognitive domains recommended by MATRICS, the CNTRICS panel selected analogous tasks for use with animals, in which the 5-CSRTT was one task chosen to measure attention (Lustig *et al.*, 2013). The 5-CSRTT was developed by Robbins and colleagues in 1983 and is now a very well-validated task, able to selectively measure sustained, focussed, divided and spatial attention, as well as inhibitory control (also referred to as impulsivity and compulsivity; Robbins, 2002). Much investigation has been carried out to show that it does indeed probe equivalent behavioural and neural mechanisms in the rodent and human brain under healthy conditions and also following pharmacological manipulation (Robbins, 2002; Chudasama & Robbins, 2004), which reinforces its applicability for new drug development. Importantly, patients of various neuropsychological disorders (such as ADHD, schizophrenia, drug addiction, Alzheimer's, Parkinson's and Huntington's disease) and their corresponding animal models show a similar combination of the different types of attentional deficits in the 5-CSRTT and CPT (Bari *et al.*, 2008).

5.1.1 The 5-choice Serial Reaction Time Task (5-CSRTT)

During the 5-CSRTT, rodents are required to respond via nose-poke to brief flashes of light that appear psuedorandomly within 1 of 5 small apertures in an operant

box (**Fig. 5.1**), therefore assessing spatially divided attention. Each session, which occurs daily, has a large number of trials (usually 100), thus being able to assess sustained attention by observing performance over time. Many other aspects of rodents' performance are automatically recorded (see **Materials and Methods** for more information). They are rewarded with palatable food (usually Yazoo® strawberry milkshake) when they make a correct response, and are punished by a time-out period and illumination of the main "house light" if they nose-poke the wrong hole or miss the trial by failing to poke a hole within the given time period. Mice typically take about 30 daily sessions after initial training to be able to perform at around 80% correct, and, once acquired, the 5-CSRTT can then be used to probe the many distinct aspects of attention by introducing manipulations which alter task demands. These would occur throughout separate, daily sessions and include: altering the stimulus duration length (which shifts general attentional demand), changing the length of time between trials (to investigate inhibitory control) and also the observation of behaviour following pharmacological manipulations (Bari *et al.*, 2008; Humby *et al.*, 2005).

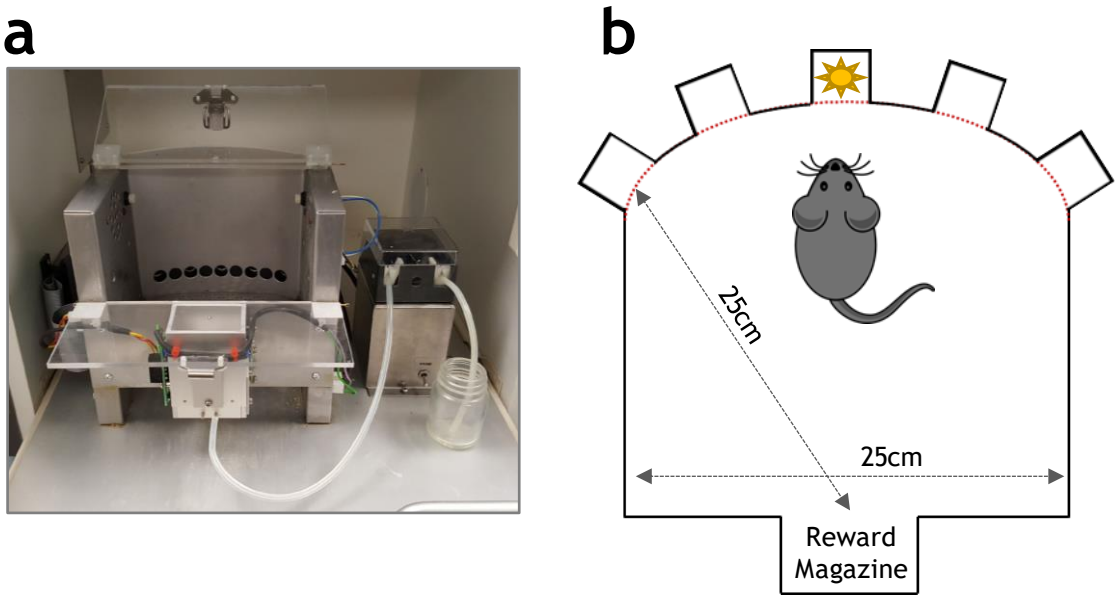


Figure 5.1. a) The 9-hole operant box. b) A schematic diagram of the 5-CSRTT equipment. Mice tend to utilise a "scanning" strategy where they face the holes during the inter-trial interval (see **Materials and Methods** for more information) in order to focus properly on the task. Holes 2, 4, 6 and 8 are blocked off during the 5-CSRTT. The food magazine is located opposite the holes, which delivers a set amount (usually 20µl) of strawberry milkshake (Yazoo®) when the mouse makes a correct response. Adapted from Bari *et al.*, 2008.

5.2 Aims

In this chapter, various aspects of the attentional function of *Map2k7*$^{+/-}$ mice compared to their wildtype littermates will be investigated by examining performance in the 5-CSRTT. Following training to a particular level, task manipulations will be carried out to 1) assess inhibitory response control by varying the inter-trial interval, and 2) performance following overall increased task demand by changing the stimulus duration length randomly throughout a session.

Additionally, performance following an acute dose of ketamine in the 5-CSRTT will be assessed in order to determine whether this drug induces an altered behavioural response in *Map2k7* deficient mice compared to their WT littermates: NMDAR antagonists, such as ketamine, are able to induce schizophrenia-like symptoms in healthy subjects (Breier *et al.*, 1997; Krystal *et al.*, 1994) as well as exacerbate symptoms in patients with schizophrenia (Lahti *et al.*, 1995, 2001; Malhotra *et al.*, 1997), and administration of ketamine in rodents has been shown to produce some symptoms relevant to schizophrenia (Miyamoto *et al.*, 2000; van den Buuse, 2010).

Finally, we were interested to find out if minocycline, a tetracycline antibiotic currently showing promise in clinical trials for schizophrenia (Levkovitz *et al.*, 2010; Oya *et al.*, 2014; Zhang & Zhao, 2014), could ameliorate any deficits seen in *Map2k7*$^{+/-}$ mice in the 5-CSRTT.

5.3 Materials and Methods

5.3.1 Subjects

16 *Map2k7*$^{+/-}$ mice (HZ; 7 female, 9 male) and 15 WT littermates (7 female, 8 male) were used in the experiment. At the start of the experiments, males weighed 29.5 ± 0.5g and females weighed 23.0 ± 0.7g, and mice were 18.9 ± 0.6 weeks of age. All mice were singularly housed in a temperature and humidity-controlled room (21 °C, 45–65 % humidity) with a 12-hour light/dark cycle (lights on at 08:00). Mice were food restricted to 85–90 % of their free-feeding weight and had ad libitum access to water throughout the experiment. All testing was carried out between 09:00 and 15:00, Monday to Friday and in accordance with the Animals (Scientific Procedures) Act, 1986.

5.3.2 Drug administration

Concentrations/doses were chosen based on pilot data in our laboratory: dose and timing of ketamine were chosen so that cognitive effects could occur but performance on the 5-CSRTT would not be confounded due to hyperlocomotion, sedation, or de-motivation. Oliver *et al.* (2009), also administered 20mg/kg 15 minutes before testing in C57Bl/6 mice and did not see effects on latencies to make a correct response or to collect the reward, suggesting that their locomotor activity and motivation are unaffected. Ketamine (as a racemic mixture of its two enantiomers; Sigma-Aldrich Co. K2753, St. Louis, MA, USA) was given at 20mg/kg intraperitoneally (i.p.) and mice were tested in the 5-CSRTT 15 minutes after administration.

Minocycline was administered in a similar manner to McKim *et al.*, 2016: mice received minocycline in their standard drinking water (0.5mg/ml; protected from light; Sigma-Aldrich Co. M9511, St. Louis, MA, USA) for a 7 day period. Fresh minocycline solution was prepared every second day and provided at room temperature. Mice were tested on the 5-CSRTT on days 4 and 7 after the start of minocycline treatment. Consumption of minocycline treated water was monitored daily for each mouse; they received an average of 81.6 ± 3.1mg/kg/day of minocycline.

5.3.3 Apparatus

Eight mouse 9-hole operant chambers with dimensions of 12 by 13cm (Campden Instruments Ltd., Cambridge Cognition Limited) were used for the experiment, which were enclosed in separate noise-attenuation outer cabinets with a ventilator fan providing low-level, constant background noise. Mice were allocated an operant box randomly and were always tested in the same operant box throughout the experiment. Care was taken to ensure that the same box was not being used for all mice of one particular group; for example, all WT/*Map2k7*$^{+/-}$ or all male/female. 9 circular holes are evenly spaced along a curved side, of which 4 holes (holes 2, 4, 6 and 8) were blocked off leaving 5 available for use in the task. The operant chambers were controlled by Campden BNC Control software.

5.3.4 The Task

A schematic of the basic structure the full 5-CSRTT takes is shown in **Fig. 5.2**. All mice were trained for 61 sessions on a fixed inter-trial interval (ITI; according to the methods outlined in Bari *et al.*, 2008; Thomson *et al.*, 2011) until they reached a stable level of performance. Mice were then trained on a variable ITI (vITI), to avoid the use of a temporal strategy to complete the 5-CSRTT, until their performance stabilised once again. Then, 5 days testing occurred which was taken as "Baseline" before they were subjected to various manipulations in order to provide information about different aspects of attentional functioning and/or motivation and impulsivity (Bari *et al.*, 2008, Humby *et al.*, 2005 and Robbins, 2002). See **Fig. 5.3** for a timeline of this experimental design.

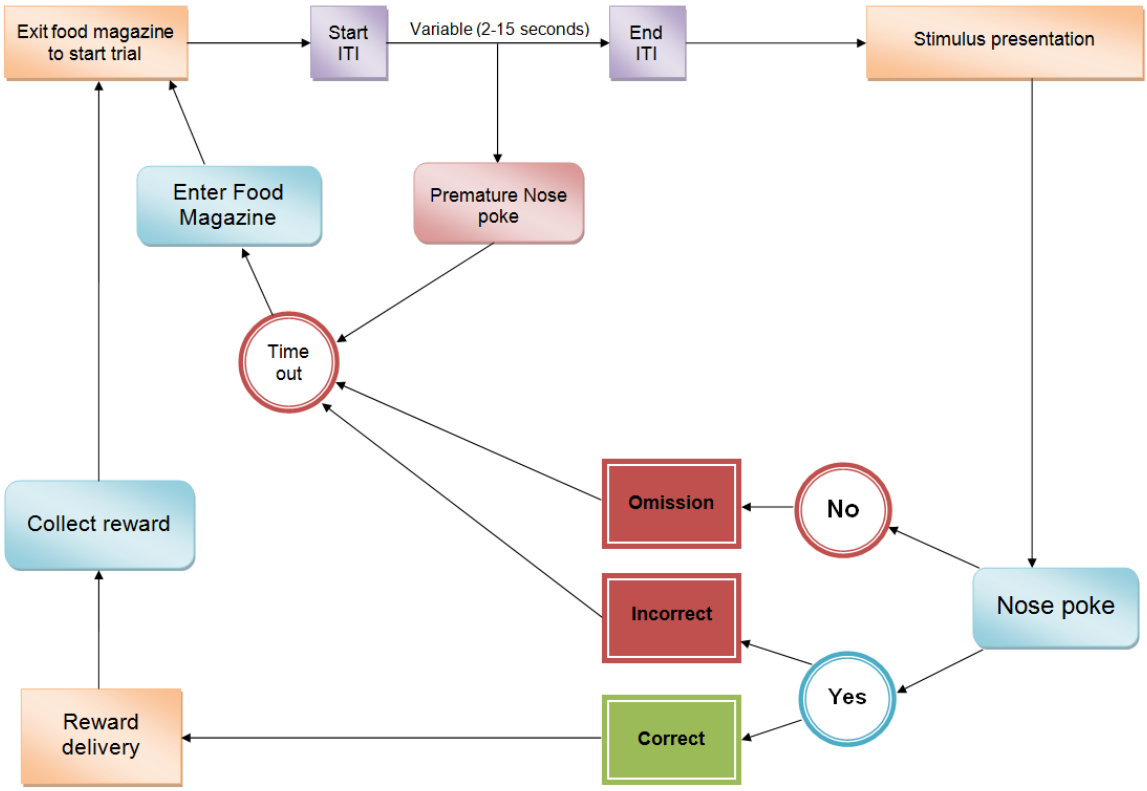


Figure 5.2. Schematic diagram showing the basic structure of the full 5-CSRTT. Each trial is discrete, separated by the inter-trial interval (ITI) period, and is initiated by the mouse entering and leaving the food magazine. A correct response is rewarded and an incorrect or premature response, or missed trial, is punished by a time out (TO) period and illumination of the house light for 5 seconds before the trial can be re-initiated. The session ends when 100 trials have been completed or 45 minutes has passed, whichever comes first. *Adapted from 5-CSRTT User Manual*.

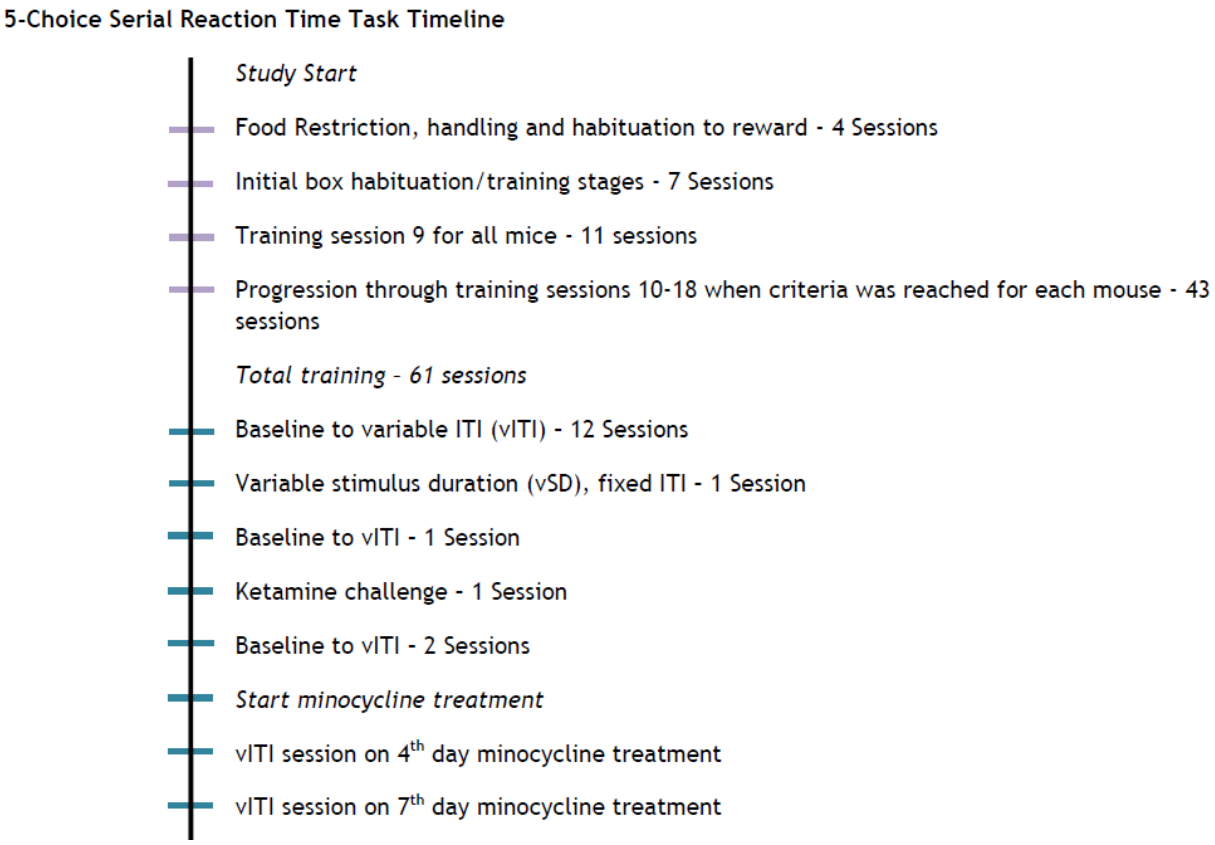


Figure 5.3. Experimental Timeline showing the order in which experimental manipulations were carried out.

Liquid reinforcer (Yazoo® Strawberry Milkshake) was used throughout the experiment. The training stages are designed to gradually introduce the mice to the operant boxes and to nose poke the lit hole in order to obtain the reward. As Stages 9 - 18 progress, the length of time the stimulus is lit for (stimulus duration) and limited hold (LH; the length of time the mice have to respond within, from the beginning of the presentation of the stimulus) decrease and the criteria become more stringent until the mice are performing at a level deemed appropriate to see differences in other aspects of performance measures (e.g. % missed) without being confounded by differences in task ability (Bari *et al.*, 2008). These details are shown in **Table 5.1** and explained in more detail below:

During the 7 sessions of initial box habituation/training, the mice are gradually introduced to the operant boxes and learn to nose poke the lit hole in order to obtain the reward. During the first session, a mouse is placed in each operant box for 30 minutes. They receive an initial "free" reward of 100µl and then a smaller 50µl free reward delivery every 40 seconds in the magazine (light always on) in

order to train the mice to learn where they can find the reward. This was repeated the next day to ensure all mice consumed all the strawberry milkshake, and then the reward amount was decreased for the following 2 sessions (50µl initially and then 20µl every 40 seconds), and the magazine light was extinguished when the mouse exits. For the next session, mice were in the box for 30 minutes or until they had completed 100 trials. They obtained an initial reward delivery of (50µl) in the magazine, in which the light is illuminated and is extinguished when the reward had been collected. One of the 5 stimulus lights are pseudorandomly illuminated for 10 seconds, and then a reward is delivered (20µl) during the final second of stimulus light illumination in order to pair the turning off of the stimulus light with the delivery of reward. From here on, whenever a reward is delivered, the magazine light illuminates and then extinguishes when the mouse has collected the reward. This session occurred 3 times and throughout, the mice were all moved to the next session as a group.

The mice progressed individually through the next training stages (10 in total; details shown in **Table 5.1**) when they reached the criteria for that stage. Once an individual mouse had reached the final training stage (Stage 18), their training was halted until the rest of the mice caught up, and were given a reminder session of the final stage twice a week (Mondays and Thursdays). If their performance dropped below criteria on a reminder session, they were trained on the following days until they reached criteria again. This training regime is encouraged for operant-based training (Oomen *et al.,* 2013) because "over"-training the mice that pick up the task more quickly than others could have confounding effects on results (Sanchez-roige *et al.,* 2012).

Training Stage #	Stimulus Duration (s)	ITI (s)	LH (s)	Time Out (s)	Session Duration (min)	Criterion to move to next stage (Bari *et al.*, 2008)
9	32	5	37	5	30	>30 Correct trials
10	16	5	21	5	30	>30 Correct trials
11	8	5	13	5	30	>50 Correct trials
12	4	5	9	5	30	>50 Correct trials, >80% Accuracy
13	2	5	7	5	30	>50 Correct trials, >80% Accuracy
14	1.8	5	6.8	5	30	>50 Correct trials, >80% Accuracy, <20% Omissions
15	1.6	5	6.6	5	30	>50 Correct trials, >80% Accuracy, <20% Omissions
16	1.4	5	6.4	5	30	>50 Correct trials, >80% Accuracy, <20% Omissions
17	1.2	5	6.2	5	30	>50 Correct trials, >80% Accuracy, <20% Omissions
18	1	5	6	5	30	>50 Correct trials, >80% Accuracy, <20% Omissions Task complete within 30 minutes
Full task (vITI)	1	2-15	6	5	45	>50 Correct trials, >80% Accuracy, <20% Omissions Task complete within 45 minutes
vSD	0.2-0.8	5	6	5	30	N/A
Ketamine	1	2-15	6	5	45	N/A
Minocycline	1	2-15	6	5	45	N/A

Table 5.1: 5-CSRTT training schedule details.

Once all the mice reached the final training stage, they were moved onto the full task (variable ITI) conditions which are the same as training Stage 18 apart from the ITI was not fixed at 5 seconds; instead it pseudorandomly varied between 2, 5, 10 or 15 seconds. As the overall ITI length was increased, the session duration was increased from 30 to 45 minutes. They were trained on the vITI until their performance stabilised (which took 11 sessions) and the final 5 days' stable data was taken as "Baseline" performance. It is this Baseline performance that is compared with the ensuing manipulations.

Task manipulations then occurred separated by normal vITI conditions (see **Fig. 5.3**) in order to check the mice were performing back to normal good level of performance. Manipulation conditions are outlined in **Table 5.1**.

5.3.5 Statistical Analysis

All mice were included in all analyses with two exceptions: one significantly atypical wildtype mouse was removed from the study (and all analyses) because of consistent abnormal repetitive behaviour (hyperactive rotational movements) that prevented the mouse from completing the task properly, disguising its true cognitive ability. One heterozygous mouse was removed from ketamine analysis because it had an adverse reaction to the ketamine and did not take part in the task following ketamine administration.

All statistical analyses were carried out using Minitab® 17 Statistical Software. Results were considered significant if $p<0.05$. All error bars are expressed as ± standard error of the mean (SEM). Bar and line graphs were created using GraphPad Prism 7. Box plots were created using BoxPlotR, an application available at http://shiny.chemgrid.org/boxplotr/ and described in Nature Methods Editorial "Kick the bar chart habit" 2014, p113.

Comparison of the last 5 days' stable performance between two experimental groups is a method generally utilised to examine group differences (Sanchez-roige *et al.*, 2012): the last 5 days of well-trained, stable performance were analysed between genotypes and, where appropriate, were compared with performance on day 4 and 7 of the 7 days' minocycline treatment.

Many of the parameters throughout this experiment yielded non-normal data. Although it is possible that parametric statistical tests give more power when

analysing non-normal data, there is no non-parametric statistical test available to accurately represent the data here. This is because a repeated measures design with multiple factors is appropriate, with nesting of each individual mouse within genotype. As non-parametric tests do not allow nesting and multiple factor repeated measures, and ANOVA is considered robust against non-normality (Laan & Verdooren, 1987), an ANOVA was decided to be the most suitable test to use here. Unless stated otherwise, results were analysed using a 2-way repeated measures ANOVA, with daily session as a within subjects factor, genotype as a between subjects factor and each individual mouse nested within genotype. Minocycline treatment data were analysed by a 3-way repeated measures ANOVA with session (i.e. day of minocycline treatment) and treatment as within subject factors, genotype as a between subjects factor and each individual mouse nested within genotype. Before data from males and females were grouped, effects of sex were investigated and were non-significant for all measurements excluding Correct Response Latency. Therefore, "sex" was included in the statistical analysis as a between subjects factor for this measure. *Post hoc* tests were conducted using Tukey's method for multiple comparisons where appropriate. As there were so many irrelevant significant effects, results for main effects and interactions are only shown and discussed when a significant effect was observed.

The performance measures for each session analysed were the following:

- % accuracy - calculated by the formula: (number of correct responses/number of correct responses + number of incorrect responses)*100
- % omissions – calculated by the formula: (number of missed trials/total number of trials completed)*100
- Number of commission errors (nose-poke during the LH period into a hole where the stimulus had not been presented)
- Number of premature responses (nose poke before the stimulus has appeared, i.e. during the ITI period)
- Number of perseverative responses (repeat nose-poke following a correct response before collecting the food reward earned)
- Total number of nose pokes (throughout the session)
- Total number of entries into the food magazine during the ITI

- Total number of trials completed
- Mean reward collection latency
- Mean correct response latency
- Mean incorrect response latency
- Vigilance decrement (the extent to which performance declined over the course of each session). Calculated by subtracting the % omissions, or % accuracy or # incorrect responses made during the final 20 trials completed from the first 20 trials completed by each mouse, for each session.
- Intra-individual variability of correct response times (IIV; the variability of response times for each mouse over the course of each session). Calculated by the standard deviation of response times for each mouse per session, then averaged for each group.
- Intra-individual variability of incorrect response times: see IIV of correct response times above.
- Overall variability of correct response times – calculated by the standard deviation of response times over the course of 5 Baseline sessions
- Overall variability of incorrect response times: see variability of correct response times above.

5.4 Results

5.4.1 Both WT and Map2k7$^{+/-}$ *mice took the same amount of time to complete training stages*

During training, each mouse was advanced to the next stage individually whenever they reached criteria for that stage until they reached stage 18 (final stage; **Table 5.1**). Overall, mice learned to complete the 5-choice task well, with WTs and *Map2k7*$^{+/-}$ mice taking a similar number of sessions to complete training (p=0.440, ns): WT mice took an average of 20.6 ± 2.22 and *Map2k7*$^{+/-}$ mice an average of 23.5 ± 2.44 (**Fig 5.4;** defined as completing >50 trials within 45 minutes, with >80% accuracy and <20% omissions over two consecutive sessions). As not all mice completed all 100 trials per session, the number of trials to complete training were also analysed: WT and *Map2k7*$^{+/-}$ mice took a similar number of trials to complete training (p=0.490, ns). WT mice took an average of 1798.6 ± 146.70 and *Map2k7*$^{+/-}$ mice an average of 1938 ± 135.39 trials.

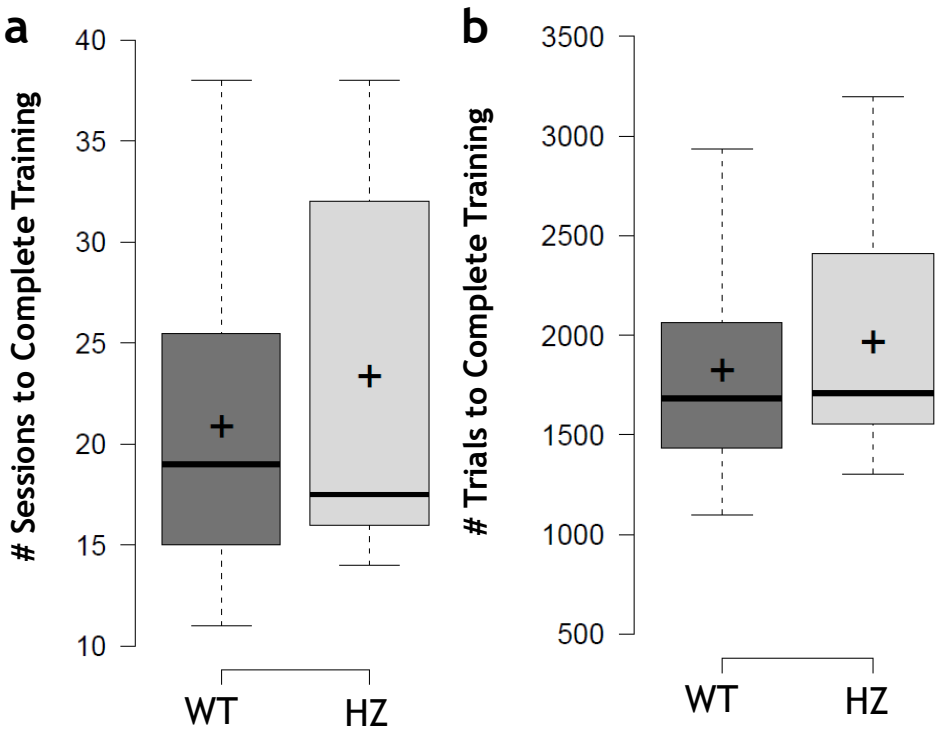
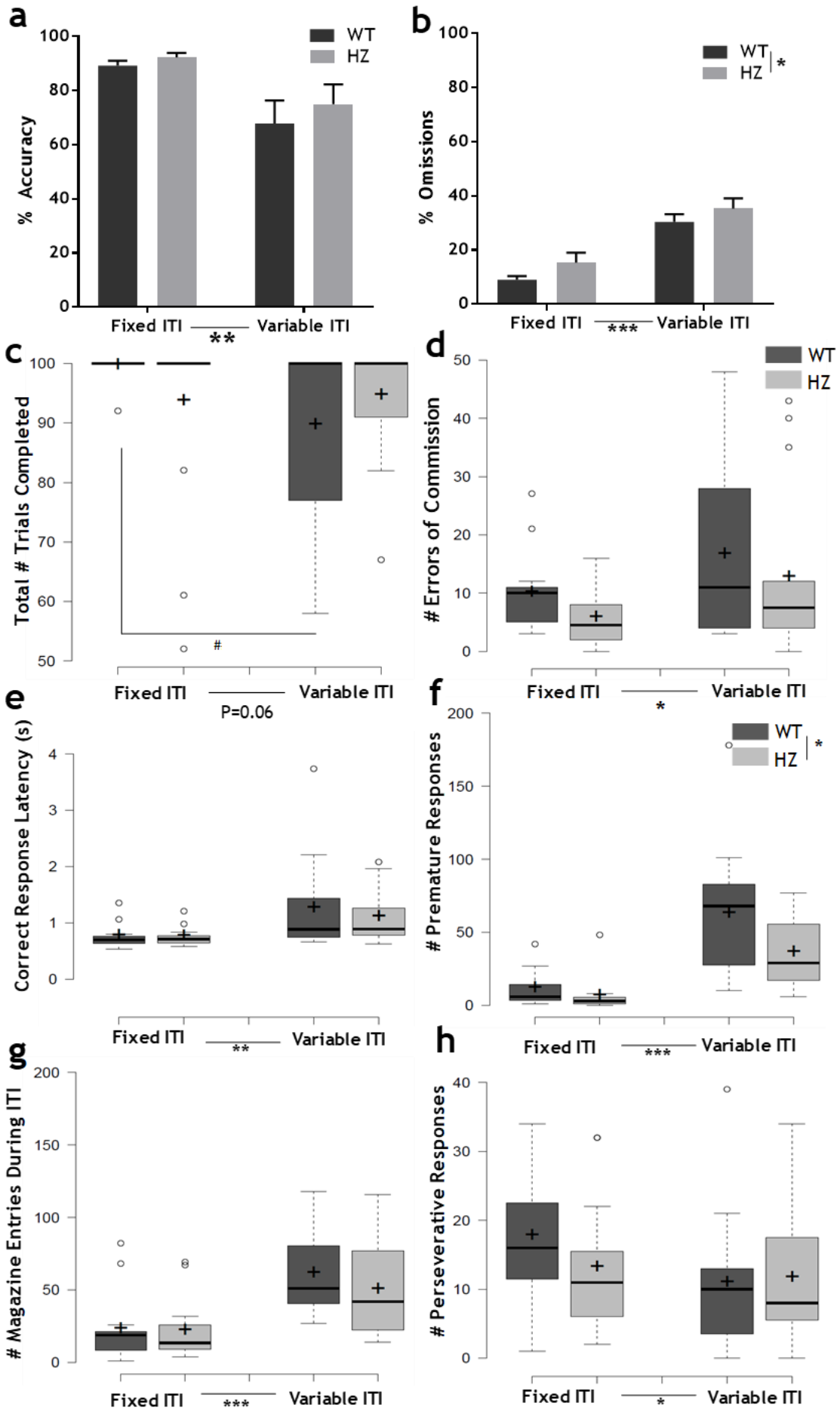


Figure 5.4. WT and *Map2k7*$^{+/-}$ mice took a similar number of sessions (a) and trials (b) to complete training for initial acquisition of the 5-CSRTT. The ITI was fixed at 5 seconds and stimulus duration fixed at 1 second. Data were analysed separately by one-way ANOVA between genotypes. No significant effect of genotype: p=0.440 and p=0.490 for **a** and **b**, respectively. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; crosses represent sample means. Data for **a** and **b** analysed separately by one-way ANOVAs between genotypes. *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

5.4.2 The transition from fITI to vITI had a brief detrimental effect on performance

Once all mice had reached criteria (>50 correct responses, >80% accuracy and <20% omissions) for training using a fixed ITI (fITI; aka final training stage), they were moved onto the standard 5-CSRTT session with a variable ITI (vITI; ranging from 2-15 seconds) to minimise confounds due to any temporal strategies they may have. All other aspects of the task remained the same (**Table 5.1**). When the last day of fixed ITI training was compared with the first day of standard session with a vITI, performance worsened (**Fig. 5.5**): % accuracy decreased ($p=0.002$, $F_{(1,27)}=11.36$), total number of trials completed showed a trend towards decreasing ($p=0.063$, $f_{(1,27)}=3.76$), and % omissions ($p<0.0001$, $F_{(1,27)}=106.89$), errors of commission ($p=0.035$, $F_{(1,27)}=5.00$), latency to correct response ($p=0.002$, $F_{(1,27)}=11.83$), premature responses ($p<0.0001$, $F_{(1,27)}=39.20$) and the number of food magazine entries during the ITI ($p<0.0001$, $F_{(1,27)}=48.32$) increased for all mice. Perseverative responses, however, decreased ($p=0.023$, $F_{(1,27)}=5.78$).

In addition to effects of the vITI on performance, there were significant effects of genotype. Overall, *Map2k7*$^{+/-}$ mice missed more trials than WTs (**Fig. 5.5b**; $p=0.011$, $F_{(1,27)}=7.54$) and made fewer premature responses than WT mice (**Fig. 5.5f;** $p=0.023$, $F_{(1, 27)}=5.84$). Also, WT mice completed significantly less trials with the vITI session compared to the fITI session whereas the *Map2k7*$^{+/-}$ mice did not (genotype x session interaction: $p=0.039$, $F_{(1, 27)}=4.72$; **Fig. 5.5c**).


a
% Accuracy
WT
HZ
Fixed ITI
Variable ITI
**
b
% Omissions
WT
HZ
*
Fixed ITI
***
Variable ITI
c
Total # Trials Completed
#
Fixed ITI
P=0.06
Variable ITI
d
Errors of Commission
WT
HZ
Fixed ITI
*
Variable ITI
e
Correct Response Latency (s)
Fixed ITI
**
Variable ITI
f
Premature Responses
WT
HZ
*
Fixed ITI
***
Variable ITI
g
Magazine Entries During ITI
Fixed ITI
***
Variable ITI
h
Perseverative Responses
Fixed ITI
*
Variable ITI

< Figure 5.5. The transition from fITI to vITI had a brief (1 session) detrimental effect on performance. Following a switch from a fixed ITI to a variable ITI: (**a**) % Accuracy was significantly decreased overall (**b**) % omissions increased for both WT and HZ mice, and HZ mice missed more trials than WTs overall. (**c**) The number of trials completed, although not significant, showed a trend towards being decreased slightly overall. WTs completed significantly less trials with the vITI whereas the HZ mice did not. (**d**) Errors of commission were significantly elevated. (**e**) All mice took significantly longer to respond correctly to stimuli. (**f**) Premature responses were significantly increased and overall, HZ mice made fewer premature responses than WT mice. (**g**) Mice made increased entries into the food magazine during the ITI. (**h**) Mice made less perseverative responses. Data analysed by a two-way ANOVA with genotype and sex as between subjects factors, session (fixed or variable ITI) as a within subjects factor, and each individual mouse nested within genotype. Post hoc tests were conducted using Tukey's method for multiple comparisons where appropriate. Bar charts represent sample mean ± SEM. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Lines between groups show the significance between those groups: *p< 0.05; **p< 0.01; ***p< 0.001 (ANOVA); #p<0.05 (Tukey's). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

5.4.3 Map2k7$^{+/-}$ *mice exhibit impaired attention in the 5-CSRTT*

Overall performance of *Map2k7*$^{+/-}$ and WT mice are given in **Figs. 5.6** and **5.7**. Throughout training and once stable performance had been attained, *Map2k7*$^{+/-}$ mice consistently showed a similar level of accuracy as WT mice (**Fig. 5.6a**), in fact, they performed marginally better at 94.6% ± 0.9 accuracy compared to WT littermates at 93.5% ± 0.7 ($p=0.052$, $F_{(1,116)}=3.87$), and made fewer commission errors overall (*Map2k7*$^{+/-}$: 4.063 ± 0.67; WT: 5.33 ± 0.48) ($p=0.0001$, $F_{(1,116)}= 17.24$) (**Fig. 5.6b**). Even when challenged with shorter stimulus durations, *Map2k7*$^{+/-}$ mice were still able to perform to a similar extent to WT mice ($p<0.0001$, $F_{(3,84)}=30.63$) (**Fig. 5.8**). However, *Map2k7*$^{+/-}$ mice showed impaired attentional performance, as indicated by an elevated number of omissions made compared to wildtype littermates ($p=0.0001$, $F_{(1,116)}=42.36$), which was consistent throughout training on the vITI, (**Fig. 5.6c** line graph). Inhibitory control measures showed that *Map2k7*$^{+/-}$ mice were not impaired compared to WTs: they made significantly fewer perseverative responses ($p=0.001$, $F_{(1,116)}=12.13$) (**Fig. 5.6d**) and exhibited a similar number of premature responses ($p=0.463$, ns) (**Fig. 5.6e**).

Parameters that give an indication of motivation levels in order to rule out lack of motivation as reasons for performance deficits showed that *Map2k7*$^{+/-}$ mice were highly motivated to perform the task: they had similar correct response latencies to WTs ($p=0.22$, ns) (**Fig. 5.7a**), were quicker to collect the reward ($p=0.0001$, $F_{(1,116)}=240.97$) (**Fig. 5.7b**) and completed almost all trials (99.6 ± 0.4 trials completed on average over the 5 days' baseline, compared to WTs completing all 100; $p=0.011$, $F_{(1,116)}=6.64$) (**Fig. 5.7d**). *Map2k7*$^{+/-}$ mice were slower to make an incorrect response (explored in more detail below) ($p<0.0001$, $F_{(1,116)}=21.21$) (**Fig. 5.7c**) and also entered the reward magazine more frequently during the ITI period (i.e. when there is no reward there to collect) than WTs ($p=0.0001$, $F_{(1,116)}=51.92$;) (**Fig. 5.7e**).

Collectively, these results indicate that *Map2k7*$^{+/-}$ mice exhibit an attentional deficit displayed as increased omissions in the 5-CSRTT. Furthermore, this deficit is not due to incapability of learning or carrying out of the task, as shown by good accuracy, even when challenged with shorter stimulus durations and when going through initial task acquisition. The deficit is also not due to motivational/motoric impairment: they displayed clear motivation to perform the task in several aspects of performance, and they had slightly faster reward collection latencies, which

indicates that measurements such as the number of omissions are not confounded for reasons such as being physically incapable of reaching the stimulus during the limited hold period.

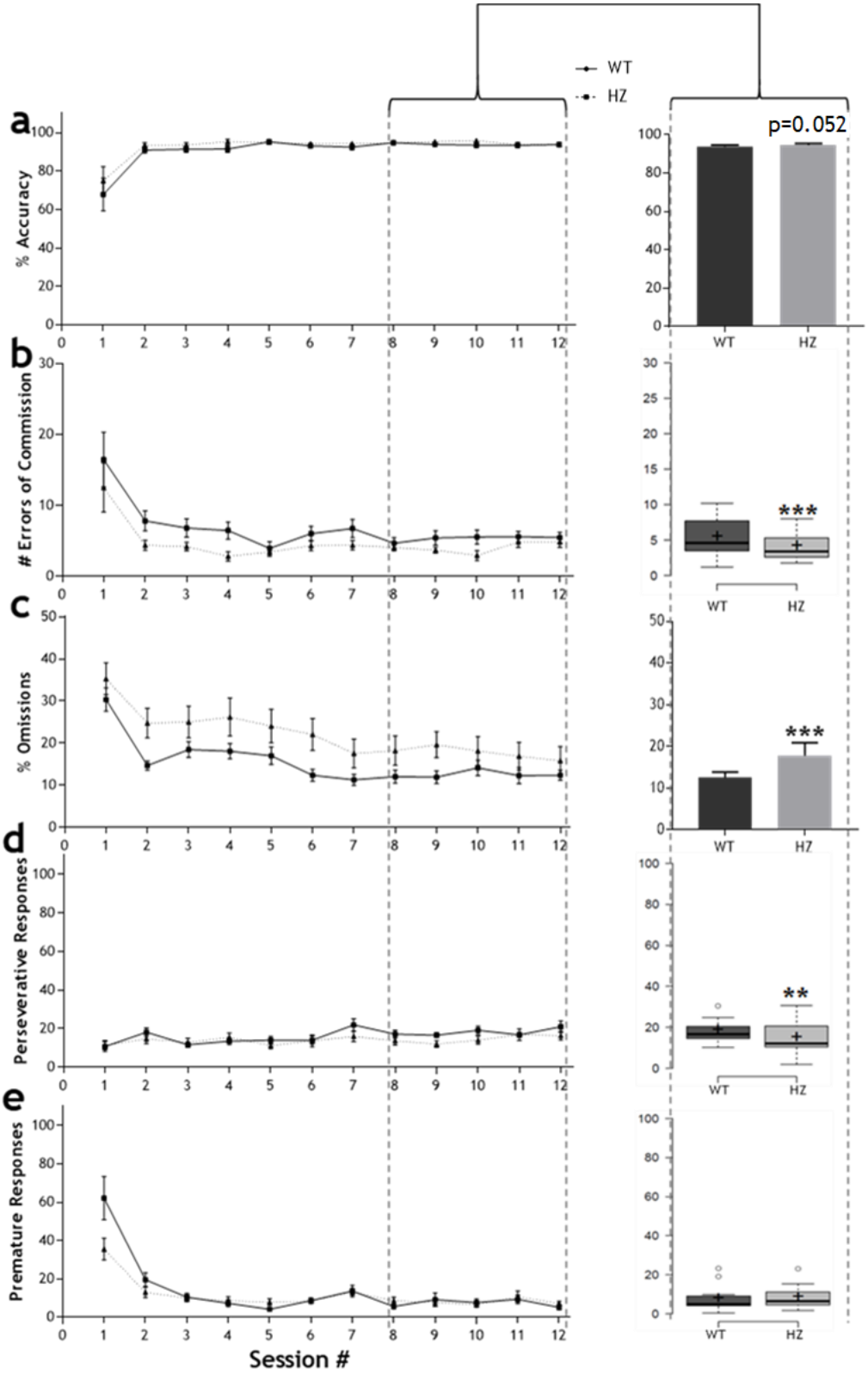


Figure 5.6. Overall performance of mice on the 5-CSRTT – *Map2k7*$^{+/-}$ mice display an attentional deficit. Each day of vITI training performance from day (Session) 1 (x-axis): (**a**) *Map2k7*$^{+/-}$ mice consistently showed a similar level of accuracy as WT mice, and (**b**) made fewer commission errors overall. (**c**) *Map2k7*$^{+/-}$ mice made an increased number of omissions, (**d**) perseverative responses and (**e**) a similar number of premature responses. Line graph points show data for each session (numbered from beginning of the first vITI session). Bar graphs: bars represent the sample mean ± SEM

of the last 5 days' stable vITI performance. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles. Data analysed by a 2-way repeated measures ANOVA (with daily session as a within subjects factor, genotype as a between subjects factor and each individual mouse nested within genotype) with Tukey's post hoc, and are presented as the mean ± standard error of the mean (SEM). **p<0.01; ***p<0.001 (ANOVA). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

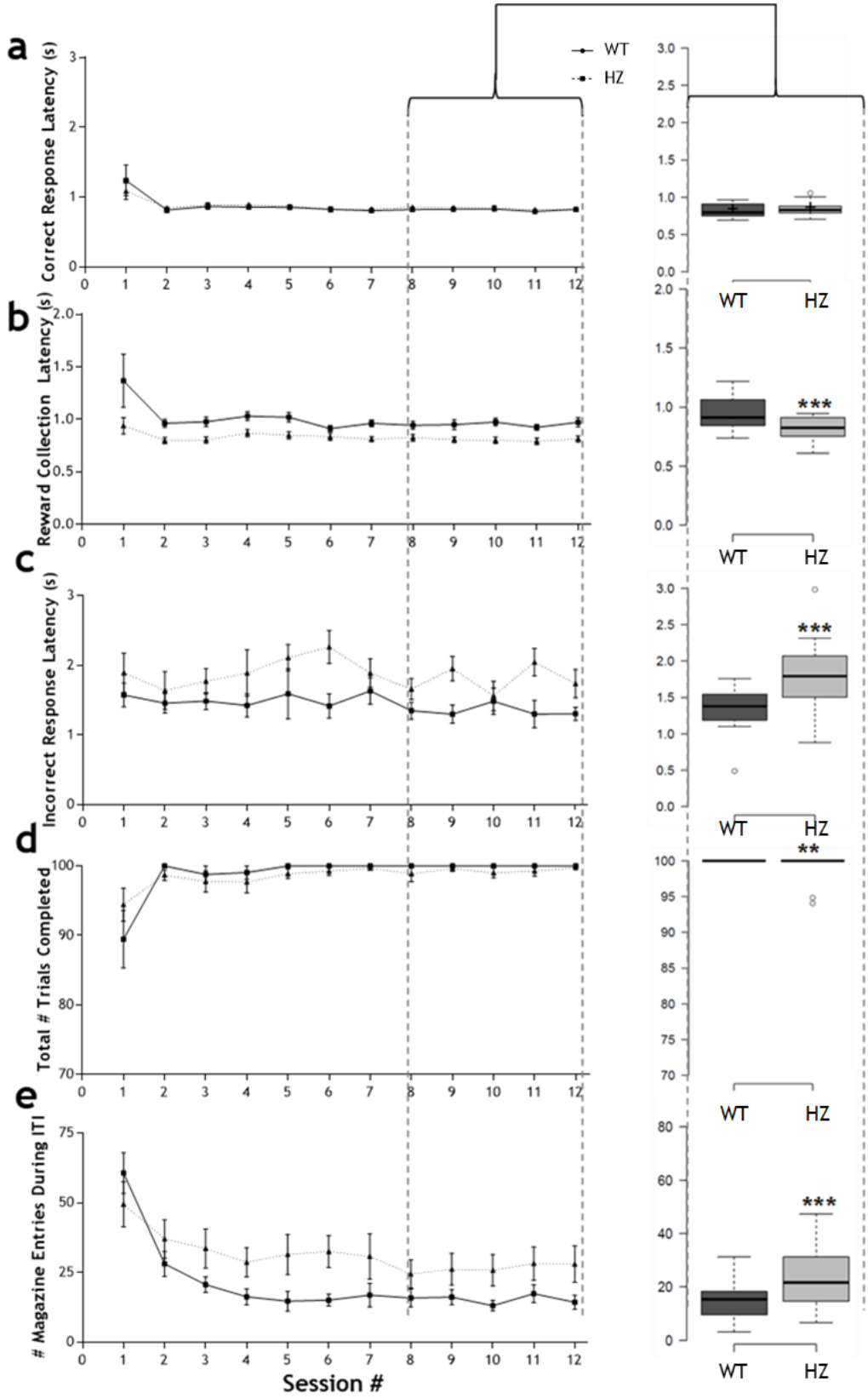


Figure 5.7. *Map2k7*$^{+/-}$ mice are highly motivated to perform the 5-CSRTT. Each day of vITI training performance from day (Session) 1 (x-axis): (**a**) *Map2k7*$^{+/-}$ mice had similar correct response latencies to WTs, (**b**) were quicker to collect the reward and (**d**) completed almost all trials. (**c**) *Map2k7*$^{+/-}$ mice were slower to make an incorrect response and (**e**) entered the reward magazine more frequently during the ITI period (i.e. when there is no reward there to collect) than WTs. Line graph points show data

for each session (numbered from beginning of vITI). Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles. Data analysed by a 2-way repeated measures ANOVA (with daily session as a within subjects factor, genotype as a between subjects factor and each individual mouse nested within genotype) with Tukey's post hoc, and are presented as mean ± standard error of the mean (SEM). **p<0.01; ***p<0.001 (ANOVA). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

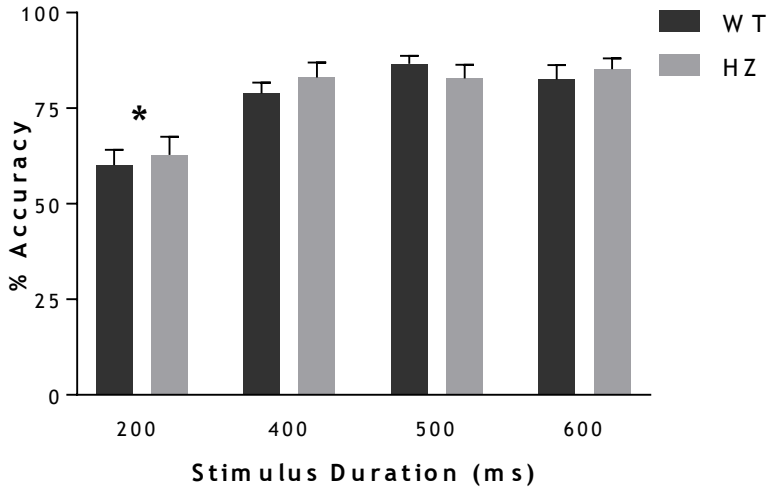


Figure 5.8. Performance of *Map2k7*$^{+/-}$ mice in the 5-CSRTT remained similar to WTs after being challenged with shorter, variable stimulus durations (SD). Throughout a single session, mice were subjected to variable stimulus durations with a fixed ITI of 5 seconds. Stimulus durations varied pseudorandomly across the session between 0.2, 0.4, 0.5 and 0.6 seconds in order to challenge accuracy. All mice performed with significantly decreased accuracy at 200ms compared to all other stimulus durations, but performance remained similar between WT and *Map2k7*$^{+/-}$ mice (p=0.47, ns). Data analysed by a repeated measures ANOVA, with genotype as a between subjects factor, stimulus duration as a within subjects factor and each individual mouse nested within genotype, with Tukey's post hoc. Data are presented as mean ± SEM. *p<0.0001, vs all other stimulus durations (Tukey's). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

5.4.4 Ketamine had a detrimental effect on 5-CSRTT performance with Map2k7$^{+/-}$ *and WT mice*

Ketamine (20mg/kg) was administered 15 minutes prior to testing in the 5-CSRTT in order to determine whether this drug induces an altered behavioural response in *Map2k7* deficient mice compared to their WT littermates: NMDAR antagonists, such as ketamine, are able to induce schizophrenia-like symptoms in healthy subjects (Breier *et al.*, 1997; Krystal *et al.*, 1994) as well as exacerbate symptoms in patients with schizophrenia (Lahti *et al.*, 1995; Lahti *et al.*, 2001; Malhotra *et al.*, 1997), and administration of ketamine in rodents has been shown to produce some symptoms of relevance to schizophrenia (van den Buuse, 2010).

Ketamine administration significantly decreased % accuracy for WT mice but not *Map2k7*$^{+/-}$ mice ($p=0.002$, $F_{(1,28)}=11.54$), although this difference was small (see **Fig. 5.9a**; WT baseline: 93.8 ± 0.8 % vs. WT ketamine: 90.3 ± 1.8 %). In a comparable way to Baseline performance, overall *Map2k7*$^{+/-}$ mice showed a trend towards an increased % accuracy with ketamine, compared to WTs, although this was not significant ($p=0.06$, $F_{(1,28)}=3.77$). Ketamine had no significant effect on % omissions for both WT and *Map2k7*$^{+/-}$ mice ($p=0.206$, ns): % omissions remained elevated for *Map2k7*$^{+/-}$ mice compared to WTs (**Fig. 5.9b**; $p<0.0001$, $F_{(1,26)}=22.80$). Inhibitory response control measures were altered after ketamine: premature responses increased overall (**Fig. 5.9c**; $p=0.002$, $F_{(1,28)}=12.24$), but post hoc analyses indicated that the increase was significantly different between WT mice but not *Map2k7*$^{+/-}$ mice, and perseverative responses significantly increased overall following ketamine administration (**Fig. 5.9d**; $p=0.006$, $F_{(1,28)}=9.01$). The number of errors of commission were significantly increased overall following an acute dose of ketamine ($p=0.001$, $F_{(1,28)}=14.30$) and overall, *Map2k7*$^{+/-}$ mice made fewer errors of commission than WTs ($p=0.006$, $F_{(1,28)}=8.86$; **Fig. 5.9e**).

Measures of motivation levels showed some differences with ketamine. There was no overall effect on correct (**Fig. 5.10a;** $p=0.864$, ns) or incorrect (**Fig. 5.10b;** $p=0.783$, ns) response latencies; however, under the influence of ketamine, *Map2k7*$^{+/-}$ mice were significantly slower to make a correct response than WTs (**Fig. 5.10a;** $p=0.002$, $F_{(1,28)}=12.30$), and *Map2k7*$^{+/-}$ mice remained, like at baseline, significantly slower to make an incorrect response than WTs (**Fig. 5.10b;** $p<0.0001$, $F_{(1,28)}=25.07$). Reward collection latency slightly but significantly increased for *Map2k7*$^{+/-}$ mice but not WTs (**Fig. 5.10e;** genotype*session

interaction: p=0.007, $F_{(1,28)}$=8.62): before ketamine administration, *Map2k7*$^{+/-}$ mice were quicker to collect the reward but following ketamine administration both WT and *Map2k7*$^{+/-}$ mice had similar reward collection latencies; however, the overall significant effect of genotype remained (p=0.006, $F_{(1,28)}$=8.99). The total number of pokes throughout the session (**Fig. 5.10c;** p<0.0001, $F_{(1,28)}$=26.75) and the number of magazine entries during the ITI (**Fig. 5.10f;** p=0.003, $F_{(1,28)}$=10.40) increased for both groups of mice, although similar to at baseline, *Map2k7*$^{+/-}$ mice entered the magazine throughout the ITI more than WTs (**Fig. 5.10f;** p=0.041, $F_{(1,28)}$=4.60). Ketamine did not affect the number of trials completed (**Fig. 5.10d;** p=0.532, ns).

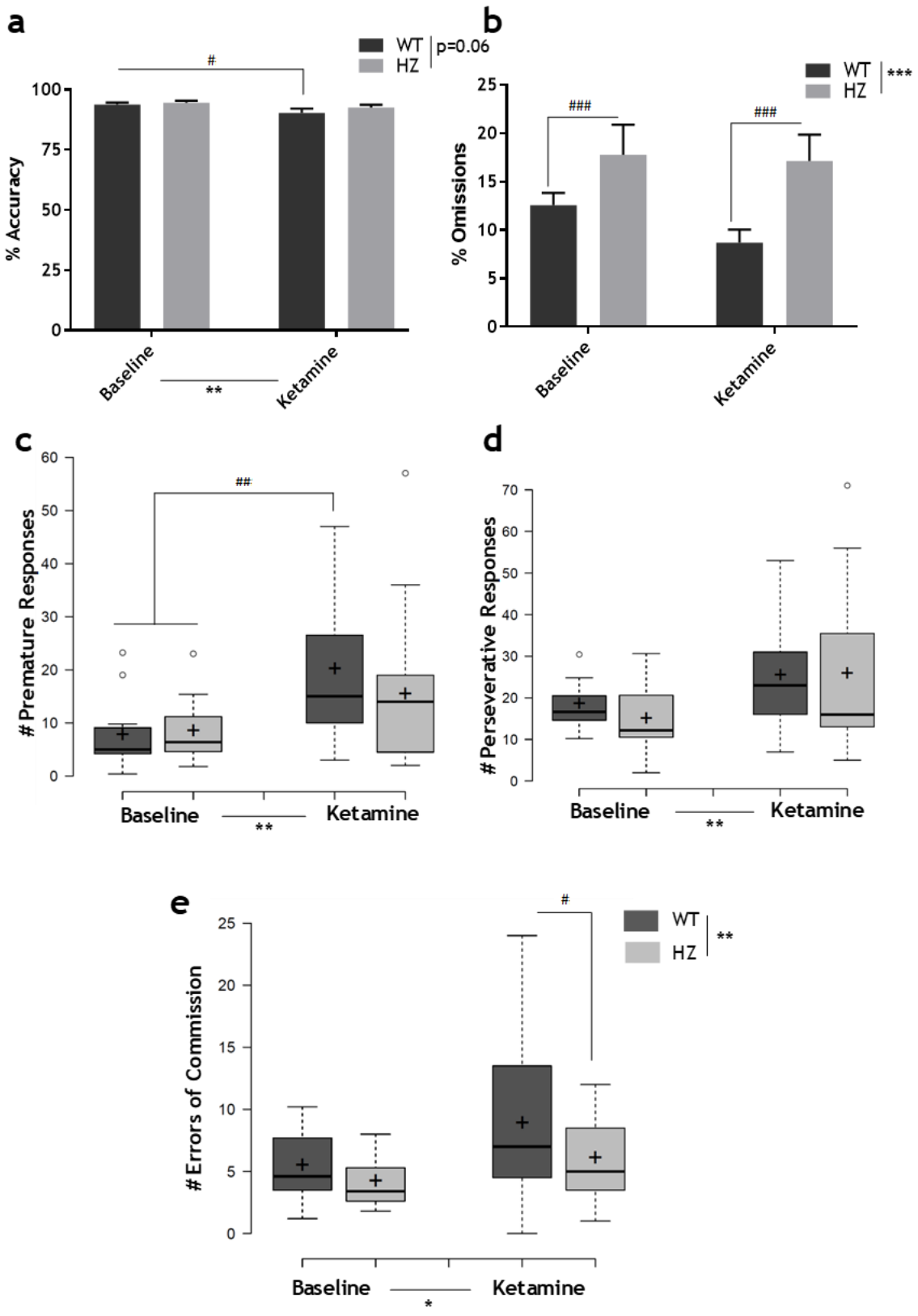


Figure 5.9. Effects of an acute dose of ketamine on mouse performance in the 5-CSRTT. Ketamine was administered at 20mg/kg, i.p. (**a**) Ketamine administration significantly decreased % accuracy for WT mice but not *Map2k7*$^{+/-}$ mice. (**b**) Ketamine had no significant effect on % omissions for both WT and *Map2k7*$^{+/-}$ mice. % omissions remained elevated for *Map2k7*$^{+/-}$ mice compared to WTs. (**c**) Ketamine significantly

increased premature responses for WT mice but not *Map2k7*$^{+/-}$ mice. (**d**) Perseverative responses significantly increased overall following ketamine administration. (**e**) The number of errors of commission were significantly increased overall following an acute dose of ketamine. Bar graphs: each bar represents the average of the last 5 days' stable performance, as mean ± SEM. Box plots: Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles. Data analysed by a three-way repeated measures ANOVA with daily session and ketamine treatment as within subject factors, genotype as a between subject factor and each individual mouse nested within genotype with Tukey's post hoc. Lines between groups show the significance between those groups: *p<0.05, **p<0.01, ***p<0.001 (ANOVA); #p<0.05, ##p<0.01, ###p<0.001 (Tukey's). *Map2k7*$^{+/-}$: n=15 (8M, 7F), WT: n= 15 (8M, 7F).

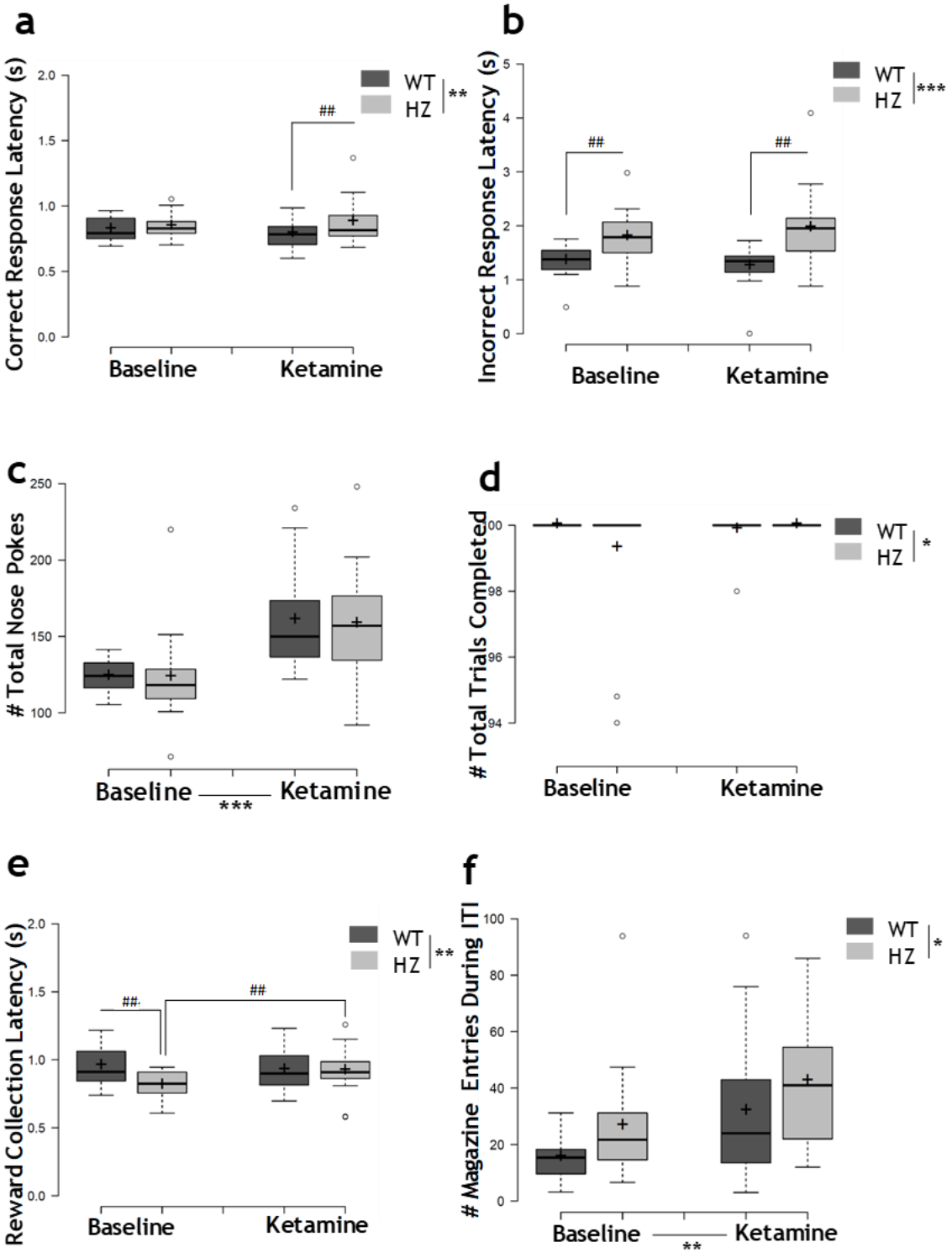


Figure 5.10. Effects of an acute dose of ketamine on measurements of motivation in the 5-CSRTT. Ketamine was administered at 20mg/kg, i.p. (**a**) Under the influence of ketamine, *Map2k7*$^{+/-}$ mice were significantly slower to make a correct response than WTs, however, there was no effect of session. There was a significant effect of genotype overall. (**b**) Ketamine had no effect on incorrect response latency; *Map2k7*$^{+/-}$ mice remained significantly slower to make an incorrect response, similar to at baseline. (**c**) Ketamine increased the total number of nose pokes made throughout the

session for both groups of mice. (**d**) The total number of trials completed remained at almost maximum for both groups of mice under the influence of ketamine. (**e**) Reward collection latency slightly but significantly increased for *Map2k7*$^{+/-}$ mice but not WTs. Before ketamine administration, *Map2k7*$^{+/-}$ mice were quicker to collect the reward but following ketamine administration both WT and *Map2k7*$^{+/-}$ mice had similar reward collection latencies. (**f**) Ketamine significantly increased the number of magazine entries during the ITI. Overall, *Map2k7*$^{+/-}$ mice entered the magazine more times during the ITI than WTs. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles. Data analysed by a three-way repeated measures ANOVA with daily session and ketamine treatment as within subject factors, genotype as a between subject factor and each individual mouse nested within genotype with Tukey's post hoc. Lines between groups show the significance between those groups: *p<0.05, **p<0.01, ***p<0.001 (ANOVA); ##p<0.01 (Tukey's). *Map2k7*$^{+/-}$: n=15 (8M, 7F), WT: n= 15 (8M, 7F).

5.4.5 Map2k7$^{+/-}$ *omissions deficit shows signs of being improved by minocycline*

Minocycline is a tetracycline antibiotic which is currently showing promise in clinical trials for treatment of the negative and cognitive symptoms of schizophrenia (Reviewed in Chaves *et al.*, 2015). Overall, minocycline, administered for 7 days, improved the % omissions score for both *Map2k7*$^{+/-}$ and WT mice through the week so that by the 4th day of treatment there was a small significant effect of session, but by the 7th day of treatment there was a larger significant effect (effect of session: p=0.006, $F_{(3,80)}$=4.53), suggesting improvement as minocycline treatment goes on. By the 7th day of treatment, the significant genotype difference from baseline had disappeared (**Fig.5.11**; ns, p>0.05). Furthermore, mice committed fewer omissions in the 5-CSRTT by the 7th day of minocycline treatment than they had ever achieved before the treatment (from 16.9 ±1.7 at baseline compared to 11.8 ±2.9 by the 7th day of minocycline treatment).

5.4.6 Map2k7$^{+/-}$ *mice display a deficit in ability to sustain attention that is not alleviated by minocycline treatment*

For each daily session, the number of omissions, commission errors or correct responses performed by each mouse in the first 20 trials they completed were subtracted from those performed during the final 20 trials to give a difference score that shows the extent to which performance for each mouse declines with session progress, which is known as a "vigilance decrement" (Parasuraman *et al.*, 1987; Robbins, 2002). For omission and commission errors, a more positive score indicates a larger vigilance decrement. For correct responses, a more negative score indicates a larger vigilance decrement. On average, *Map2k7*$^{+/-}$ mice show a vigilance decrement compared to WT mice (who did not) at baseline, manifesting as a significantly higher increase in the number of omissions at the end of each session than the beginning, compared to WT mice ($p=0.012$, $F_{(1,184)}=6.51$) (**Fig. 5.12a**). Minocycline improved the number of omissions difference score by the 7th day of treatment overall, whilst, again, removing the statistical significance between genotype groups (**Fig. 5.12a**) ($p=0.009$, $F_{(2,184)}=4.79$). At 4 days' minocycline treatment, again, it appeared to have an intermediate significance level, as if the mice are improving over time with minocycline treatment. Like % accuracy seen at Baseline (**Fig. 5.6a**), the % accuracy difference scores were similar in WTs and HZs (no effect of genotype: $p=0.826$, ns). Surprisingly, all mice showed a significantly increased vigilance decrement in this measurement on the 7th day of minocycline treatment as compared to Baseline ($p=0.008$, $F_{(2,184)}=4.92$) (**Fig. 5.12b**). This was also true for the number of incorrect responses difference score: there was no significant effect of genotype ($p=0.455$, ns) but there was a significant effect of session ($p=0.001$, $F_{(2,184)}=7.84$) (**Fig. 5.12c**).

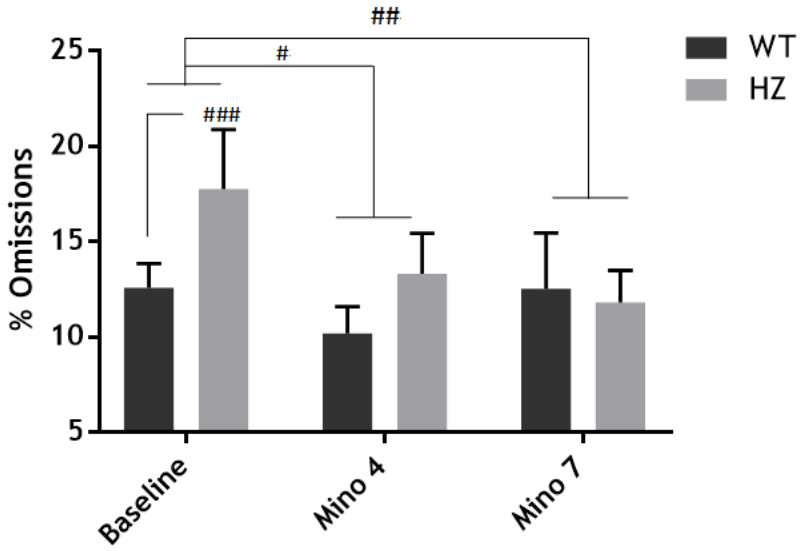
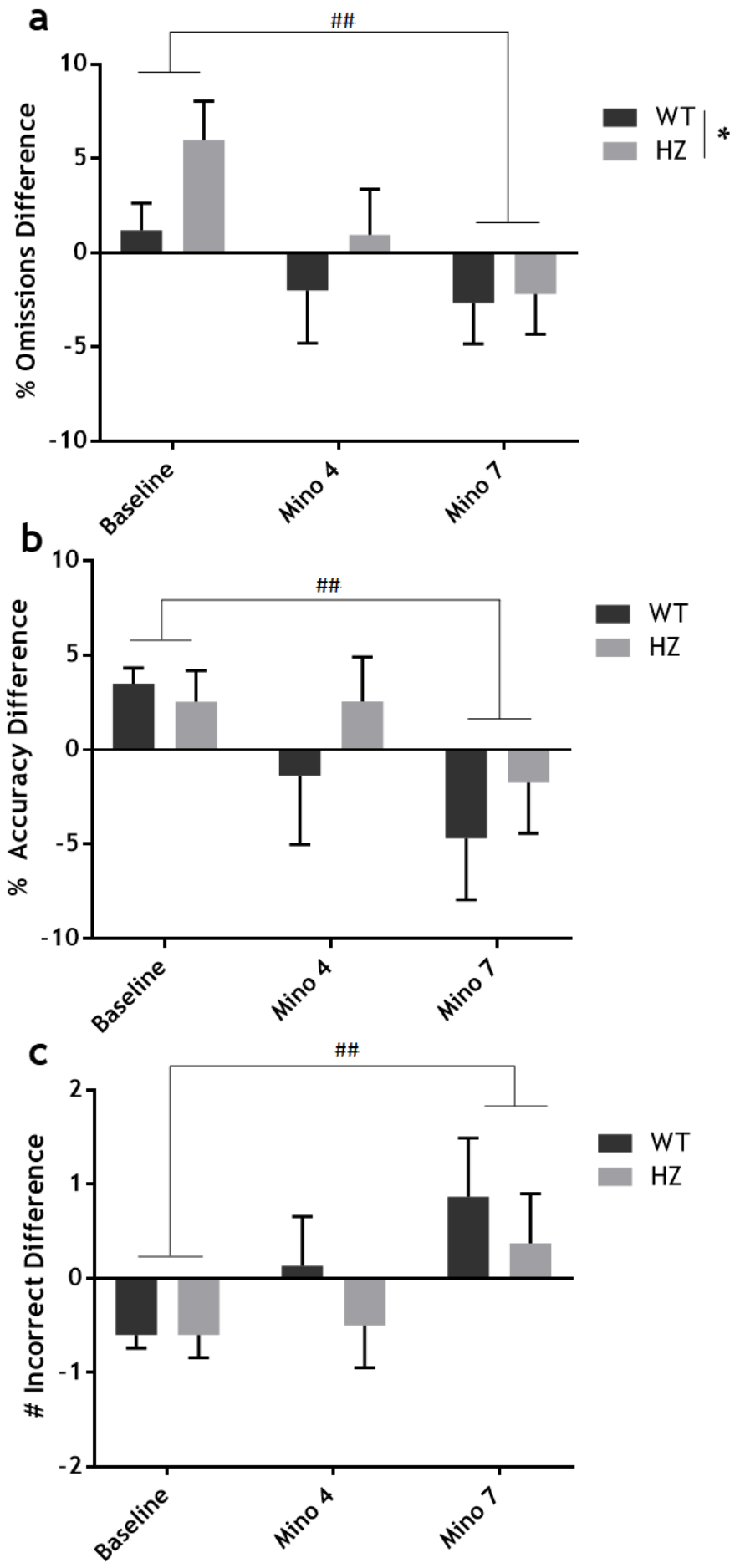


Figure 5.11. *Map2k7*$^{+/-}$ omissions deficit shows signs of being improved by minocycline. Overall, minocycline, administered for 7 days (Mino 7), improved the % omissions score for both Map2k7$^{+/-}$ and WT mice by the 7th day of treatment, by which time the significant genotype difference at baseline had disappeared. The 4th day of minocycline treatment (Mino 4) showed intermediate significance between baseline and the 7th day of treatment. Mice received 81.6mg/kg/day minocycline on average. Data analysed by a three-way repeated measures ANOVA with daily session and minocycline treatment as within subject factors, genotype as a between subject factor and each individual mouse nested within genotype with Tukey's post hoc. Data presented as mean ± SEM. #p<0.05, ##p<0.01, ###p<0.001 (Tukey's). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).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< Figure 5.12. *Map2k7*$^{+/-}$ mice display a sustained attention deficit compared to WT mice. (**a**) *Map2k7*$^{+/-}$ mice exhibit a significantly higher increase in the number of omissions at the end of each session than the beginning, compared to WT mice. Minocycline improved the number of omissions difference score by the 7th day of treatment (Mino 7) overall, whilst, again, removing the statistical significance between genotype groups. (**b**) Like % accuracy as seen at Baseline (**Fig. 5.6a**), the % accuracy difference scores were similar in WTs and HZs (no effect of genotype: p=0.826, ns). All mice showed a significantly increased vigilance decrement in this measurement on the 7th day of minocycline treatment as compared to Baseline. (**c**) This was also true for the number of incorrect responses difference score: there was no significant effect of genotype but there was a significant effect of session. The 4th day of minocycline treatment (Mino 4) showed intermediate significance between baseline and the 7th day of treatment. Mice received 81.6mg/kg/day minocycline on average. Data analysed by a three-way repeated measures ANOVA with daily session and minocycline treatment as within subject factors, genotype as a between subject factor and each individual mouse nested within genotype with Tukey's post hoc. Data presented as mean ± SEM. Lines between groups show the significance between those groups: *p<0.05 (ANOVA); ##p<0.01 (Tukey's). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

5.4.7 Map2k7$^{+/-}$ *mice are more varied in their response times than WT mice that is not altered by minocycline treatment*

Intra-individual reaction time variability (IIV) is a measure of variability in response times of a subject carrying out a task over the course of a single session, thus quantifying short-term fluctuations in an individual's performance, and gives an indication of the stability of cognitive processing (Kanai & Rees, 2011). IIV is perturbed in a number of neuropsychiatric disorders including schizophrenia (Geurts *et al.,* 2008; Kaiser *et al.,* 2008). We assessed IIV by measuring the variability of reaction times of each mouse to make a correct and incorrect response over the course of each daily session for every day of Baseline (stable) performance. Interestingly, *Map2k7*$^{+/-}$ mice show significantly higher variability in their reaction times when making incorrect responses (**Fig. 5.13a;** p=0.034, $F_{(1,125)}$=4.58) but not when making correct responses (**Fig. 5.13b;** p=0.491, ns). This effect was maintained when looking at group reaction time variability, averaged for each mouse over the course of the vITI training, including the 5 days' stable performance, i.e. Sessions 1-12: see **Fig. 5.7** (**Fig. 5.13c, d**): incorrect response times of *Map2k7*$^{+/-}$ mice vary significantly more on a day-to-day basis than WTs (**Fig. 5.13c;** p=0.049, $F_{(1,4)}$=6.43) and group reaction time variability for correct responses remained the same for all mice (**Fig. 5.13d;** p=0.453, ns). Minocycline did not have a significant effect on the standard deviations of either correct or incorrect response times of mice compared to baseline performance (**Fig. 5.13a, b**).

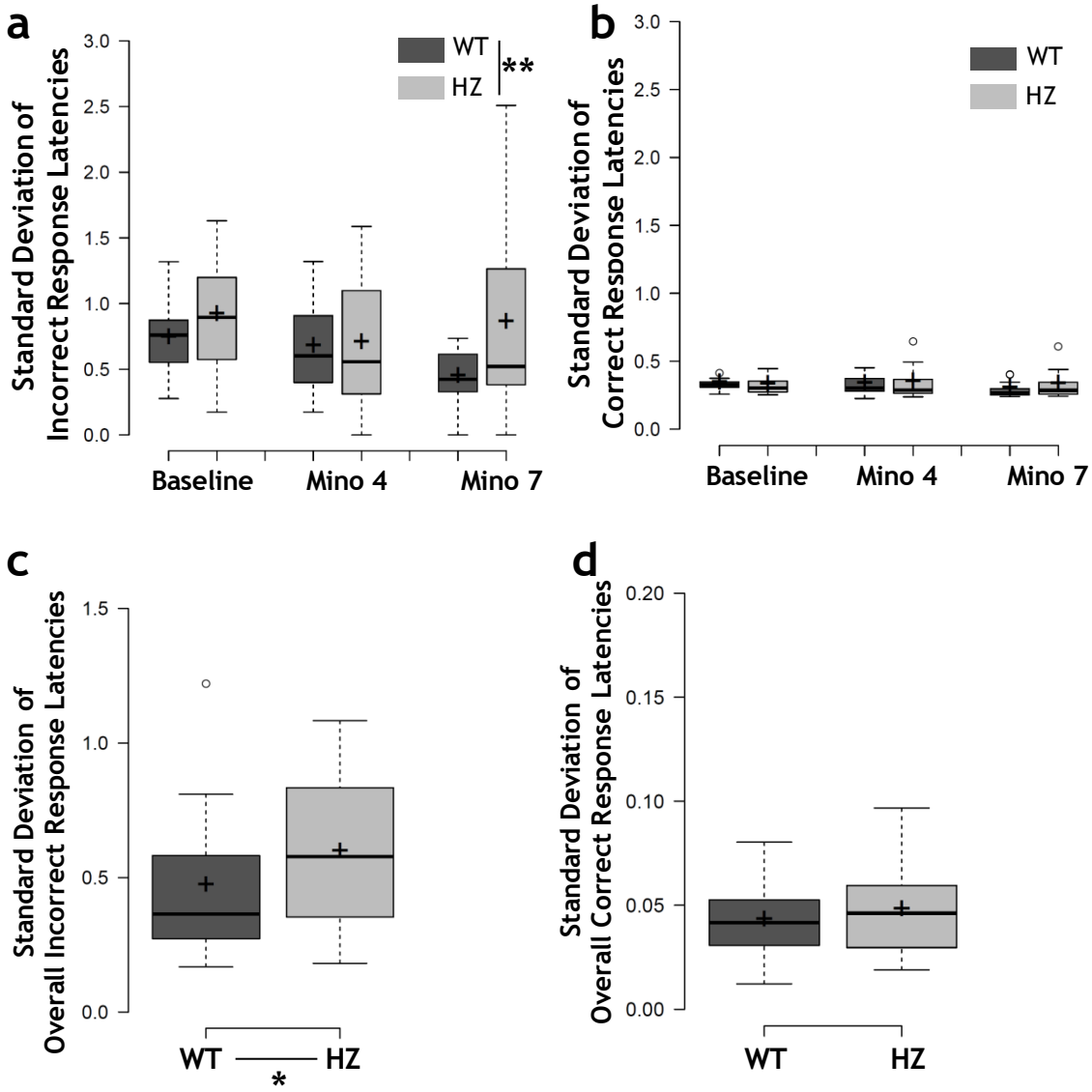

Figure 5.13. *Map2k7*$^{+/-}$ mice show more unstable cognitive processing than WT mice. *Map2k7*$^{+/-}$ mice display a higher intra-individual reaction time variability for (**a**) incorrect, but not (**b**) correct, response times than WT mice throughout Baseline that was not significantly alleviated by minocycline treatment. Increased variability also occurred for *Map2k7*$^{+/-}$ mice compared to WTs on average on a day-to day basis, seen during training to a vITI and Baseline performance for latency to (**c**) incorrect but not (**d**) correct responses. (See **Figs. 5.7 c** and **a** to visualise this). **a** and **b** represent each individual mouse's IIV; **c** and **d** represent the standard deviations of each group over the course of the last 5 days' stable performance (Baseline). The 4th day of minocycline treatment (Mino 4) showed intermediate significance between baseline and the 7th day of treatment. Data were analysed by a two-way repeated measures ANOVA with daily session as a within subjects factor, genotype as a between subjects factor, with each individual mouse nested within genotype (data from **a** and **b**), or as a one-tailed *t* test between genotypes (data from **c** and **d**). Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers

extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles. Lines between groups show the significance between those groups: *p<0.05, **p<0.01 (ANOVA or one-tailed *t* test). *Map2k7*$^{+/-}$: n=16 (9M, 7F), WT: n= 15 (8M, 7F).

5.5 Discussion

5.5.1 Map2k7$^{+/-}$ *mice display a variety of different attentional deficits in the 5-CSRTT*

Map2k7$^{+/-}$ mice show attentional deficits in accordance with those seen in psychiatric patients carrying out similar tasks, some of which shows signs of being alleviated by minocycline. Additionally, throughout training in this experiment, all mice appeared to be using a temporal strategy (reported previously: Nikiforuk & Popik, 2014) which was prevented post-training by utilising a variable ITI. Finally, ketamine administration had a detrimental effect on performance, but did not appear to disrupt attentional performance specifically, or to exacerbate deficits seen in *Map2k7*$^{+/-}$ mice.

5.5.2 Training

Map2k7$^{+/-}$ mice took the same number of sessions and trials to reach criterion than WT mice, which allows the interpretation of results post-training to be unconfounded. When their training regime switched from a fixed ITI to variable ITI, the performance of all mice was worsened. This is expected if mice were using a temporal strategy as explained in Humby *et al*. (1999) and Bruin *et al*. (2006): mice have learned when they should begin to pay attention, as paying attention before the 5 second ITI will not have any beneficial effect on results. As the variable ITI spanned a range that was above and below the 5-second fixed ITI, the mice were caught out, reflected by their worsened performance. This behaviour is similar to rats in Nikiforuk & Popik (2014) when introduced to a variable ITI, who showed increased % omissions and correct response latency.

Map2k7$^{+/-}$ mice missed more trials than WTs over the two sessions (worse performance) but made fewer premature responses than WT mice (less impulsive; Robbins, 2002). Also, WT mice completed significantly fewer trials with the vITI session compared to the fITI session whereas the *Map2k7*$^{+/-}$ mice did not (thus appearing less affected by the change in ITI length). The fact that *Map2k7*$^{+/-}$ mice are performing better than WT controls is not expected. It may be because they are hyperactive compared to WTs (see **Chapter 4**), however, their performance presumably would only have a detrimental effect on results if they were worse than WTs with respect to behaviour following the change in ITI length, so this is probably not a cause for concern regarding interpretation of results post-training.

From this point on the mice are now required to pay attention across the course of each session because stimuli appearances will be unpredictable. The detrimental effect of the variable ITI appeared to only last for a single session, showing how quickly the mice can adapt and change their strategy (see line graphs in **Fig. 5.6** and **5.7**). It is beneficial to have the vITI as the standard as this verifies that it is attention being measured as opposed to the ability to keep to time.

5.5.3 Map2k7$^{+/-}$ *mice display an attentional deficit*

Once stable performance had been attained, attentional performance and motivation parameters were looked at as a whole rather than as singular parameters, in order to determine whether *Map2k7*$^{+/-}$ mice exhibited a deficit. Accuracy in the 5-CSRTT was unimpaired in *Map2k7*$^{+/-}$ mice, indicating that they are able to acquire the basic principles of the task to normal levels of performance. Equally, there was no evidence that these mice showed increased levels of impulsivity (premature responses) or compulsivity (perseveration). Response latencies (with the exception of incorrect response latencies, discussed below), together with the number of magazine entries throughout the ITI, indicated that they had good motivation to perform the task. Strikingly, there was a very specific deficit in that rates of missed responses were substantially raised. The % omissions deficit showed signs of diminishing over time (**Fig. 5.6c**) and it is possible that if the mice had been trained for longer on this task that the number of omissions in WT and *Map2k7*$^{+/-}$ mice may have eventually reached the same level. However, the deficit remains for some length of time which could reasonably be attributed to a lack of attention, as long-term repetition of the same task could tap into other domains such as long-term habit formation. It is also arguable that rodents (and humans) can learn to pay attention.

This combination of results (increased omissions, good accuracy and faster response latencies) suggests that *Map2k7*$^{+/-}$ mice are unable to maintain the same levels of attention as WT littermates, despite the fact they appear to be highly motivated and understand how to carry out the task (Humby *et al.*, 1999; Robbins, 2002). Changes in accuracy levels as opposed to omissions are frequently looked at as the main measure of attentional function in the 5-CSRTT; however, several studies confirm that increased omissions with the absence of an accuracy deficit probably result from stimulus detection failures as a consequence of inattention,

so long as motoric / motivational impairments can be ruled out (Cordova *et al.*, 2006; Fletcher *et al.*, 2007; Inglis *et al.*, 2001; Risbrough *et al.*, 2002; Tzanoulinou *et al.*, 2015; Young *et al.*, 2004, 2007).

As well as an omission deficit, *Map2k7*$^{+/-}$ mice also display a worsened vigilance decrement that manifests as an increased amount of missed trials at the end of each session than at the beginning, compared to WT mice, whose performance did not decline over the course of a session. Patients with schizophrenia consistently show a similar vigilance deficit *(*Nestor *et al.*, 1990, 1991; Hahn *et al.*, 2012; Lysaker *et al.*, 2010, Mass *et al.*, 2000), as do other rodent models relevant to schizophrenia (Barnes *et al.*, 2012, 2014). Young *et al.* (2013) went one step further and took the 5-choice Continuous Performance Test (a variation on the 5-CSRTT) and back-translated it to humans so that the human version was based on the rodent version. They found patients with schizophrenia presented with increased omissions and no corresponding decrease in accuracy. The authors also found that the number of missed trials committed by patients with schizophrenia increased to a worse extent over time than healthy controls, along with increased variability in response times – remarkably similar to what is seen in the current study. The variability in response times is discussed further in the following paragraph.

Map2k7$^{+/-}$ mice also showed another form of attention deficit: increased intra-individual reaction time variability. IIV is a measure of variability in response times of a subject carrying out a task over the course of a single session. Originally seen merely as "noise" in experimental data, researchers now realise that it also reflects the stability of cognitive processing and short term fluctuations in performance over a session (Kaiser *et al.,* 2008) and it has been suggested that IIV, as well as average task performance levels, are good predictors for real-world functioning (Stuss *et al.,* 2003). IIV is consistently increased in schizophrenia (Kaiser *et al.,* 2008), ADHD (reviewed in Kuntsi & Klein, 2012) and several other psychiatric/cognitive disorders (Musso *et al.*, 2015; Camicioli *et al.*, 2008; Geurts *et al.,* 2008). Moreover, it is under investigation as a reliable predictor for those who are at risk of developing schizophrenia (Shin *et al.,* 2013), ADHD (Henríquez-Henríquez *et al.,* 2015) and intrinsic deficits in cognitive function (Grand *et al.*, 2016). Here, we looked at the distribution of response times for each mouse over the course of each daily session to give a measure of IIV. *Map2k7*$^{+/-}$ mice have

increased IIV for incorrect responses but not correct responses. They also respond more variably on a day-to-day basis as a group when making incorrect responses compared to WT mice. IIV is an intriguing indicator of cognitive function because of its sensitivity, reliability and robustness across different tasks that involve reaction times (Kuntsi & Klein, 2012). Establishing the underlying neural mechanisms to increased IIV have been the focus of many studies which have shown that increased IIV is correlated with disruption of dopamine regulation in the PFC and subsequent increase of neural signal-to-noise (MacDonald *et al.*, 2006, 2009; Stefanis *et al.*, 2005). Nevertheless, other neural systems, hitherto unexplored are likely to be involved.

In addition to *Map2k7*$^{+/-}$ mice showing increased variability in making incorrect responses, they also show altered latencies in other measurements. They are quicker or similar in all other latency measurements recorded: *Map2k7*$^{+/-}$ mice are faster to collect and consume the reward and respond just as quickly as WT mice when making a correct response. As well as showing high motivation to complete the task, this set of results suggests that when the *Map2k7*$^{+/-}$ mice have noticed the stimulus, they are just as quick to respond correctly, but when they miss a stimulus (probably due to inattention), they have slower processing times than WTs before deciding to take a guess. This may manifest as increased, and more variable, incorrect response reaction times.

Map2k7$^{+/-}$ mice also make an increased number of magazine entries during the ITI period compared to WTs. The ITI is the period of time after they have collected the reward (if they responded correctly), or after the time out period (if they responded incorrectly or missed the stimulus). Therefore, *Map2k7*$^{+/-}$ mice are showing signs of anticipating wrongly when they should receive a reward. Two possible explanations for this are either because they are applying an increased amount of salience to the reward, or that they "like" it more than WT mice. Previous studies in our lab have suggested that *Map2k7*$^{+/-}$ mice do not experience increased preference for sucrose (Thompson, 2013); *Map2k7*$^{+/-}$ mice may therefore apply more salience to the reward magazine than WTs. Furthermore, *Map2k7*$^{+/-}$ mice appear to exhibit greater entrainment to the light stimulus as they do not show decreased accuracy in conjunction with their increased % omissions (Amitai & Markou, 2010). Throughout any given trial, if they fail to detect the stimulus, instead of guessing which hole to poke (thus decreasing their % accuracy score),

they withhold responding and consequently present with a missed trial. The decreased numbers of commission errors made by *Map2k7*$^{+/-}$ mice is also indicative of this. Frequent and mistaken trips to the reward magazine, and strong entrainment to the light stimuli may both be examples of *Map2k7*$^{+/-}$ mice applying increased salience to some aspects of the task, which is interesting in relation to psychiatric disorders that include cognitive impairment, especially schizophrenia, because one of the symptoms in patients is applying too much salience to particular, often irrelevant, aspects of the environment (Kapur, 2003).

5.5.4 Ketamine had a detrimental effect on Map2k7$^{+/-}$ *and WT performance in the 5-CSRTT*

NMDAR dysfunction as a contributor to schizophrenia pathophysiology was proposed three decades ago (Javitt, 1987) and it was based on observations that NMDAR antagonists, such as ketamine, are able to induce schizophrenia-like symptoms in healthy subjects (Breier *et al.*, 1997), as well as exacerbate symptoms in patients with schizophrenia (Lahti *et al.*, 1995). Importantly, administration of ketamine to healthy human subjects induced attentional deficits in the form of increased omissions (missed responses) and commission errors (incorrect responses) in the CPT without affecting reaction times (Krystal *et al.*, 1994).

Mice were given an acute dose of 20mg/kg ketamine 15 minutes prior to testing in the 5-CSRTT. Overall, ketamine showed signs of altering task latencies: *Map2k7*$^{+/-}$ mice made slightly slower correct response latencies and reward collection latencies compared to WT mice. However, incorrect response latencies were completely unaffected compared to baseline. Despite some alterations in some response latencies, all mice did not appear to have lost motivation to perform the task as their total nose pokes increased, along with number of magazine entries during the ITI (*Map2k7*$^{+/-}$ mice in particular for this measure) and the number of trials completed overall were unaffected. Performance-wise, ketamine was disruptive but not specifically for measurements of attention as % omissions were unaffected for both groups of mice. However, % accuracy decreased, and premature responses, perseverative responses and commission errors increased. Of these, % accuracy, premature responses and commission errors were less disrupted by ketamine in *Map2k7*$^{+/-}$ mice compared to WT mice.

In general, where ketamine administration in rodents has been previously reported, the worsening of performance in cognitive tasks appears inconsistent and not specific to any particular element of cognition (Amitai & Markou, 2010; Nikiforuk & Popik, 2014). However, in rats, administration of the NMDAR antagonist MK-801 consistently increases premature responses (Fletcher *et al.*, 2011; Higgins *et al.*, 2003; Paine & Carlezon, 2009). One study (Oliver *et al.,* 2009) administered ketamine in mice at 20mg/kg i.p. and tested in the 5-CSRTT 15 minutes after, which is the same regime used here. The authors compared C57Bl/6 mice to CD1 mice and found an increase in premature responses in CD1 mice but not C57Bl/6, and an increase in perseverative responding in C57Bl/6 but not CD1 mice, as well as unaffected % omissions and % accuracy. In the current study there was an increase in premature responses in WT (C57Bl/6) but not in *Map2k7*$^{+/-}$ mice, increased perseverative responses in all mice, unaltered % omissions, but decreased % accuracy in WT mice. Comparison of these results highlights the fact that even different strains of the same species can present with differing or opposite results under the same conditions, and also highlights the differences that can occur under similarly controlled conditions in different laboratory environments, which may be what is occurring here. It is also possible that ketamine has un-reproducible effects because of relatively low affinity for the NMDAR (Amitai & Markou, 2010). Studying the effects of NMDA dysfunction on mice with disruption in a schizophrenia risk gene may benefit from using an agent that more potently antagonises NMDAR, such as MK801 (Wong *et al.,* 1986).

Further to this, acute, systemic administration of ketamine has been shown to increase glutamate release in the medial PFC, as well as increasing dopamine release (Lorrain *et al.,* 2003). Acetylcholine is also increased in the PFC in response to acute ketamine administration in rodents, but not when under a repeated pre-treatment regime with ketamine *(*Nelson *et al.,* 2002). As monoaminergic, cholinergic and glutamatergic neurotransmission are closely interconnected with each other and are critically involved in cognitive functions (Amitai & Markou, 2010), it is perhaps not surprising that ketamine has varying effects on cognitive functions. It would be interesting to investigate the effects of administering ketamine in a chronic or sub-chronic way rather than acute administration on 5-CSRTT performance in *Map2k7*$^{+/-}$ mice. This regime has been used before in rats but not mice, and the authors showed differential deficits

between acute and repeated doses of ketamine, e.g. ketamine appeared to have a more drastic effect on incorrect responses when given acutely as opposed to repeated administration (Nikiforuk & Popik, 2014). Thomson *et al.* (2011) also found differential effects with acute PCP compared to repeated PCP administration in rats.

Following an acute dose of ketamine, *Map2k7*$^{+/-}$ mice appear to be more distracted by obtaining a reward (nose poking more frequently, even when not relevant, and making more entries into the reward magazine when there is not reward there to collect) rather than focussing on the task, and they seem to be less affected by performance parameters related to attention. Despite nose poking prematurely and to an increased extent, being slower to respond to stimuli or a reward delivery, *Map2k7*$^{+/-}$ mice did not miss more trials than they did at baseline (although still to an increased extent compared to WT mice) but they did present with a modest decrease in % accuracy. Ketamine appears to have a slightly decreased effect overall in *Map2k7*$^{+/-}$ mice compared to WT mice for some parameters but not others, and ketamine disrupts certain aspects of performance in all mice. Future experiments (discussed above) could look into this further to verify whether this is a robust finding, or a variable effect of acute ketamine administration. On the whole, ketamine does not cause behavioural observations as might be expected based on results from patients with schizophrenia: it does not exacerbate previous deficits in *Map2k7*$^{+/-}$ mice (i.e. cause a further increase in % omissions), nor does it induce a specific attentional deficit in WT mice.

5.5.5 Minocycline shows signs of improving some aspects of performance in the 5-CSRTT

Minocycline is a semi-synthetic tetracycline antibiotic showing promise in current clinical trials for the treatment of the negative and cognitive symptoms of schizophrenia; importantly, it has shown ability to improve attentional deficits (Liu *et al.*, 2014). It is an ideal candidate for schizophrenia treatment because it is already deemed safe for human consumption, and readily crosses the blood-brain barrier (Zink *et al.*, 2005). Minocycline was administered to *Map2k7*$^{+/-}$ mice for one week in their drinking water, with testing on the 5-CSRTT on days 4 and 7 of treatment. Minocycline improved the % omissions score of the mice overall, but the *Map2k7*$^{+/-}$ mice in particular showed signs of continual improvement of their % omissions score throughout minocycline treatment, performing better on the 7th

day of treatment than they had ever performed beforehand. Although minocycline treatment did not have a significant effect on the IIV of *Map2k7*$^{+/-}$ mice, in the same way as it affected % omissions, the treatment improved performance of all mice overall with respect to the vigilance decrement, including having a particular influence on *Map2k7*$^{+/-}$ mice.

As minocycline has previously been shown to improve attention deficits in human patients with schizophrenia (Liu *et al.*, 2014), these results further warrant the refinement of *Map2k7*$^{+/-}$ mice as a model of attentional, and possibly other cognitive impairments because the ability to maintain and focus attention inevitably has an impact on performance in other cognitive domains.

Minocycline has also been shown to improve reaction times in healthy volunteers in a sustained attentional task (Sofuoglu *et al.*, 2011). Additionally, in a mouse model of relevance to schizophrenia produced by administration of an NMDAR antagonist, MK801, minocycline improved deficits in prepulse inhibition and visuo-spatial memory (Levkovitz *et al.*, 2007) and also improved phencyclidine-induced novel object recognition deficits in mice (Fujita *et al.*, 2008). Also, in a developmental two-hit mouse model of schizophrenia, presymptomatic minocycline treatment was able to alleviate multiple behavioural abnormalities relevant to schizophrenia (Giovanoli *et al.*, 2016). Despite minocycline having been shown to have beneficial effects in both healthy and pathological (cognitive deficits) human and rodent studies, the exact mechanism of action of minocycline is still unknown, although two main mechanisms have been proposed with relation to cognition: inhibition of activated microglia and/or enhancing glutamate release via NMDARs (Liu *et al.*, 2014; Lisiecka *et al.*, 2015). It is entirely conceivable that either, or a combination of both of these mechanisms are relevant in the current study because of the potential for them both to interact with the MKK7/JNK pathway. The JNK pathway is essential for pro-inflammatory functions of microglia (Waetzig *et al.*, 2005) and NMDARs are located upstream of the MKK7/JNK pathway (Centeno *et al.*, 2007), suggesting that altering microglia and/or NMDAR activation states via minocycline have potential to affect regulation of the MKK7/JNK pathway in order to produce a cognitive enhancing effect. In **Chapter 3**, Western blotting of prefrontal MKK7/JNK pathway components from mice that have received 7 days' worth of minocycline treatment in the same way as administered here are compared with mice that had standard drinking water and

discussed. Overall, there was no difference in protein levels of MKK7/JNK signalling pathway components with mice that had received minocycline and mice that had not; however, gaining knowledge of the mechanism of minocycline and how it has acted to improve attention in the current experiment will benefit from more sensitive methods of protein quantification and longer-term studies. In general, more molecular and clinical evidence on minocycline's potential as an agent to improve cognition is needed. However, our data support the concept that this drug is effective in improving some aspects of attentional function.

5.5.6 Conclusion

The results presented here demonstrate the importance of MKK7/JNK signalling for attentional processes. Mice haploinsufficient for the *Map2k7* gene show deficits in attention, a core cognitive impairment in many neuropsychiatric diseases (Millan *et al.*, 2012) and show signs of improvement in attentional performance with minocycline treatment. Importantly, dissection of attentional processes revealed that *Map2k7*$^{+/-}$ mice present with impaired vigilance/sustained attention as evidenced by a significantly higher (than WT) increase in the number of omissions at the end of each test session compared to the beginning. Additionally, *Map2k7*$^{+/-}$ mice exhibit impaired cognitive stability, as evidenced by significantly more varied response times over the course of a test session than WT mice when attending to incorrect responses, which is highly relevant to 'real-world' functioning. Hence, the data may be important for understanding the mechanisms of cognitive dysfunction, and highlight the possibility of treating some of these deficits with minocycline.

Chapter 6 The Rodent Gambling Task

6.1. Introduction

The ability to make sound decisions that incorporate aspects of emotion, memory of past events and projections to future events is a crucial element of normal cognitive functioning. Patients with schizophrenia have altered decision-making abilities, evident from behaviour in real-world circumstances. For example, many patients make poor decisions regarding their treatment-taking and in spending money (thus are more likely to have financial problems). Moreover, many exhibit addictive behaviours and are involved with interpersonal conflicts (Fond *et al.*, 2013). One way in which decision-making abilities are assessed in patients with schizophrenia under laboratory conditions is by the Iowa Gambling Task (IGT). The IGT is a touchscreen-based task originally developed by Bechara *et al.* in 1994 to detect decision-making impairments in patients with frontal lobe damage. Subjects are presented with $2000 of fake money and four virtual decks of cards, each associated with a different monetary win/loss probability, and they are instructed to try and win as much money as possible in the time available by virtually "turning over" cards from the different decks. 100 "turns" generally occur per session, but the participants are not told this. Two decks have small, frequent wins and small losses, making them advantageous overall, whereas the other two decks give higher wins that occur less often but have larger consequential loss. Healthy participants tend to first sample all of the decks and by about the 50th turn, they have learned which decks are advantageous and mainly turn over these cards (i.e. small, frequent wins with small losses). The IGT has been used extensively in clinical and research studies to investigate the decision-making impairments seen in neurological disorders such as pathological gambling behaviour and addiction, obesity, obsessive compulsive disorder, depression, psychopathy, bipolar disorder and schizophrenia (Li *et al.*, 2010).

The brain regions required for the IGT are also areas which are highly dysfunctional in schizophrenia (e.g. the PFC; Bechara *et al.*, 1999; Li *et al.*, 2010), and dysfunction in the ability to make sound decisions is one of the core cognitive symptoms of schizophrenia; therefore, patients with schizophrenia have been well studied in the IGT although they present with conflicting results. Some studies show that patients with schizophrenia make more disadvantageous decisions

(Cella *et al.*, 2012; Fond *et al.*, 2013; Kester *et al.*, 2006; Sevy *et al.*, 2007; Shurman *et al.*, 2005); some studies show that patients on atypical drugs make poorer decisions than patients on typical drugs (Beninger *et al.*, 2003), and some show there is no difference in decision-making between patients and healthy controls (Evans *et al.*, 2005; Rodríguez-Sánchez *et al.*, 2005). This lack of consensus is thought to reflect the complexity and heterogeneity of schizophrenia cases (chronic or first episode schizophrenia, schizoaffective disorder, etc), including the fact that patients have different dominating symptoms (Bark *et al.*, 2005), and also because patients are receiving differing medication (Beninger *et al.*, 2003). However, even this concept is debatable; for example, Shurman *et al.* (2005) found no correlation between severity of schizophrenia or treatment type with performance in the IGT.

An aspect of performance in the IGT that appears to have consensus in the literature is that patients with schizophrenia do not improve over the course of the task. Healthy participants will begin by picking cards from each deck and then quite quickly begin to preferentially pick the advantageous decks (i.e. the two decks with small, frequent wins and small losses), but patients with schizophrenia take longer to adopt this strategy (Kester *et al.*, 2006; Kim *et al.*, 2016; Kim *et al.*, 2009; Shurman *et al.*, 2005; Turnbull *et al.*, 2006). This slowness to alter their responding based on negative feedback is thought to be caused by increased perseverative behaviour, a well-established trait in patients with schizophrenia and frontal lobe damage (Pantelis *et al.*, 1999). Perseverative behaviour in schizophrenia and the IGT was further investigated by Turnbull *et al.* in 2006, who created a version of the IGT where reward/punishment contingencies associated with each deck were altered over the course of a session, thus investigating adaptive decision-making. Patients with schizophrenia that had high negative symptoms scores, despite performing just as well as controls in learning the normal IGT, showed difficulty in shifting their responses when the reward/punishment contingencies of the cards were completely reversed. This is an example of perseverative behaviour: the over-reliance on decks that had previously been learned to be favourable (Turnbull *et al.*, 2006).

A rodent version of the IGT (rGT) has been developed (reviewed in Van Den Bos, 2014), which, along with the IGT for humans, has been validated for cross-species investigation of cognition within RDoC/CNTRICS cognitive constructs (Cope *et al.*,

2016). In a comparable way to the IGT, the rGT presents mice with four options on a touchscreen apparatus that differ in frequency and magnitude of reward/punishment possibilities. Rodents learn the different contingencies over a lengthy training period, and once learned, have shown to consistently stick to the advantageous choices and avoid the disadvantageous choice (i.e. the choice which has the least frequent wins but has the largest reward amount) in rats (Zeeb *et al.*, 2009) and mice (van Enkhuizen *et al.*, 2013) in the corresponding 5-hole operant box version. Although the rGT differs from the human task in that it is acquired over an extensive training period as opposed to a single session, neural circuitry in both rodent and human versions of the gambling task are reported to be similar, requiring the PFC, striatum and amygdala (de Visser *et al.*, 2011), giving it potential to be a good translational task for measuring decision-making abilities in rodent models of disorders involving deficits in decision-making, including schizophrenia.

6.2. Aims

In the current study, the aim is to investigate the role of the MKK7/JNK pathway in decision-making processes by studying *Map2k7*$^{+/-}$ mice and their WT littermates in the rGT and their ability to learn the rGT in the touchscreen apparatus. It will also be investigated whether mice make optimal decisions in the rGT and, in a comparable way to Turnbull *et al*. (2006) with the IGT, to adjust responding when rGT choice contingencies are switched. The effects on performance of an acute dose of amphetamine will also be investigated. Acute amphetamine administration produces an enhanced dopaminergic response in patients with schizophrenia compared to healthy controls (Laruelle *et al.*, 1996), and disrupts performance in the 5-hole operant box version of the rGT by causing rodents to opt for less advantageous options (Zeeb *et al.*, 2009). Based on this, it is hypothesised that *Map2k7*$^{+/-}$ mice will show an altered behavioural response to amphetamine compared to WT mice, and that choice behaviour for all mice may be disrupted.

6.3. Materials and Methods

6.3.1 Subjects

12 *Map2k7*$^{+/-}$ mice (6 Male, 6 Female) and 10 wildtype (WT) (5 Male, 5 Female) littermates were used (two WT mice were removed from the experiment due to constant abnormal repetitive movements). Mice were 15-16 weeks of age at the start of the study, male mice weighing 31.1 ± 0.49g on average and female mice weighing 23.5 ± 0.22g on average. All mice had experienced no previous procedures (naïve to drugs and testing), and were pair-housed in a temperature and humidity-controlled room (21°C, 45–65% humidity) with a reversed 12-hour light/dark cycle (lights off at 07:00). Mice were food restricted to 85-90% of their individual free-feeding weight and had *ad libitum* access to water throughout the experiment. Testing was carried out daily between 08:00 and 13:00, Monday to Friday and in accordance with the Animals (Scientific Procedures) Act 1986.

6.3.2 The Rodent Gambling Task (rGT)

In the rGT, mice are able to freely choose between four options that differ in magnitude and ratios of reward/punishment possibilities (Choices 1-4; see **Fig. 6.3**). Mice undergo up to 100 trials per daily session, each thirty-minutes long. Overall, Choice 2 is the most advantageous option and Choice 4 is the least advantageous, because they give the most and least reward per unit time, respectively (see **Fig. 6.3**). Choice 1 is the next most advantageous option, followed by Choice 3. Mice were trained on the rGT until they reached stable performance. Manipulation 1 then occurred (see below) until stable performance was re-attained and then mice underwent Manipulation 2.

Once stable performance had been once again re-attained, D-Amphetamine (1.5mg/kg, 2ml/kg i.p.) was administered 5 minutes prior to re-testing.

A timeline of the whole experimental procedure is given in **Fig. 6.1.**

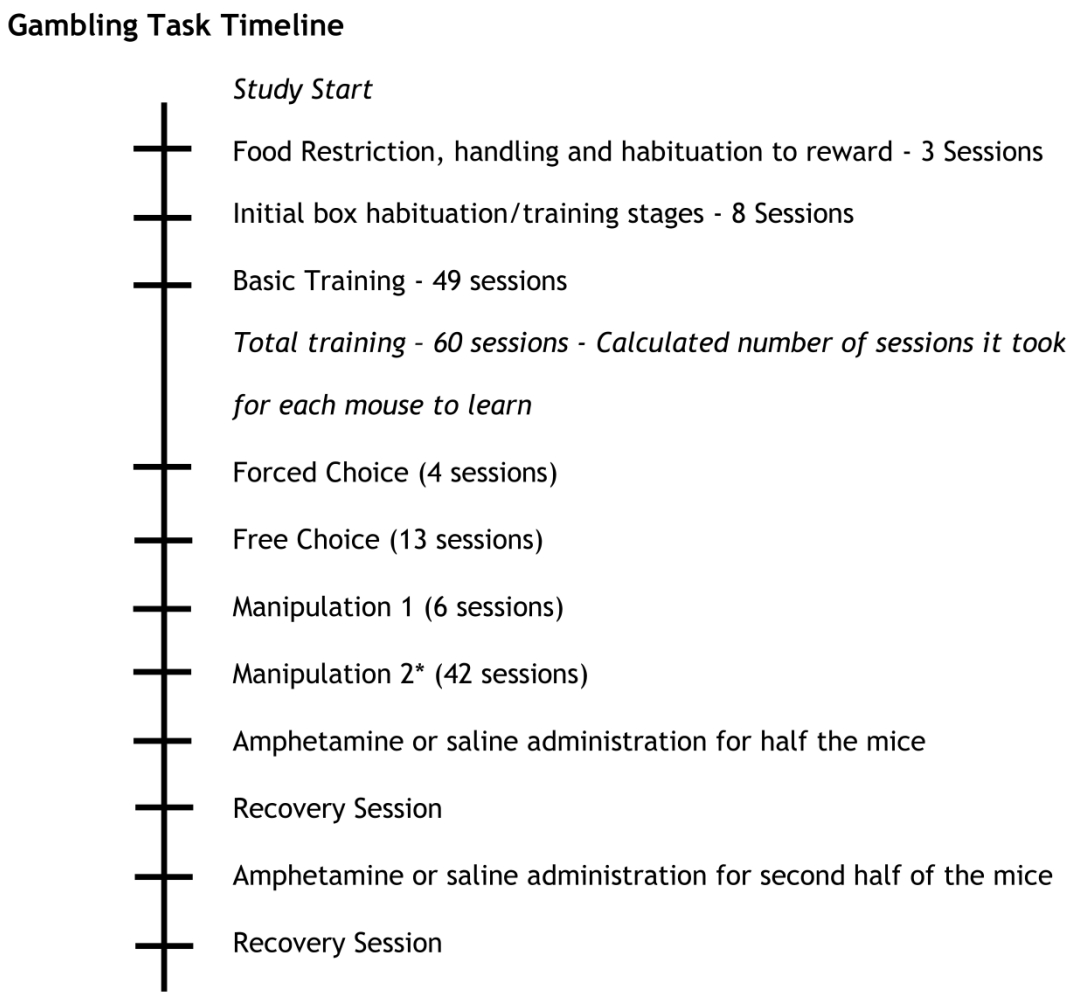

Figure 6.1. A timeline showing the experimental procedure. *42 sessions were analysed for Manipulation 2, however, 56 sessions were actually run. This is because the mice underwent one session per day for the first 28 sessions and two sessions per day for the next 14 days, but only the morning sessions were analysed when mice were run twice per day. The reason for this was to speed up learning, and as time of day may have an influence on task parameters the afternoon sessions were not included in the analysis

6.3.3 Apparatus

Mice were tested in four identical touchscreen operant chambers (**Fig. 6.2**) within a sound and light attenuating box with a silent extractor fan (80614; Bussey-Saksida Mouse Touchscreen Chamber; Campden Instruments, UK). Mice were singularly always tested in the same operant box and were allocated a box randomly; however, a check was made to ensure that no experimental group (genotype or sex) would exclusively be carrying out the rGT at the same time of day or in a particular operant box. Each chamber was trapezoidal in shape (narrow

end: 4.6cm, wide end: 23.8cm, height: 23cm) and contained a liquid reward dispenser at the narrow end, a house light, a tone generator, a perforant metal floor and a touchscreen at the wide end. During testing, the touchscreen was covered by a black Plexiglas mask with five square windows (3.5 x 3.5cm), each separated by 0.4 cm and located at a height of 3.6 cm from the floor of the chamber. Through these windows, visual stimuli in the form of white squares the same size as the windows in the mask could be shown on the screen; however, the centre square was never lit. Infra-red light beams were positioned at the rear (close to the food magazine) and front (close to the touchscreen) of each box and allowed quantifying the horizontal locomotor activity of each animal. In addition, infra-red beams covered the area very close to the touchscreen so that the mice only had to come into close proximity to the screen rather than actually touch the screen in order for it to be sensed, so that sniffing behaviour was also counted. The operant chambers were controlled by ABET II Touch software on the Whisker® operating system (Campden Instruments, UK).

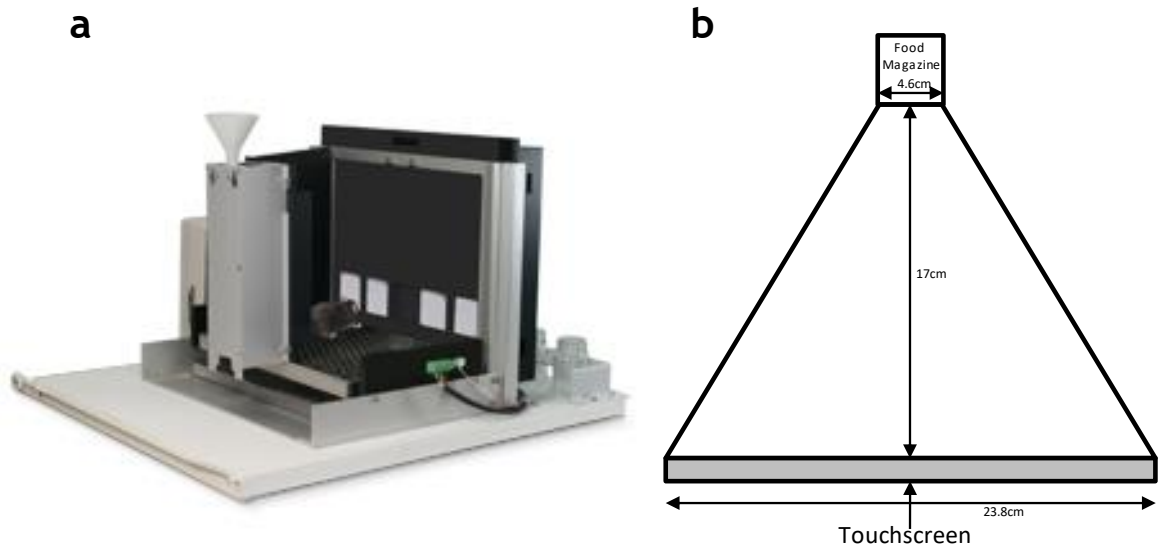

Figure 6.2. (a) The touchscreen operant chamber and (b) a bird's eye schematic diagram of the touchscreen layout. (a) In order to focus the rodents' attention towards particular parts of the touchscreen, most of it is covered by a black Plexiglas mask. This leaves 5 square windows, the middle of which is never used in the task. (**b**) The shape of the chamber is such that it focusses rodents' attention towards the screen and the reward magazine but does not have any corners in which the mice can spend time investigating rather than focussing on the task (see the 9-hole operant box, **Fig. 5.1** for comparison). Mice initiate each trial by entering the reward magazine; when they turn around they are faced with the whole screen, which enables the mice to make an unbiased selection throughout the task. The food magazine delivers strawberry milkshake (Yazoo®) when they "win" the trial, the amount varies depending on which choice they select.

6.3.4 Habituation and training

Prior to the beginning of each subjects' test session, the subject was transported to the test room in the home cage and allowed to acclimatize for thirty minutes before testing commenced. Mice were fed half an hour after their session had completed to ensure that each mouse had a similar level of motivation to complete the task each day. Inner chambers were cleaned with diluted antibacterial spray after the testing of each mouse to eliminate odour cues from previous mice. All testing was carried out with the main "house" light in the operant box turned off.

During the initial box habituation/training, the mice are gradually introduced to the operant boxes and learn to touch the screen in order to obtain reward. During the first session, a mouse was placed in their allocated operant box for 20 minutes. They received an initial "free" reward of 150µl strawberry milkshake (SM; Yazoo®) and the tray light was illuminated. Once the mouse had entered and left the food dispenser, the reward tray light was turned off. There was a 10s delay before the dispenser light was turned on and SM was then delivered for 280ms (7µl). If the mouse was in the reward tray at the end of the 10s delay, an extra 1s was added to the delay. The procedure was repeated until the end of the session.

This procedure was repeated the next day but with a 40-minute session instead of 20 minutes, ensuring that all mice had consumed all the strawberry milkshake.

Next, the mice were trained to touch the screen and were in the box for 30 minutes or until they had completed 100 trials. The stimulus (a white square) was displayed psuedorandomly in one of the 4 windows (grid positions 1, 2, 4 or 5) whilst the others remained blank. After 30 seconds, the stimulus was removed and 7µl reward was delivered, accompanied by illumination of the food dispenser light, which turned off when the mouse entered the dispenser to collect the reward. Exiting the reward dispenser automatically triggered the inter-trial interval (ITI) period of 5 seconds, at the end of which a stimulus was presented in one of the grid squares. A stimulus would not appear in the same grid square more than 3 times in a row. If the mouse was to touch the stimulus whilst it was illuminated, the stimulus was removed and 3x 7µl SM reward was given along with illumination of the dispenser light. This session occurred once, and then the same procedure was repeated for the following five sessions, apart from a stimulus appeared in every one of the four grid squares (1, 2, 4 and 5) and 3x 7µl reward was only delivered when the mouse touched one of the stimuli. No reward was given if the mouse touched grid position 3 where no stimulus was displayed. If a mouse touched the grid during the ITI period (i.e. before the stimulus was presented), this resulted in a premature response to be recorded, followed by a time out of 5 seconds.

The next sessions were aimed at building on the training for the mouse to touch the touchscreen at every trial and were still run in darkness, but illumination of the main house light for 5 seconds occurred if the mouse made an incorrect

response (touching an unlit square), or missed the stimulus and did not respond (omission). Each session began with a delivery of 7µl SM and illumination of the dispenser light. Once the mouse had consumed the reward and exited the dispenser, the ITI of 5 seconds began and a stimulus appeared psuedorandomly in one of the grid positions (1, 2, 4 or 5), and the limited hold (LH) period began: the period in which the mouse has to respond. If the mouse touched the stimuli, it was recorded as a correct response and was given 7µl SM. If the mouse did not respond within the LH period, it was recorded as an omission and no reward was given, along with illumination of the main house light for 5 seconds (time out period; TO). After the TO, the mouse had to make an entry into the reward dispenser to start the next trial: the dispenser light was turned on to indicate this period. As these sessions progressed from 1-4, the length of time for which the stimulus is lit (stimulus duration; SD) and LH period were decreased from 30 - 10 and 37 – 10 seconds, for SD and LH, respectively. Mice were moved on individually to the next stage when they achieved criteria of >80% accuracy and <20% omissions for two consecutive sessions. Mice that had completed the 4th stage were rested without daily training, whilst mice not at criteria of stage 4 continued. Mice on rest were given a reminder training session twice per week and if they fell below criteria on a reminder session, they were trained daily until criteria was re-attained. This training regime is encouraged for operant-based training (Oomen *et al.*, 2013) because "over"-training the mice that learn the task more quickly than others could increase variability in performance and have confounding effects on the interpretation of the results. These stages of training took 49 sessions in total.

6.3.5 The full rGT

Next, the mice were given four sessions to learn each of the reward-punishment contingencies. These sessions lasted for 30 minutes or 100 trials, whichever came first. The contingencies were counterbalanced across mice so that each quarter of mice were trained on one of four different combinations of locations of contingencies: **A**= Choice 1, 4, 2, 3; **B**=Choice 2, 1, 3, 4; **C**= Choice 3, 2, 4, 1; **D**= Choice 4, 3, 1, 2 in grid squares 1, 2, 4 and 5, respectively. The mice were forced to choose a particular square (i.e. only one square was lit at each trial) so that each mouse had equal prior exposure to each of the contingencies. A white square was presented in one of the four stimulus locations and the LH and stimulus

duration length were fixed at 10 seconds. Incorrect responses (not at the lit square) resulted in no action but were recorded. Failure to respond within the LH period was recorded as an omission, and then the stimulus was removed. A correct response at each square resulted in the following (see also **Fig. 6.3**):

- Choice 1: Rewarded 90% of the time with 1 x 7µl strawberry milkshake; punished 10% of the time with a 5s timeout.
- Choice 2: Rewarded 80% of the time with 2 x 7µl strawberry milkshake; punished 20% of the time with a 10s timeout.
- Choice 3: Rewarded 50% of the time with 3 x 7µl strawberry milkshake; punished 50% of the time with a 30s timeout.
- Choice 4: Rewarded 40% of the time with 4 x 7µl strawberry milkshake; punished 60% of the time with a 40s timeout.

Choice 2 delivers the optimum reward per unit time (411 SM deliveries in 30 minutes).

If the trial was rewarded (win), then the square was removed, the appropriate number of SM deliveries occurred and the tray light was illuminated. Once the reward had been consumed and the mouse had left the reward dispenser, the ITI period began and the dispenser light was extinguished.

If the trial was punished (loss) then the house light was turned on, the stimuli were removed and the square touched flashed (0.2s on and then 0.2s off) for the TO period of 10 seconds. At the end of the TO, the house light was turned off, the tray light was illuminated and the flashing image was removed. The next trial was initiated when the mouse had entered and left the reward dispenser (which extinguishes the light), by starting the next ITI period.

A premature response was recorded when a touch was made in one of the response grid areas (1-5) during the ITI and resulted in a TO of 5 seconds with the house light turned on. At the end of the TO the house light was switched off and the tray light illuminated. The next trial was initiated when the mouse entered and left the reward dispenser by starting the next ITI period.

After four sessions of exposure to the different reward contingencies, mice were moved onto the full task where they had the option to pick whichever square they

liked at each trial. Everything remained the same as for when they were forced to pick a choice, apart from all four of the stimulus squares appeared at each trial in grid positions 1, 2, 4 and 5 instead of just one. Mice were trained on this free choice version until they reach stable performance; i.e., they were consistent in their choice pattern for five consecutive days, which took 13 sessions in total.

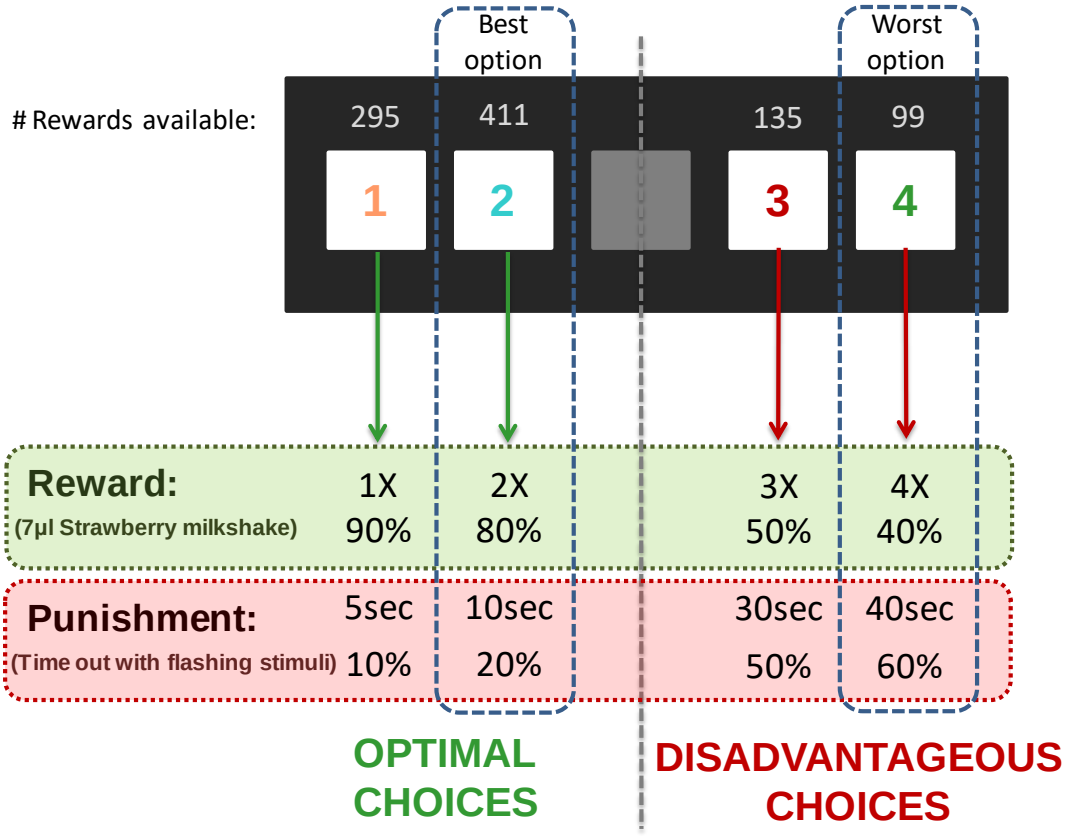

Figure 6.3. Schematic diagram depicting the reward/punishment contingencies associated with each choice on the touchscreen. Choice 2 is the most advantageous option and Choice 4 is the least, according to the number of SM reward deliveries available per unit time. Considering the probabilities and extent of the punishment period, if chosen exclusively within the 30-minute period, it is possible for mice to obtain 295 SM reward deliveries with Choice 1, 411 with Choice 2, 135 with Choice 3 and 99 with Choice 4.

6.3.6 Manipulation 1

Choice 1 and 2 were optimal choices (2 the most optimal) and 3 and 4 were the least optimal (4 the least optimal) throughout training to stable performance. The position of the two more similar choices (1 and 3) were switched in Manipulation 1, making the contingency groups resemble the following: **A**= Choice 3, 4, 2, 1; **B**= Choice 2, 3, 1, 4; **C**= Choice 1, 2, 4, 3; **D**= Choice 4, 1, 3, 2 in grid squares 1, 2, 4 and 5, respectively (see **Fig.6.4** and **Table 6.1** for an overview of each of the manipulations). Mice were tested on Manipulation 1 until they reached stable performance, which took 6 sessions in total.

6.3.7 Manipulation 2

Mice were then subjected to a second manipulation, where Choice 2 and 4 (most vs. least optimal) were switched, in addition to adjustment of the punishment period for Choice 3 and 4: it was decreased from 20 and 40 seconds to 15 and 20 seconds, respectively (**Fig. 6.4** and **Table 6.1**). The contingency groups for this manipulation resembled the following: **A**= Choice 3, 2, 4, 1; **B**= Choice 4, 3, 1, 2; **C**= Choice 1, 4, 2, 3; **D**= Choice 2, 1, 3, 4 in grid squares 1, 2, 4 and 5, respectively. Switching of the two more extreme options could be considered an "easy" switch for the mice to learn and it could be hypothesised that mice would learn this switch more quickly than with Manipulation 1, as this is the second time they have had to switch. By making the punishment less extreme for the disadvantageous options 3 and 4, we are able to assess the sensitivity of mice to the punishment period length rather than sensitivity to reward amounts obtained per choice, or probability of reward-punishment (this was unchanged). Mice were tested on Manipulation 2 until they reached stable performance, which took 56 sessions.

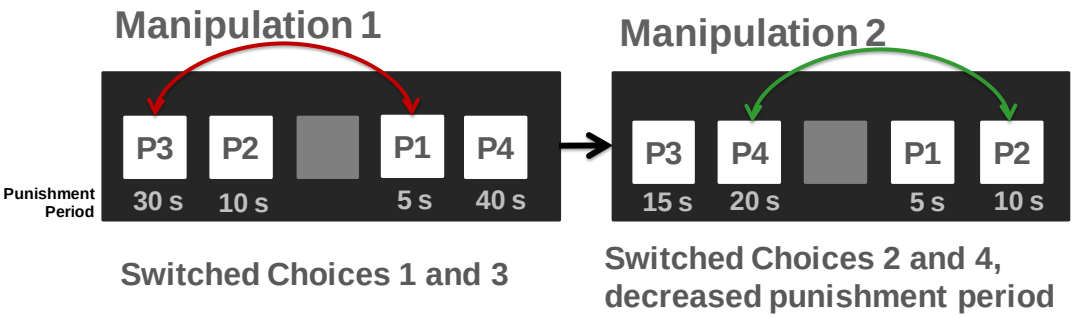

Figure 6.4. Schematic diagram depicting the nature of the manipulations. In Manipulation 1, the location of Choices 1 and 3 were switched. In Manipulation 2, the location of Choices 2 and 4 were switched, as well as decreasing the punishment periods for Choices 3 and 4.

Condition	Grid Position	Choice #	Reward (x7ul SM)	% Punished	Punishment length (s)
Baseline	1	1	1	10	5
Baseline	2	4	4	60	40
Baseline	4	2	2	20	10
Baseline	5	3	3	50	30
Manipulation 1	1	**3**	3	50	30
Manipulation 1	2	4	4	60	40
Manipulation 1	4	2	2	20	10
Manipulation 1	5	**1**	1	10	5
Manipulation 2	1	3	3	50	**15**
Manipulation 2	2	**2**	2	20	10
Manipulation 2	4	**4**	4	60	**20**
Manipulation 2	5	1	1	10	5

Table 6.1. Choice contingencies in each grid position for Baseline and Manipulations. Highlighted in bold are the alterations made when compared to Manipulation 1.

6.3.8 Amphetamine Challenge

Once stable performance had been re-acquired following Manipulation 2, the effect of an acute dose of 1.5mg/kg amphetamine was assessed. The amphetamine dose was chosen based on results by van Enkhuizen *et al*. (2013) and previous data from our lab, in order to produce disruption of cognition without the confounding increase in locomotor activity seen with higher doses. Amphetamine administration was counterbalanced such that 1.5mg/kg D-amphetamine (Sigma Aldrich, A-5880) or saline was administered i.p. at 2ml/kg on the first day. Mice were then tested without drug on the following day and then D-amphetamine or saline was administered to the other half of the group on the third day. Performance on the intervening day was analysed to verify that recovery from amphetamine had occurred before proceeding with amphetamine administration on the third day. Recovery was deemed to have occurred if any effects from amphetamine had returned to Baseline performance.

6.3.9 Statistical Analysis

All mice were included in all analyses with the exception of two significantly atypical WT mice (who were removed from study) because of consistent abnormal repetitive behaviour (hyperactive rotational movements) that prevented the mouse from completing the task properly, disguising its true cognitive ability. Following amphetamine administration, one mouse was not as active as the other mice, completing just 11 trials for that session, so was therefore removed from all amphetamine analyses.

All statistical analyses were carried out using Minitab® 17 Statistical Software. Results were considered significant if $p<0.05$. All error bars are expressed as ± standard error of the mean (SEM). Bar and line graphs were created using GraphPad Prism 7. Box plots were created using BoxPlotR, an application available at http://shiny.chemgrid.org/boxplotr/ and described in Nature Methods Editorial "Kick the bar chart habit" 2014, p113.

The performance measures analysed for each session were the following:

- **% Choice 1** (% of the choices made to the grid position rewarding 1 SM deliveries)
- **% Choice 2** (% of the choices made to the grid position rewarding 2 SM deliveries)

- **% Choice 3** (% of the choices made to the grid position rewarding 3 SM deliveries)
- **% Choice 4** (% of the choices made to the grid position rewarding 4 SM deliveries)
- **% premature responses** (responses made during the ITI before the stimuli appear on the screen; number of premature responses/total number of trials initiated × 100)
- **% omissions** (number of omissions/total number of trials initiated × 100)
- **Number of perseverative responses per loss** (repeat touches to any part of the screen; number of perseverative responses/number of lost trials)
- **Total number of responses made**
- **Reward magazine entries during the ITI**
- **Total number of beam breaks**
- **Latency to make a choice**
- **Reward collection latency**

All behavioural parameters were analysed for sex differences. Many variables (% optimal responses, % omissions, % premature responses, % responses to Choice 1, % responses to Choice 3, latency to make a choice, total number of beam breaks and number of magazine entries during the ITI) showed a significant effect of sex. As there was a difference between male and female mice in so many aspects of the task, it was more appropriate to take sex into account for all analyses in this chapter rather than only for parameters which exhibited sex differences. Genotype group sizes contained equal numbers of male and female mice; so, in addition to considering sex in analyses, effects of sex are not likely to influence the data in a misrepresentative way.

The last 5 days of stable performance from the "Free Choice" rGT were analysed between genotypes and, where appropriate, were compared with stable performance following Manipulation 1, Manipulation 2 and the Amphetamine challenge.

Some of the parameters throughout this experiment yielded non-normal data. Although it is possible that parametric statistical tests give more power when analysing non-normal data, there is no non-parametric statistical test available to accurately represent the data here. This is because a repeated measures design

with multiple factors is appropriate, with nesting of each individual mouse within genotype. As non-parametric tests do not allow nesting and multiple factor repeated measures, and ANOVA is considered robust against non-normality (Laan & Verdooren, 1987), an ANOVA was decided to be the most suitable test to use here. Unless stated otherwise, results were analysed using a 3-way repeated measures ANOVA, with daily session as a within subjects factor, genotype and sex as between subjects factors and each individual mouse nested within genotype and sex. Amphetamine treatment data were analysed by a 3-way repeated measures ANOVA with session (i.e. amphetamine treatment or normal test session) as a within subjects factors, genotype and sex as between subjects factors and each individual mouse nested within genotype and sex. Post hoc tests were conducted using Tukey's method for multiple comparisons where appropriate. As there were so many irrelevant significant effects, results for main effects and interactions are only shown and discussed when a significant effect was observed.

6.4. Results

6.4.1. Map2k7$^{+/-}$ *mice were quicker to complete training stages*

Following initial training to touch the screen, the mice progressed onto training to touch the screen at every trial. For this section of training, mice were moved on to the next sub-stage when they reached criteria. Criteria were defined as performing at >80% accuracy (touching the square that was lit), and <20% omissions (not making a response during a trial) for two consecutive sessions. The number of sessions and trials taken for each mouse to reach criteria was recorded. *Map2k7*$^{+/-}$ mice learned the task more quickly than WT mice: they took a fewer sessions on average (23.91 ± 2.71 compared to WT mice taking 32.80 ± 3.45 sessions; $p=0.038$, $F_{(1,18)}=5.02$) and fewer trials on average (1154.67 ± 84.78 compared to WT mice taking 1325.90 ± 61.96 trials; $p=0.05$, $F_{(1,18)}=4.25$) to complete training (**Fig. 6.5**).

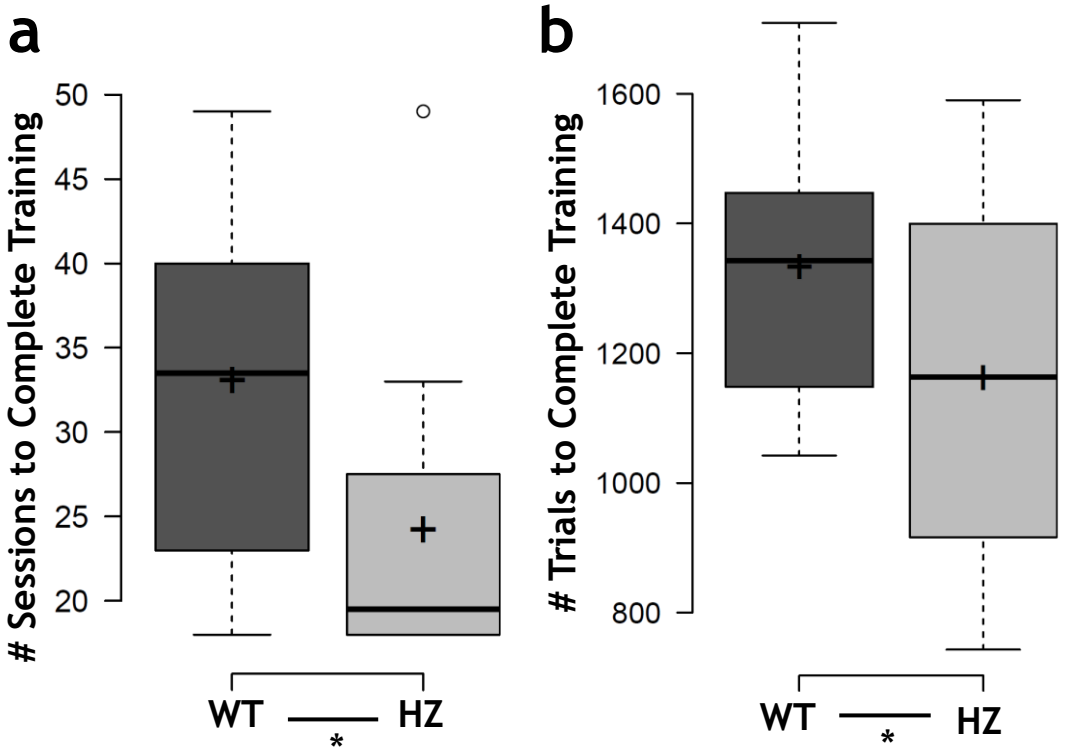

Figure 6.5. *Map2k7*$^{+/-}$ mice completed training more quickly than WT mice. *Map2k7*$^{+/-}$ mice took (**a**) fewer sessions and (**b**) fewer trials on average to complete Gambling Task training. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Data for **a** and **b** analysed separately by a two-way ANOVA with genotype and sex as factors. Lines linking groups represent significant differences between those groups: *p<0.05 (ANOVA). *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

6.4.2 Map2k7$^{+/-}$ *mice show slightly less-risky choice behaviour in the rGT than WTs*

6.4.2.1 Choice Performance

Mice were then trained on the Free Choice rGT until they reached a stable level of choice performance, which took 13 sessions in total. The last 5 days of stable performance (Baseline) were analysed for differences between genotypes. *Map2k7*$^{+/-}$ mice showed enhanced performance on the rGT at Baseline compared to WTs with decreased % Choice 4 (least optimal option; $p<0.0001$, $F_{(1,80)}=15.85$; **Fig. 6.6d**), and a slight increase in responding for the most optimal choice, 2

(p=0.011, $F_{(1,80)}$=6.73; **Fig. 6.6b**). The % of choices to 1 (**Fig. 6.6a**) and 3 (**Fig. 6.6c**) were similar between WT and *Map2k7*$^{+/-}$ mice (p=0.795 and p=0.237, respectively).

6.4.2.2 Other performance and motivation parameters

Looking at other performance parameters, WT and *Map2k7*$^{+/-}$ mice made similar % omissions (**Fig. 6.7a**) and % premature responses (**Fig. 6.7b**) (p=0.192 and p=0.651, respectively). However, *Map2k7*$^{+/-}$ mice showed signs of having enhanced performance in some measurements: they made less perseverative responses per loss (p<0.0001, $F_{(1,80)}$=50.53; **Fig. 6.7c**) and more choices per session on average (p<0.0001, $F_{(1,80)}$=19.16; **Fig. 6.7d**). *Map2k7*$^{+/-}$ mice were also hyperactive whilst carrying out the task: they made significantly more beam breaks than WT mice (p<0.0001, $F_{(1,80)}$=461.80; **Fig. 6.8a**). Despite this, *Map2k7*$^{+/-}$ mice made less entries into the magazine throughout the ITI period (p=0.047, $F_{(1,80)}$=4.06; **Fig. 6.8b**) and collected the reward just as quickly as WT mice (p=0.138, ns; **Fig. 6.8d**); however, they were quicker to make a choice on average (p<0.0001, $F_{(1,80)}$=34.96; **Fig. 6.8c**). Overall, *Map2k7*$^{+/-}$ mice seemed highly motivated to complete the rGT: they are hyperactive and are quicker to make a choice, made more choices overall, did not miss more trials, and collected the reward just as quickly as WT mice. They also exhibited slightly more focussed behaviour than WT mice in the sense that they made less perseverative responses, and less entries into the reward magazine throughout the ITI.

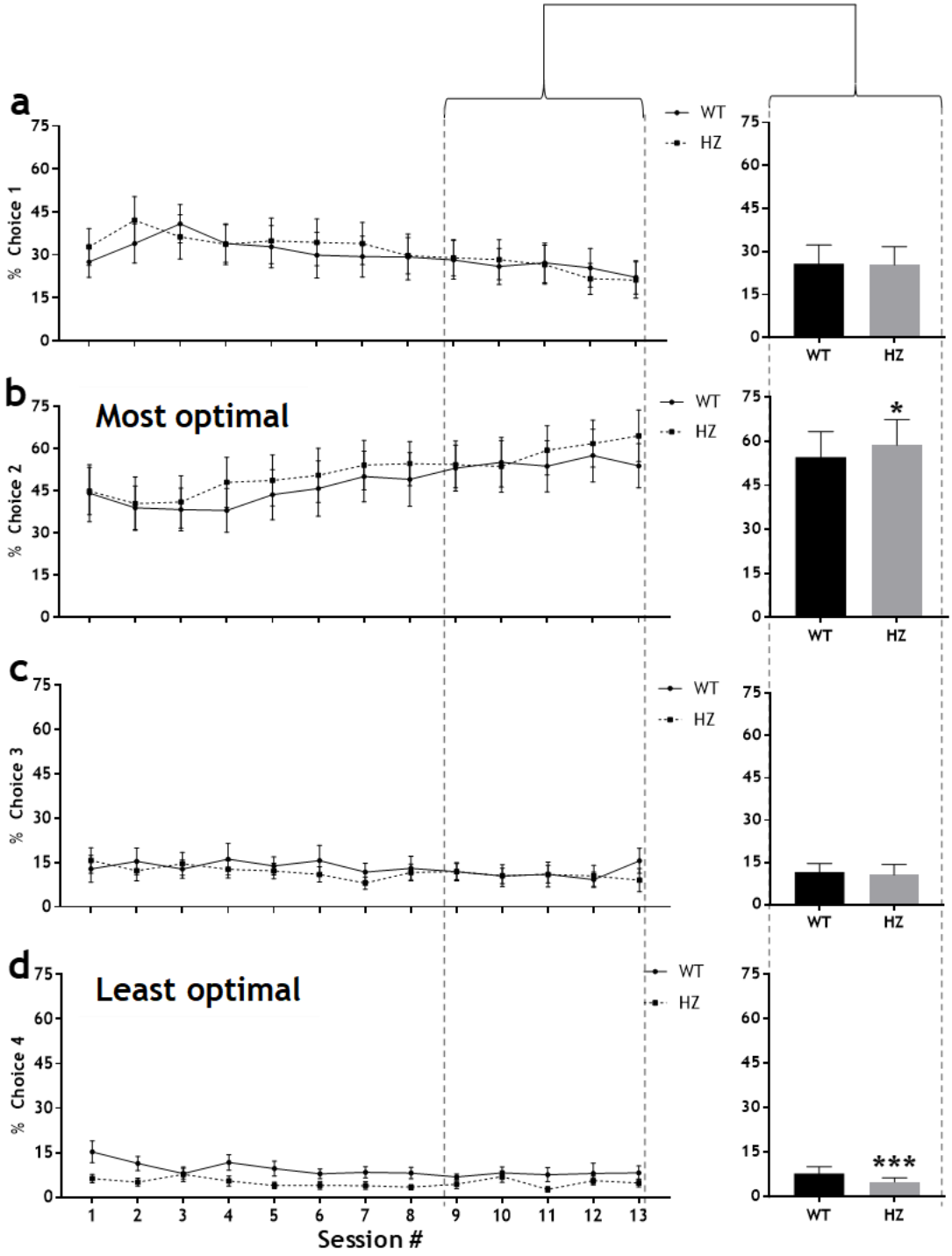

Figure 6.6. *Map2k7*$^{+/-}$ mice show slightly less-risky choice behaviour in the rGT. *Map2k7*$^{+/-}$ mice made similar number of Choices 1 (**a**) and 3 (**c**) as WT mice but made significantly more choices to 2 (**b;** the "most optimal" choice) and significantly less choices to 4 (**d;** the "least optimal" choice). Line graphs show data for each session (numbered from beginning of Free Choice). Data points (line graphs) or bars represent the mean; error bars represent the SEM. Data analysed by a 3-way repeated measures ANOVA with the last 5 days' session as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Tukey's post hoc analysis was used where appropriate. *p<0.05, ***p<0.0001 (ANOVA). *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

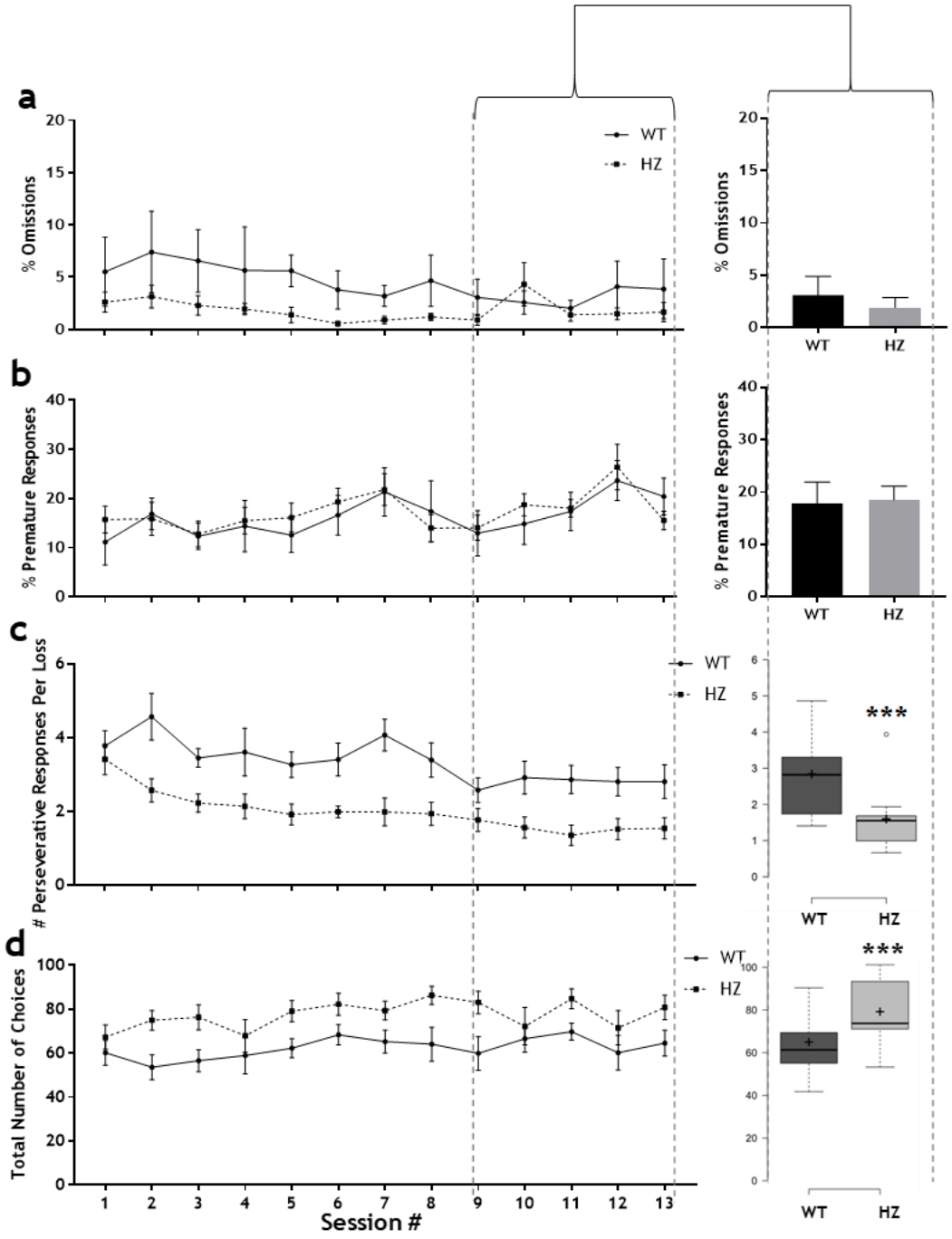

Figure 6.7. *Map2k7*$^{+/-}$ mice show enhanced performance in some aspects of the rGT. (**a**) % omissions and (**b**) % premature responses were similar between *Map2k7*$^{+/-}$ mice and WT mice. However, *Map2k7*$^{+/-}$ mice made significantly less perseverative responses per loss (**c**) and more choices (**d**) each daily session than WTs. Line graphs show data for each session (numbered from beginning of Free Choice). Data points (line graphs) or bars represent the mean; error bars represent the SEM. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Data analysed by a 3-way repeated measures ANOVA with the last 5 days' session as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Tukey's post hoc

analysis was carried out where appropriate. ***p<0.0001 (ANOVA). *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

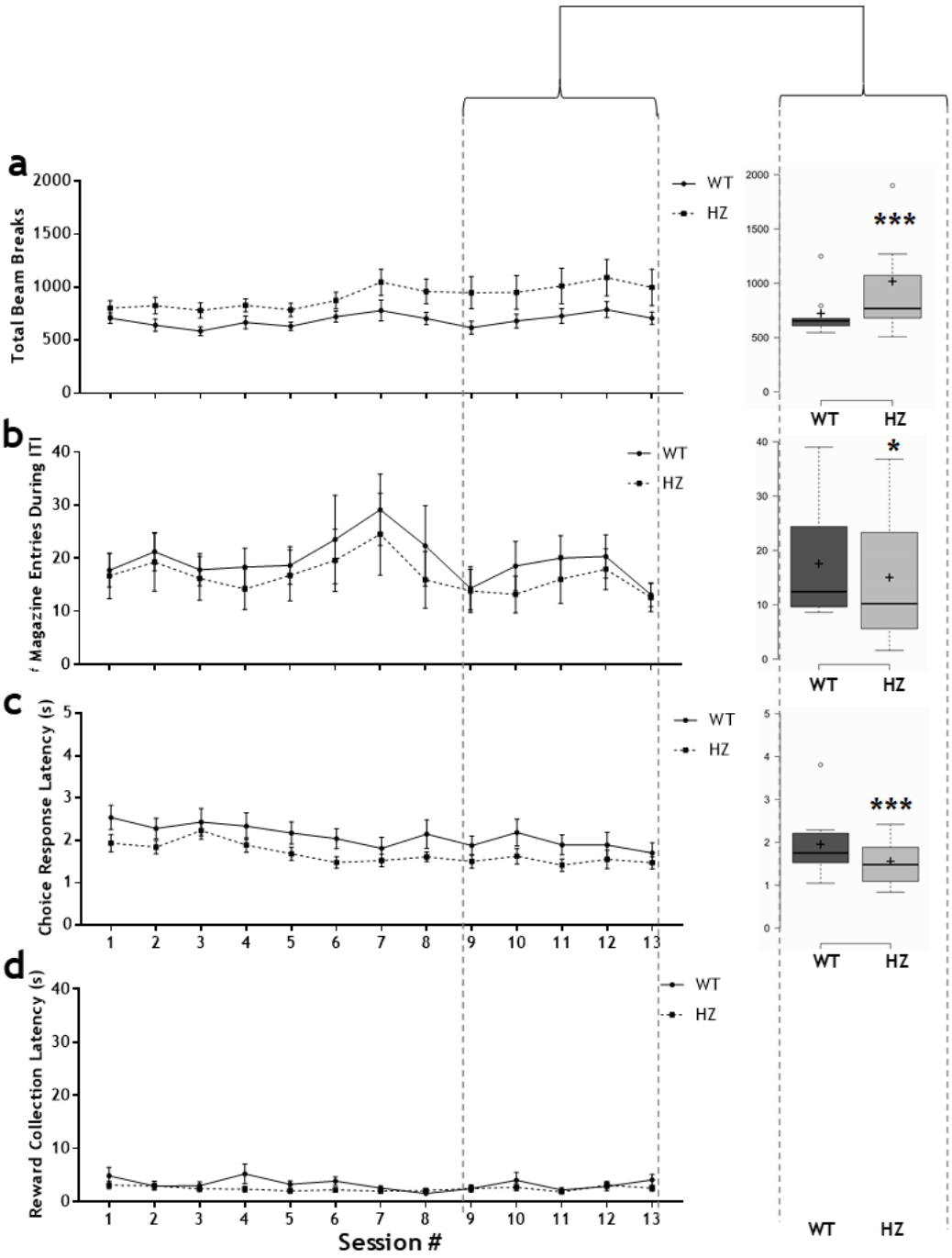

Figure 6.8. *Map2k7*$^{+/-}$ mice show good motivation to carry out the rGT task in most parameters. *Map2k7*$^{+/-}$ mice made significantly more beam breaks (**a**), more magazine entries during the ITI (**b**) and made choices more quickly (**c**) than WT mice. Reward collection latency was similar between *Map2k7*$^{+/-}$ and WT mice (**d**). Line graphs show data for each session (numbered from beginning of Free Choice). Data points represent the mean; error bars represent the SEM. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Data analysed by

a 3-way repeated measures ANOVA with the last 5 days' session as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Tukey's post hoc analysis was carried out where appropriate. *p<0.05, ***p<0.0001 (ANOVA). *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

6.4.3 Manipulation 1 affected performance in the rGT to the same extent in WT and Map2k7$^{+/-}$ *mice*

6.4.3.1 Choice Performance

Manipulation 1 involved a switch between options 1 and 3 (**Fig. 6.4**), which are the two more subtly-different options in that neither were the most or least optimal. As is expected, overall performance for all mice during the first session following this manipulation dropped, reflected in a significantly decreased % response for the better option, 1, and an increased % response for the more disadvantageous option, 3, because they were previously located in each other's position (effect of session: p<0.0001 for both choices; $F_{(5,100)}$=6.71 (Choice 1), 7.42 (Choice 2); **Fig. 6.9a** and **c,** respectively). % choices for 2 and 4, which were not switched, remained unchanged (no significant effect of session: p=0.132 and p=0.315; **Fig. 6.9b** and **d,** respectively). Remarkably, the mice detected the manipulation and rapidly altered their responding, such that by the fourth session post-switch they were already performing close to previous baseline levels of performance. By the time stable performance had been achieved for three consecutive sessions (which took six sessions in total), the mice had returned to the same pattern of responding as before. Comparing the last 3 days of Manipulation 1 between genotypes showed that *Map2k7*$^{+/-}$ mice had enhanced performance compared to WTs: slightly increased responding for Choice 2 (p=0.001, $F_{(1,40)}$=12.47; **Fig. 6.9b**), decreased responding for Choice 4 (p<0.001; $F_{(1,40)}$=56.28; **Fig. 6.9d**), and similar responding for Choices 1 (p=0.325, ns) and 3 (p=0.478, ns).

6.4.3.2 Other performance and motivation parameters

Analysis of Baseline performance compared with the first day of Manipulation 1 showed other performance parameters were not significantly altered by Manipulation 1: no significant effect of session for % omissions (**Fig. 6.10a**;

p=0.456), number of perseverative responses per loss (**Fig. 6.10c**; p=0.732) and total number of choices (**Fig. 6.10d**; p=0.234). There was an overall significant effect of session for % premature responses (**Fig. 6.10b;** p=0.025, $F_{(5,100)}$=2.70); however, post hoc analyses confirmed the significant difference was not between Manipulation 1 and the other 5 sessions.

Motivation parameters were altered by Manipulation 1 to some extent: there was an overall decrease in total beam breaks (**Fig. 6.11a;** p<0.0001, $F_{(5,100)}$=11.94) and magazine entries during the ITI (**Fig. 6.11b**; p=0.004, $F_{(5,100)}$=3.78); reward collection latency was increased (**Fig. 6.11d**; p=0.029, $F_{(5,100)}$=2.62), but choice response latency remained the same (**Fig. 6.11c**; p=0.075). Post hoc analyses showed that there was a significant effect between Manipulation 1 and some, but not all, of the last 5 days' stable performance parameters (indicated on the line graphs of **Fig. 6.11**). Whereas total beam breaks were significantly decreased for the first day of Manipulation 1 compared to 3 of 5 of the days, magazine entries during the ITI were significantly decreased compared to 2 of 5 of the days and reward collection latency was increased compared to 1 of the 5 days. Overall, Manipulation 1 appeared to influence some aspects of motivation levels as the mice were slower to respond and moved around the box less. This is 'normal' for the first session after an unexpected change and suggests the mice were 'feeling defeated'; however, all mice showed signs of having good motivation to complete the task (no change in % omissions, total number of choices and latency to make a choice).

Once stable performance had been re-attained following Manipulation 1, there were some alterations in performance between WT and *Map2k7*$^{+/-}$ mice. Analysing the last 3 days of Manipulation 1 showed that *Map2k7*$^{+/-}$ mice made less % omissions than WT mice (**Fig. 6.10a;** p=0.001, $F_{(1,40)}$=13.92), which was previously similar between genotypes at Baseline. *Map2k7*$^{+/-}$ mice also made a similar number of reward magazine entries during the ITI to WTs (**Fig. 6.11b;** p=0.720), whereas at Baseline they made significantly less entries than WTs. All other performance and motivation parameters remained similarly significant or non-significant between genotypes as to what they were at Baseline: beam breaks (**Fig. 6.11a**; p<0.0001, $F_{(1,40)}$=166.16) and number of choices made (**Fig. 6.10d;** p<0.0001, $F_{(1,40)}$=23.24) were increased compared to WTs; *Map2k7*$^{+/-}$ mice were quicker to make a choice (**Fig. 6.11c**; p<0.0001, $F_{(1,40)}$=24.82); and reward collection latency (**Fig. 6.11d**;

p=0.172) and percentage of premature responses (**Fig. 6.10b**; p=0.108) remained similar. Throughout stable performance following Manipulation 1, *Map2k7*$^{+/-}$ mice continued to have good motivation to perform the task. Although significant, the decrease in % omissions and levelling of reward magazine entries during the ITI compared to WTs was very modest and should not have contributed to any significant degree on choice behaviour.

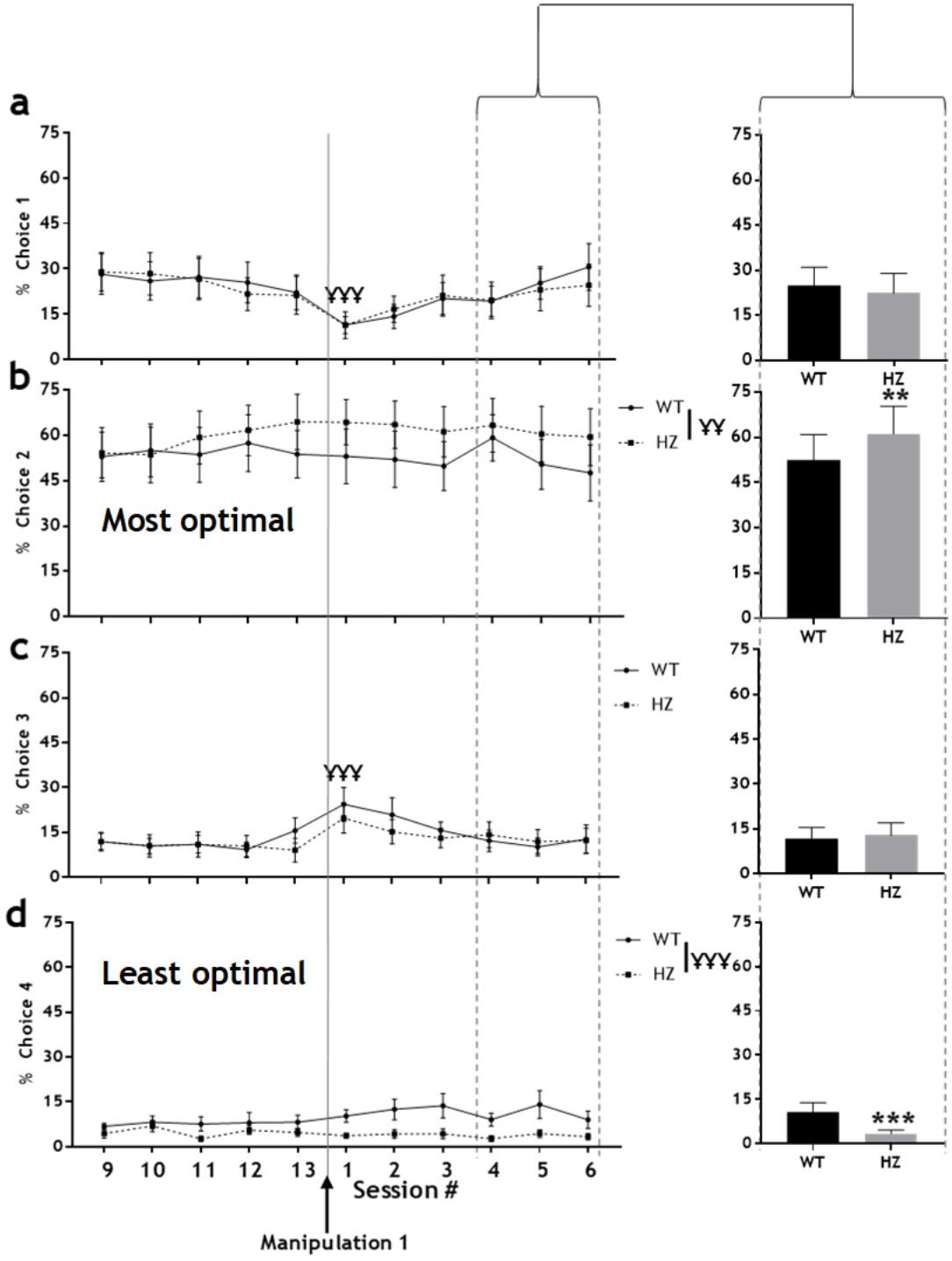

Figure 6.9. rGT performance was affected by Manipulation 1 to the same extent in WT and *Map2k7*$^{+/-}$ mice. Significant differences from the analysis of the last 5 days, plus first day of Manipulation 1 are indicated by ¥. Significant differences from the analysis of the last 3 days' Manipulation 1 are indicated by *. Comparing the first day of Manipulation 1 to the last 5 days' stable responding revealed a significant effect of session for Choices 1 (**a**) and 3 (**c**). There was no significant change for Choices 2 (**b**) and 4 (**d**). This was analysed by a three-way repeated measures ANOVA with session (last 5 days, plus first day of Manipulation 1) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups.

Significant differences from this analysis are indicated by ¥¥p<0.01, ¥¥¥p<0.0001 (ANOVA/Tukey's). Analysing the last 3 days of Manipulation 1 showed that *Map2k7*$^{+/-}$ mice were similar to WTs with their responses to 1 (**a**) and 3 (**c**) but *Map2k7*$^{+/-}$ mice responded significantly less than WTs to choice 2 (**b**) and more than WTs to choice 4 (**d**). This was analysed by a three-way repeated measures ANOVA with session (last 3 days' Manipulation 1) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by **p<0.01, ***p<0.0001 (ANOVA). Line graphs show data for each session (numbered from beginning of 5 days' stable performance). Data points represent the mean; error bars represent the SEM. Tukey's post hoc analyses were carried out throughout where appropriate. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

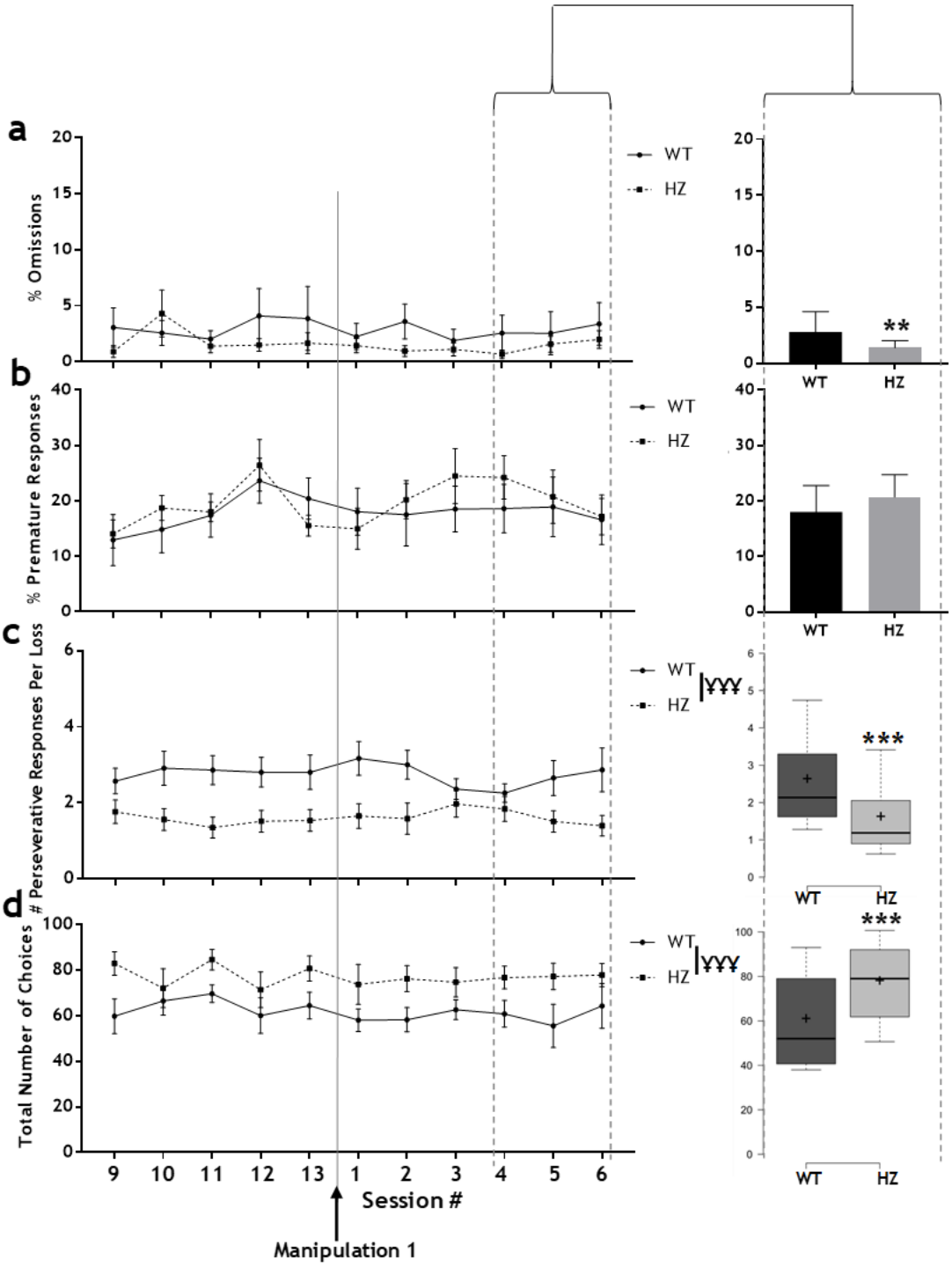

Figure 6.10. Manipulation 1 did not have an immediate significant effect on the above (a-d) performance parameters in the rGT, but performance was altered once stable performance had been re-attained. Significant differences from the analysis of the last 5 days, plus first day of Manipulation 1 are indicated by ¥. Significant differences from the analysis of the last 3 days' Manipulation 1 are indicated by *. Analysis of the last 5 days performance compared with the first day of Manipulation 1 showed no significant effect of session for % omissions (**a**), number of perseverative responses per loss (**c**) and total number of choices (**d**). There was a significant effect of session for % premature responses (**b**); however, post hoc analyses confirmed the significant difference was not between Manipulation 1 and any of the other 5 sessions.

This was analysed by a three-way repeated measures ANOVA with session (last 5 days, plus first day of Manipulation 1) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by ¥¥¥p<0.0001 (ANOVA). Analysing the last 3 days of Manipulation 1 showed that *Map2k7*$^{+/-}$ mice made a similar percentage of premature responses to WTs (**b**). *Map2k7*$^{+/-}$ mice made significantly fewer omissions (which was not the case before Manipulation 1; **a**) and perseverative responses (**c**) than WTs, and made significantly more choices (**d**). Overall, following Manipulation 1, *Map2k7*$^{+/-}$ mice were still performing slightly better than WTs in some parameters. This was analysed by a three-way repeated measures ANOVA with session (last 3 days' Manipulation 1) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by **p<0.01, ***p<0.0001 (ANOVA). Line graphs show data for each session (numbered from beginning of 5 days' stable Baseline performance). Data points represent the mean; error bars represent the SEM. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Tukey's post hoc analyses were carried out throughout where appropriate. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

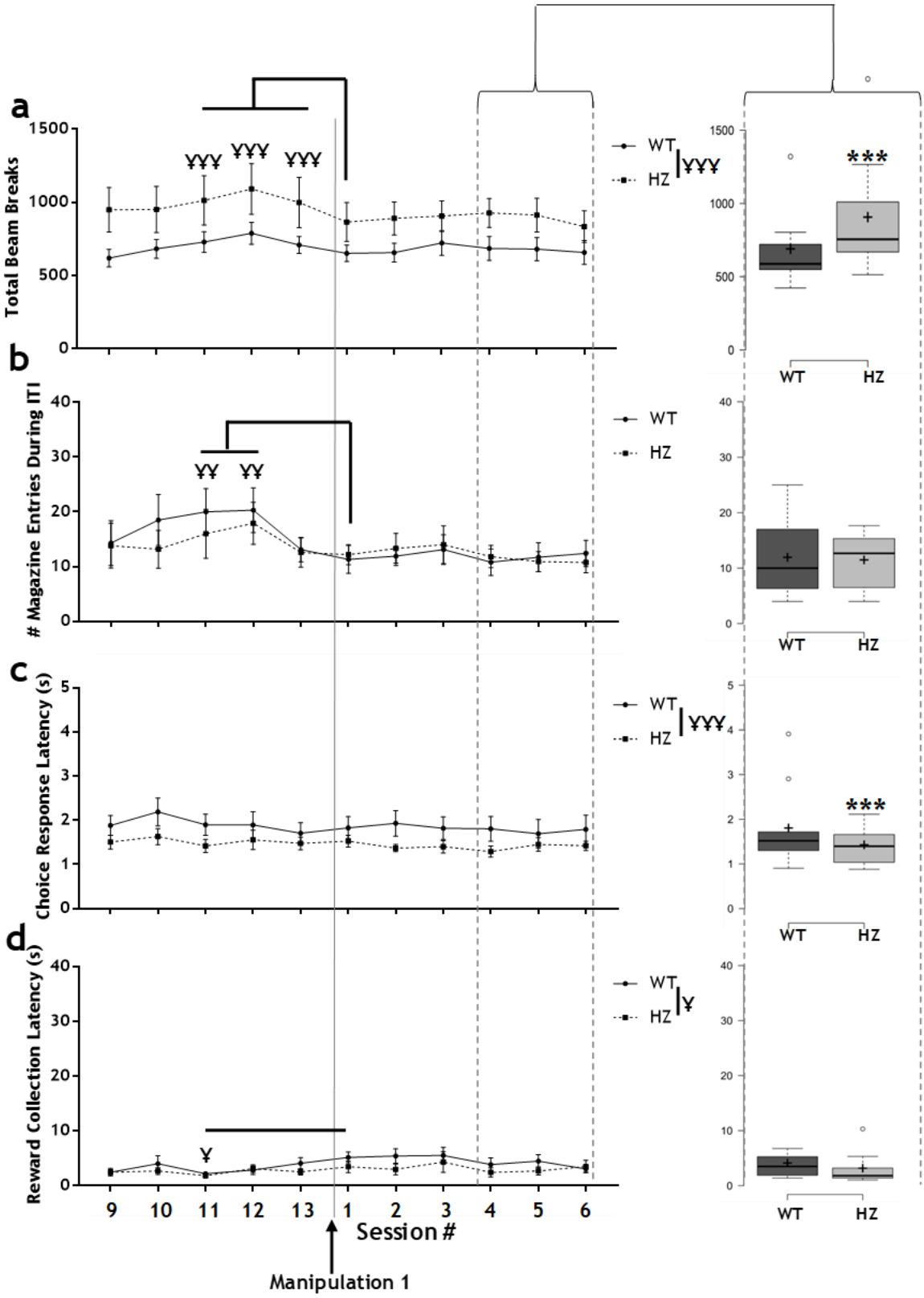

Figure 6.11. Manipulation 1 had a slight effect on motivation parameters in the rGT. Significant differences from the analysis of the last 5 days, plus first day of Manipulation 1 are indicated by ¥. Significant differences from the analysis of the last 3 days' Manipulation 1 are indicated by *. Analysis of the last 5 days performance compared with the first day of Manipulation 1 showed there was an overall significant effect of session for total beam breaks (**a**), magazine entries during the ITI (**b**) and

reward collection latency (**d**), but not for choice response latency (**c**). Post hoc analyses showed that there was a significant effect between Manipulation 1 and some, but not all, of the last 5 days' stable performance parameters (indicated on the line graphs). This was analysed by a three-way repeated measures ANOVA with session (last 5 days, plus first day of Manipulation 1) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by ¥p<0.05, ¥¥p<0.01, ¥¥¥p<0.0001 (ANOVA/Tukey's). Analysing the last 3 days of Manipulation 1 showed that *Map2k7*$^{+/-}$ mice make more beam breaks than WT mice (**a**) and are quicker to make a choice (**c**). Other motivation parameters remain the same for *Map2k7*$^{+/-}$ compared to WT mice (number of magazine entries during the ITI (**b**), reward collection latency (**d**). Overall, following Manipulation 1, *Map2k7*$^{+/-}$ mice are still showing signs of good motivation to perform the task, and in some measurements, show slightly better motivation (being quicker to make a choice and moving around more). This was analysed by a three-way repeated measures ANOVA with session (last 3 days' Manipulation 1) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by ***p<0.0001 (ANOVA). Line graphs show data for each session (numbered from beginning of 5 days' stable performance). Data points represent the mean; error bars represent the SEM. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Tukey's post hoc analyses were carried out throughout where appropriate. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

6.4.4 Map2k7$^{+/-}$ *mice took significantly longer than WT mice to re-establish stable responding following Manipulation 2*

6.4.4.1 Choice Performance

Manipulation 2 then occurred, which consisted of switching the most and least optimal options: Choice 2 with Choice 4. The severity of the punishment period was also decreased for Choice 3 and Choice 4, reducing them from 30 and 40 seconds to 15 and 20 seconds, respectively, whilst keeping the punishment likelihood the same for all options (see **Table 6.1** and **Fig.6.4**). The punishment period length now increased linearly from Choices 1-4 (from 5 – 10 – 15 – 20 seconds, respectively), as opposed to increasing disproportionately. Throughout Manipulation 1, the mice were capable of tracking even subtly different contingencies. We predicted that switching two more obviously different contingencies would likely have the effect of inducing just as much, or even more motivation to switch because the consequences are more drastic. However, the decrease of the punishment period for the disadvantageous options at the same time as this switch makes this prediction more complex and enables examination of the magnitude of effect that lesser punishment has on the mice when attempting to learn a new contingency switch, as opposed to other aspects of the task (reward amount, punishment probability), because these remain the same, just in a different location on the touchscreen. In the session following Manipulation 2, a large effect on choice performance was seen because % responses to Choice 2 and 4 switched. Hence, on average mice were choosing: WT: 54.64 ± 9.37; *Map2k7*$^{+/-}$: 58.72 ± 8.52 % Choice 2 at Baseline and WT: 9.97 ± 2.39; *Map2k7*$^{+/-}$: 5.70 ± 1.85 % Choice 2 following Manipulation 2, and WT: 7.77 ± 1.92; *Map2k7*$^{+/-}$: 4.89 ± 1.29 % Choice 4 at Baseline, and WT: 49.21 ± 8.72; *Map2k7*$^{+/-}$: 55.40 ± 9.05 % Choice 4 following Manipulation 2 (**Fig. 6.13b** and **d**). The last 3 days of Manipulation 1 were compared with the first 5 of Manipulation 2 (as they are grouped this way on the line graphs) and Choice 2 and 4 had a significant effect of session: $p<0.0001$ for both choices, $F_{(7,140)}=32.99$ (Choice 2), $F_{(7,140)}=23.57$ (Choice 4); **Fig. 6.13b** and **d,** respectively. % response for Choices 1 and 3 also changed (increased) following Manipulation 2, but not to the same extent as with Choices 2 and 4 (effect of session: $p=0.005$, $F_{(7,140)}=3.06$ (Choice 1) and $p=0.004$, $F_{(7,140)}=3.16$ (Choice 3)).

Manipulation 2 had a huge effect on mice, particularly *Map2k7*$^{+/-}$ mice, who took substantially longer to re-establish stable responding following this manipulation (**30.25** ± 4.17 sessions to stable performance as compared with **12.1** ± 4.32 sessions for WTs; p=0.007; $F_{(1,20)}$=9.05; **Fig. 6.12**).

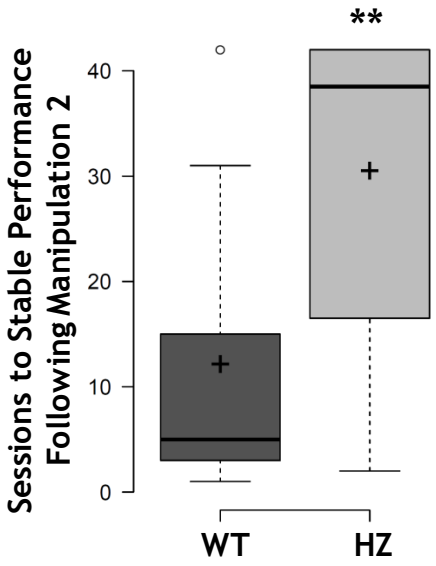

Figure 6.12. *Map2k7*$^{+/-}$ mice took substantially longer to re-establish stable responding following Manipulation 2. The number of sessions that it took for each mouse to reach more than 70% of their choices being either Choice 1 or Choice 2 (the two optimal choices) for three consecutive sessions were calculated: *Map2k7*$^{+/-}$ mice took significantly more sessions than WT mice. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Data analysed by a one-way ANOVA between genotypes. **p<0.01 (ANOVA). *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

Interestingly, when their performance was once again stable for five sessions following Manipulation 2, the choice preference of the two groups of mice settled differently to what previously occurred following Manipulation 1 (and at Baseline). When comparing stable performance at the end of Manipulation 1 (last 3 days, grouped) with the stable performance at the end of Manipulation 2 (last 5 days, grouped), both groups of mice increased their % responding for Choice 3 (effect of session: p=0.005, $F_{(1,152)}$= 7.94). % responding for Choice 4 also increased (effect

of session: $p<0.0001$, $F_{(1,152)}= 27.99$), but post hoc analyses showed that this was solely an increase that occurred for *Map2k7*$^{+/-}$ mice (genotype x session interaction: ($p<0.0001$, $F_{(1,152)}=13.61$)). Overall, there was a significant decrease in % Choice 1 following Manipulation 2 (effect of session: $p=0.001$, $F_{(1,152)}=14.60$); however, post hoc analyses indicated that the significant decrease only occurred with WT mice, whereas *Map2k7*$^{+/-}$ mice % Choice 1 remained the same (genotype x session interaction: $p=0.001$; $F_{(1,152)}=12.28$; **Fig. 6.13a**). Instead, *Map2k7*$^{+/-}$ mice decreased their preference for Choice 2, with WT mice % Choice 2 staying the same (genotype x session interaction: $p<0.0001$; $F_{(1,152)}=19.47$; **Fig. 6.13b**). The sacrifice of % Choices 1 and 2 for Choices 3 and 4 were different between the distinct groups of mice: WT mice made less % Choice 1 for more % Choice 2 and 3, and *Map2k7*$^{+/-}$ mice made less % Choice 2 for more % Choice 4 and 3.

At stable performance following Manipulation 2 (comparison of the last 5 days), *Map2k7*$^{+/-}$ mice made more % Choice 1 ($p<0.0001$, $F_{(1,80)}=79.39$) and % Choice 4 ($p=0.039$, $F_{(1,80)}=4.38$), less % Choice 2 ($p<0.0001$, $F_{(1,80)}=61.05$) and similar % Choice 3 ($p=0.771$, ns) compared to WTs (**Fig. 6.13**). Interestingly, in contrast to what occurred at Baseline, *Map2k7*$^{+/-}$ mice made more disadvantageous choices than WTs.

6.4.4.2 Other performance and motivation parameters

The last 3 days of Manipulation 1 were compared with the first 5 days of Manipulation 2 in order to see what effect this had on other performance parameters. % omissions, number of choices made, choice latency, magazine entries during the ITI, number of perseverative responses and % premature responses were all unaltered overall following Manipulation 2 compared to Manipulation 1 (**Fig. 6.14** and **6.15;** $p=0.573, 0.586, 0.237, 0.063, 0.007, 0.020$, respectively; post hoc analysis confirmed that of the significant *p*-values, there was no significance between any of the sessions separately). However, total beam breaks and reward collection latency significantly increased following Manipulation 2 ($p<0.0001$ for both, $F_{(7,140)}=7.64$ and 12.34, respectively; **Fig. 6.15a, d**) and total beam breaks were elevated for the rest of the experiment (see line graph, **Fig. 6.15a**). Manipulation 2 had a particularly large effect on reward collection latency of both mice initially (WT mice went from **3.79** ± 0.62 seconds at Manipulation 1 to **14.5** ± 3.35 seconds, and *Map2k7*$^{+/-}$ mice went from **2.85** ± 0.76 seconds to **27.23** ± 6.68 seconds), but *Map2k7*$^{+/-}$ mice were

significantly affected throughout (see line graph, **Fig. 6.15d)**. Although the genotype x session interaction term only approached significance (p=0.072, $F_{(7,140)}$=1.91), post hoc analyses showed that *Map2k7*$^{+/-}$ mice had a slower reward collection latency on each of the 5 days following Manipulation 2 than WTs.

When comparing stable performance at the end of Manipulation 1 (last 3 days, grouped) with the stable performance at the end of Manipulation 2 (last 5 days, grouped), the number of choices made (p<0.0001, $F_{(1,152)}$=19.43), number of beam breaks (p=0.002, $F_{(1,152)}$=9.48), number of magazine entries during the ITI (p<0.0001, $F_{(1,152)}$=13.72) and reward collection latency (p<0.0001, $F_{(1,152)}$=13.85) increased; % premature responses (p=0.752, ns) remained the same, and % omissions (p=0.058, $F_{(1,152)}$=3.66), latency to make a choice (p=0.005, $F_{(1,152)}$=8.24 and number of perseverative responses (p<0.0001, $F_{(1,152)}$=48.33) were decreased overall (**Figs. 6.14, 6.15**). Additionally, once mice had reached stable choice responding following Manipulation 2, significant differences between genotypes were altered compared to stable performance following Manipulation 1 in some performance parameters. Where *Map2k7*$^{+/-}$ mice made fewer % omissions compared to WTs previously, % omissions were now similar following Manipulation 2 (no effect of genotype: p=0.216, ns). *Map2k7*$^{+/-}$ mice made significantly less perseverative responses and more total choices made following Manipulation 1, but were both similar between genotypes following Manipulation 2 (effect of genotype overall: p<0.0001 for both, $F_{(1,152)}$= 19.96 and 12.90, respectively; genotype x session interaction: p<0.0001 for both, $F_{(1,152)}$=14.92 and 13.45, respectively). The number of magazine entries during the ITI period increased overall, however, post hoc analysis showed that this increase occurred for WTs but not *Map2k7*$^{+/-}$ mice (effect of genotype overall: p=0.001, $F_{(1,152)}$=11.19; genotype x session interaction: p=0.004, $F_{(1,152)}$=8.75). Some performance parameters did not alter the significance between genotypes, however: the number of beam breaks remained elevated in *Map2k7*$^{+/-}$ mice (effect of genotype: p<0.0001, $F_{(1,152)}$=76.33; no genotype x session interaction: p=0.876, ns). The latency to collect reward was elevated overall in Manipulation 2 compared to Manipulation 1, but remained similar between WT and *Map2k7*$^{+/-}$ mice (no effect of genotype: p=0.757, ns; no genotype x session interaction: p=0.315, ns). % premature responses were once again elevated in *Map2k7*$^{+/-}$ mice (effect of genotype: p=0.006, $F_{(1,152)}$=7.76). Finally, WTs were still slower to make a choice

than *Map2k7*$^{+/-}$ mice throughout stable responding following Manipulation 2 (effect of genotype: p=0.001, $F_{(1,152)}$=12.16; no genotype x session interaction: p=0.119, ns).

Overall, all mice appeared to show high motivation throughout Manipulation 2. Aside from the increase in reward collection latency, all motivation and other performance parameters changed or remained the same in a way that would, if anything, indicate increased drive to perform (the number of choices made, magazine entries and beam breaks increased, the % premature responses remained the same and % omissions, number of perseverative responses and latency to make a choice were decreased). With the exception of the increase in reward collection latency (considered in **Section 6.5**), the choice response behaviour is not confounded by performance in other aspects, including motivational aspects, of the task. There were some genotype differences that occurred: perseverative responses and number of choices made were different between genotypes following Manipulation 1, but this significant effect had disappeared by Manipulation 2, and the number of magazine entries throughout the ITI was increased for WT mice but not *Map2k7*$^{+/-}$ mice following Manipulation 2.

At stable performance following Manipulation 2 (comparison of the last 5 days), *Map2k7*$^{+/-}$ mice made more % premature responses (p=0.001, $F_{(1.80)}$=12.98), and beam breaks (p<0.0001, $F_{(1,80)}$=53.60), less magazine entries during the ITI (p<0.0001, $F_{(1,80)}$=31.30), were quicker to make choices (p=0.023, $F_{(1,80)}$=5.37) compared to WT mice. *Map2k7*$^{+/-}$ and WT mice performed similarly for % omissions (p=0.754, ns), perseverative responses (p=0.328, ns), number of choices per session (p=0.928, ns) and reward collection latency (p=0.555, ns) (**Figs. 6.14** and **6.15**).

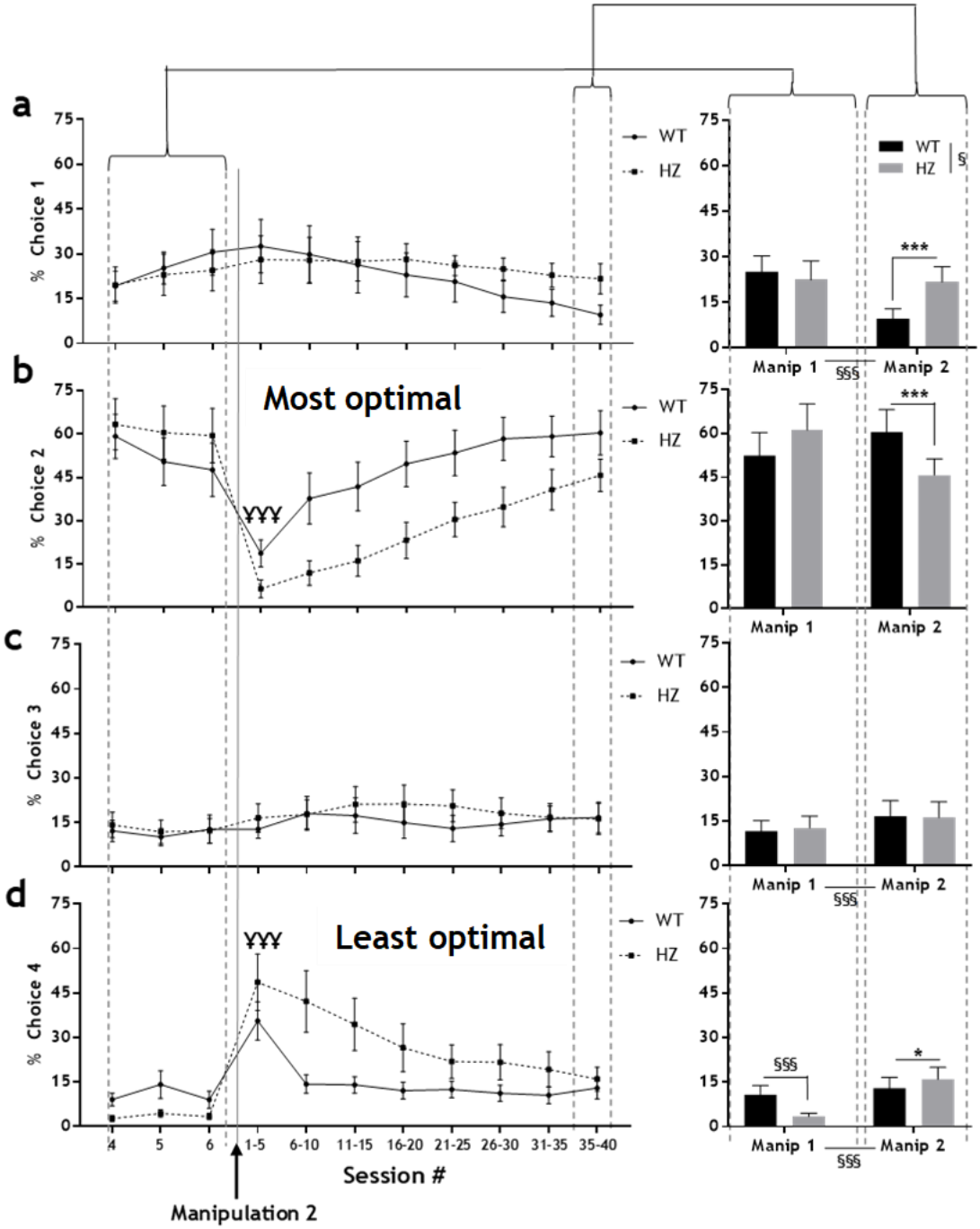

Figure 6.13. rGT performance was affected by Manipulation 2, in particular for *Map2k7*$^{+/-}$ mice, and all mice changed their pattern of choice responses when stable performance was once again achieved. Significant differences from the analysis of the last 3 days' Manipulation 1 and last 5 days' Manipulation 2 are indicated by §. Significant differences from the analysis of the last 3 days Manipulation 1, plus first 5 days of Manipulation 2 are indicated by ¥. Significant differences from the analysis of the last 5 days Manipulation 2 are indicated by *. Comparing the first 5 days of Manipulation 2 to the last 3 days' stable responding of Manipulation 1 revealed a significant decrease for Choice 2 (**b**) and increase for Choice 4 (**d**). There was no significant change for Choices 1 (**a**) and 3 (**c**). This was analysed by a three-way repeated measures ANOVA with session (last 3 days Manipulation 1, plus first 5 days of Manipulation 2) as a within subjects factor, genotype and sex as a between subjects

factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by ¥¥¥p<0.0001 (ANOVA). Analysing the last 3 days of Manipulation 1 compared with the final 5 days of Manipulation 2 showed that *Map2k7*$^{+/-}$ and WT mice had altered their stable choice pattern compared to before: WT mice made less % Choice 1 (**a**) and more % Choice 2 (**b**) and 3 (**c**), and *Map2k7*$^{+/-}$ mice made less % Choice 2 (**b**) but more % Choice 4 (**d**) and 3 (**c**) than before. This was analysed by a three-way repeated measures ANOVA with session (last 3 days' Manipulation 1 and last 5 days' Manipulation 2, both grouped) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by §p<0.05, §§§p<0.0001 (ANOVA/Tukey's). At stable performance following Manipulation 2 (comparison of the last 5 days), *Map2k7*$^{+/-}$ mice made more % Choice 1 (**a**) and % Choice 4 (**d**), less % Choice 2 (**b**) and similar % Choice 3 (**c**) compared to WTs. This was analysed by a three-way repeated measures ANOVA with session (last 5 days Manipulation 2) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by *p<0.05, ***p<0.0001 (ANOVA). Line graphs show data for each session (numbered from beginning of last 3 days' Manipulation 1 stable performance). Data points represent the mean; error bars represent the SEM. Tukey's post hoc analyses were carried out throughout where appropriate. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

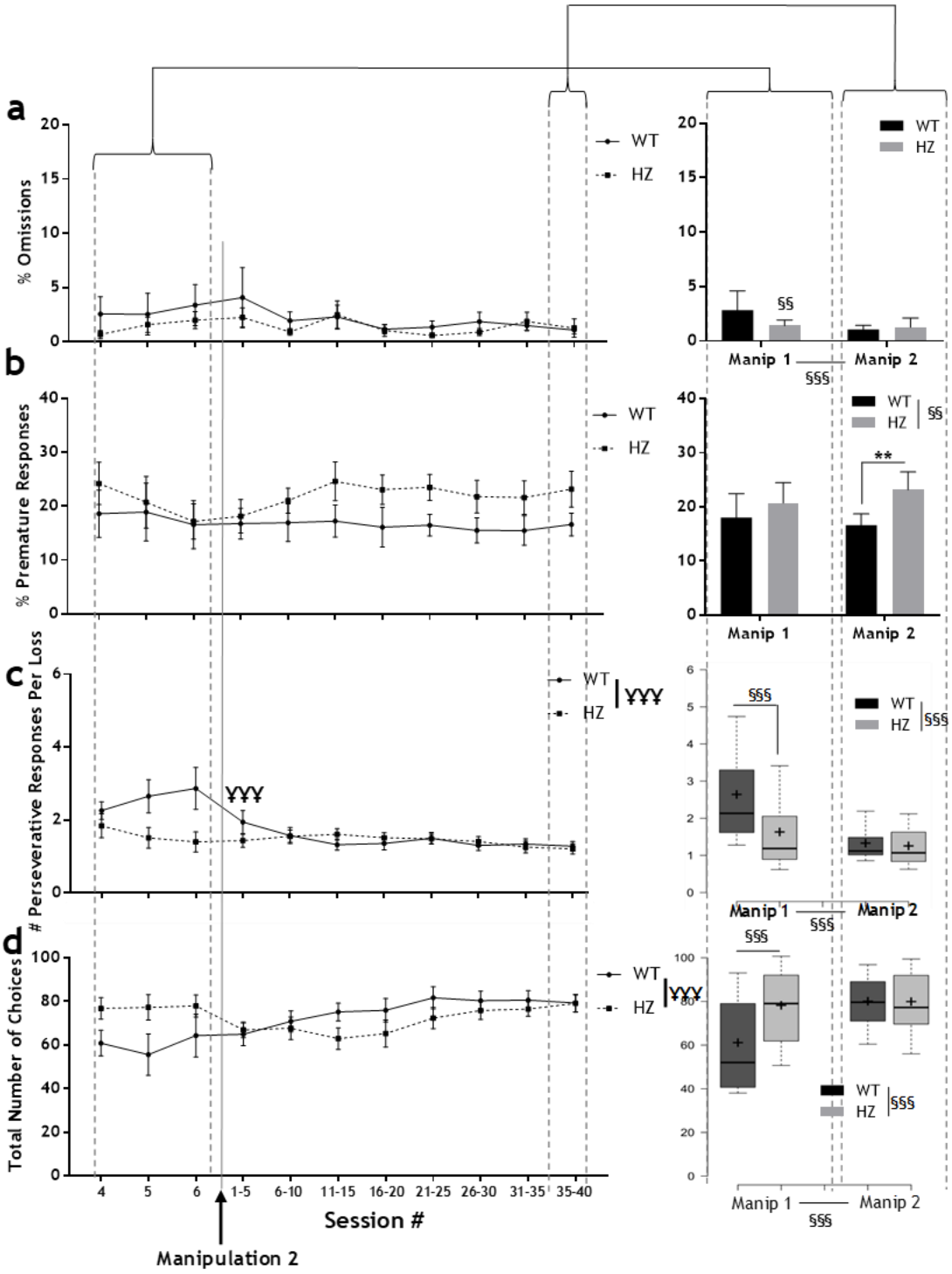

Figure 6.14. Manipulation 2 had an effect on other performance parameters in the rGT. Significant differences from the analysis of the last 3 days' Manipulation 1 and last 5 days' Manipulation 2 are indicated by §. Significant differences from the analysis of the last 3 days Manipulation 1, plus first 5 days of Manipulation 2 are indicated by ¥. Significant differences from the analysis of the last 5 days Manipulation 2 are indicated by *. Analysis of the last 3 days Manipulation 1 with the first 5 of Manipulation 2 showed % omissions (**a**), % premature responses (**b**) and total number of choices (**d**) were unaltered, whereas the number of perseverative responses were decreased (**c**) during Manipulation 2. This was analysed by a three-way repeated

measures ANOVA with session (last 3 days Manipulation 1, plus first 5 days of Manipulation 2) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by ¥¥¥p<0.001 (ANOVA/Tukey's). Analysing the last 3 days of Manipulation 1 compared with the final 5 days of Manipulation 2 showed that % omissions decreased overall (**a**); premature responses remained the same (**b**), perseverative responses decreased overall (**c**) and total number of choices increased overall (**d**). This was analysed by a three-way repeated measures ANOVA with session (last 3 days' Manipulation 1 and last 5 days' Manipulation 2, both grouped) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by §§p<0.01, §§§p<0.0001 (ANOVA/Tukey's). At stable performance following Manipulation 2 (comparison of the last 5 days), *Map2k7*$^{+/-}$ mice made similar % omissions (**a**), more % premature responses (**b**), and a similar number of perseverative responses (**c**) and choices (**d**) compared to WT mice. This was analysed by a three-way repeated measures ANOVA with session (last 5 days Manipulation 2) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by **p<0.01 (ANOVA). Line graphs show data for each session (numbered from beginning of 3 days' stable Manipulation 1 performance). Data points represent the mean; error bars represent the SEM. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Tukey's post hoc analyses were carried out throughout where appropriate. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

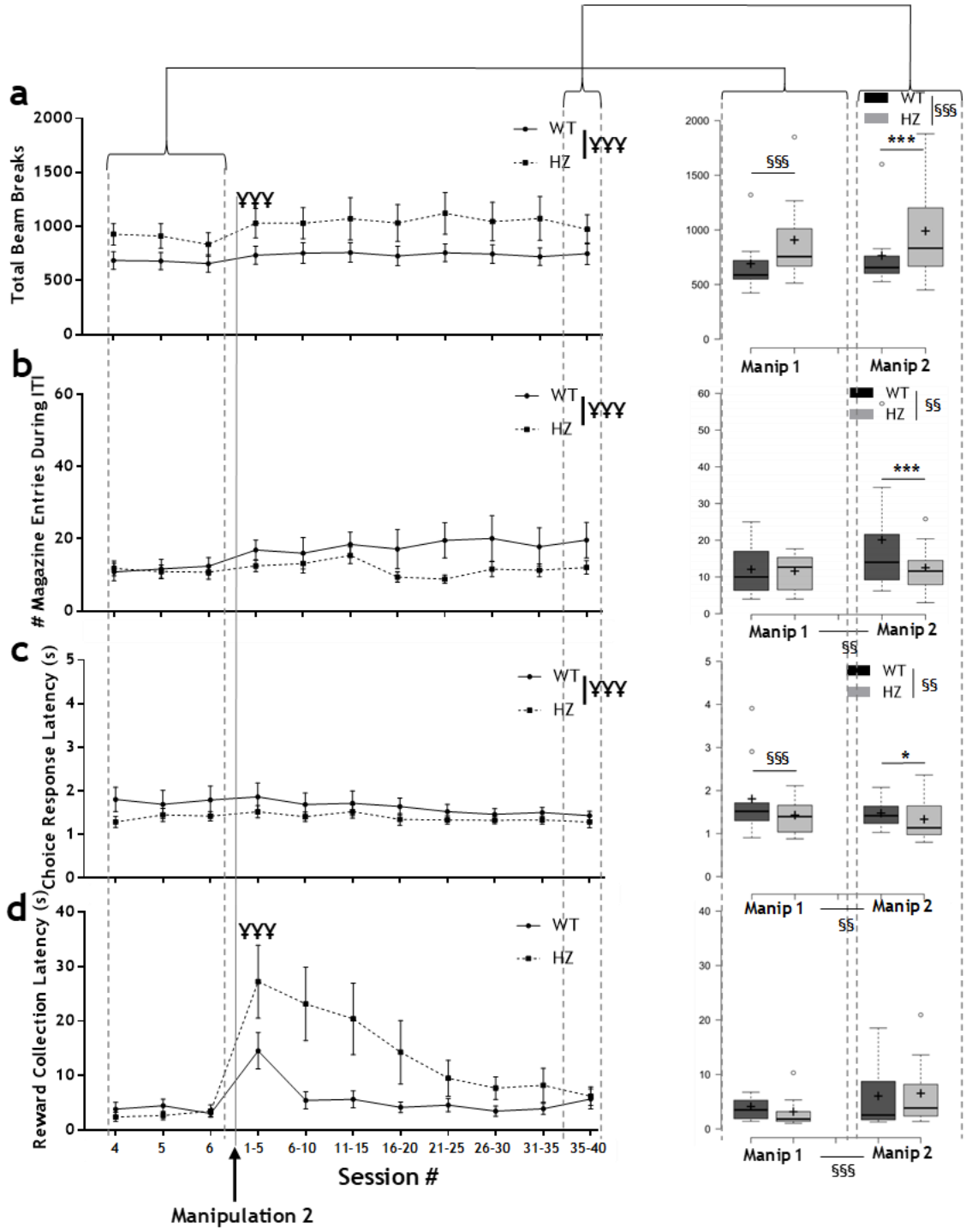

Figure 6.15. Manipulation 2 had a slight effect on motivation parameters in the rGT. Significant differences from the analysis of the last 3 days' Manipulation 1 and last 5 days' Manipulation 2 are indicated by §. Significant differences from the analysis of the last 3 days Manipulation 1, plus first 5 days of Manipulation 2 are indicated by ¥. Significant differences from the analysis of the last 5 days Manipulation 2 are indicated by *. Analysis of the last 3 days Manipulation 1 with the first 5 of Manipulation 2 showed an increase in total beam breaks (**a**) and reward collection latency (**d**), whereas the number of magazine entries during the ITI (**b**) and choice response latency (**c**) were unaltered during Manipulation 2. This was analysed by a three-way repeated measures ANOVA with session (last 3 days Manipulation 1, plus first 5 days of Manipulation 2) as a within subjects factor, genotype and sex as a

between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by ¥¥¥p<0.001 (ANOVA/Tukey's). Analysing the last 3 days of Manipulation 1 compared with the final 5 days of Manipulation 2 showed total beam breaks (**a**) were unaltered, the number of magazine entries (**b**) and reward collection latency (**d**) were increased, and choice response latency (**c**) was decreased. This was analysed by a three-way repeated measures ANOVA with session (last 3 days' Manipulation 1 and last 5 days' Manipulation 2, both grouped) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Lines linking groups represent significant differences between those groups. Significant differences from this analysis are indicated by §§p<0.01, §§§p<0.0001 (ANOVA/Tukey's). At stable performance following Manipulation 2 (comparison of the last 5 days), *Map2k7*$^{+/-}$ mice made an increased number of beam breaks (**a**), less magazine entries (**b**) and choice response latency (**c**), and an increase in reward collection latency (**d**) compared to WT mice. This was analysed by a three-way repeated measures ANOVA with session (last 5 days' Manipulation 2) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Significant differences from this analysis are indicated by *p<0.05, **p<0.01 (ANOVA). Line graphs show data for each session (numbered from beginning of 3 days' stable Manipulation 1 performance). Data points represent the mean; error bars represent the SEM. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Tukey's post hoc analyses were carried out throughout where appropriate. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

6.4.5 Amphetamine administration

6.4.5.1 Choice Performance

Risk-taking behaviour in the rGT was analysed following administration of 1.5mg/kg D-amphetamine, a dose that has previously been shown to alter the cognitive but not locomotor aspects of the task carried out in the 5-hole operant chamber (van Enkhuizen *et al.*, 2013). Amphetamine administration was carried out over 4 days: half of each genotype group were given D-amphetamine and the other half were given saline intraperitoneally on the first day. On the second and fourth days, mice were tested normally on the rGT without drug in order to verify that any effects had worn off (recovery); on the third day, saline or D-amphetamine was administered to the other half of the group. Comparing performance of animals who had received saline with animals who had received an acute dose of 1.5mg/kg amphetamine showed that overall, mice showed increased preference for Choice 1 ($p<0.006$, $F_{(1,19)}=9.59$; **Fig. 6.16a**). This increase in % Choice 1 appeared to be more evident in WT mice. Although there was no significant genotype effect overall ($p=0.893$, ns), nor was there a genotype x treatment interaction ($p=0.199$, ns), post hoc analyses revealed that there was a significant increase in % Choice 1 for WTs but not for *Map2k7*$^{+/-}$ mice. The increase in preference for Choice 1 was accompanied by a slight decrease in response for Choice 2 (**Fig. 6.16b**), although this was not significant ($p=0.172$, ns). % response for Choices 3 and 4 remained the same following amphetamine administration ($p=0.207$ and 0.114, respectively; **Fig. 6.16c** and **d**); however, *Map2k7*$^{+/-}$ mice made less % Choice 3 than WTs overall ($p=0.049$, $F_{(1,19)}=4.41$).

Overall, mice subtly switched their choice preference after receiving amphetamine to choices that were the least risky (i.e., Choice 1), with a trend towards the effect being stronger in WT than *Map2k7*$^{+/-}$ mice.

6.4.5.2 Other performance and motivation parameters

Following an acute dose of 1.5mg/kg D-amphetamine, % omissions were increased ($p=0.026$, $F_{(1,19)}=5.83$; **Fig. 6.16e**), % premature responses were decreased ($p=0.050$, $F_{(1,19)}=4.37$; **Fig. 6.16f**), and *Map2k7*$^{+/-}$ mice continued to make more premature responses than WT mice ($p=0.003$, $F_{(1,19)}=11.90$). The number of perseverative responses per loss decreased to a small extent ($p=0.018$, $F_{(1,19)}=6.77$; **Fig. 6.17a**), and total number of choices ($p=0.119$, ns) and reward collection

latency (p=0.748, ns) remained similar, which implies the dose of amphetamine was not so high that it prevented motivation to carry out the task. Interestingly, total beam breaks were actually decreased (p=0.044, $F_{(1,19)}$=4.65; **Fig. 6.17c**), but the significant hyperactivity of *Map2k7*$^{+/-}$ mice compared to WTs remained (p<0.0001, $F_{(1,19)}$=24.04), showing, again, that the dose of amphetamine was not too high, as hyperlocomotor side effects were not seen (Anisman & Kokkinidis, 1975; Chen *et al.*, 2007; Wise & Bozarth, 1987). Finally, the latency to make a choice increased overall (p<0.0001, $F_{(1,19)}$=35.06), and the number of magazine entries during the ITI decreased overall (p=0.44, $F_{(1,19)}$=4.65); however, post hoc analyses revealed that both the latency increase and the decrease in magazine entries were only significant for WT mice (genotype x treatment interaction: p=0.039, $F_{(1,19)}$=4.91 and p=0.056, $F_{(1,19)}$=4.16, respectively; **Fig. 6.17e** and **d**).

Overall, mice were affected by amphetamine in other performance parameters, some of which are consistent with previous studies (Van Enkhuizen *et al.*, 2013; increase in % omissions, decrease in perseverative responses, similar reward collection latencies), but some changes were not consistent with previous studies (% premature responses were decreased, choices made were not decreased). However, the dose of amphetamine used was effective in altering the cognitive aspects of the task (i.e. the choice preferences) without introducing confounding factors, such as an increase in locomotor activity or a reduction in motivation. Finally, there is a trend for WT mice to have increased reactions to amphetamine relative to *Map2k7*$^{+/-}$ mice in some parameters (magazine entries during the ITI and choice response latency).

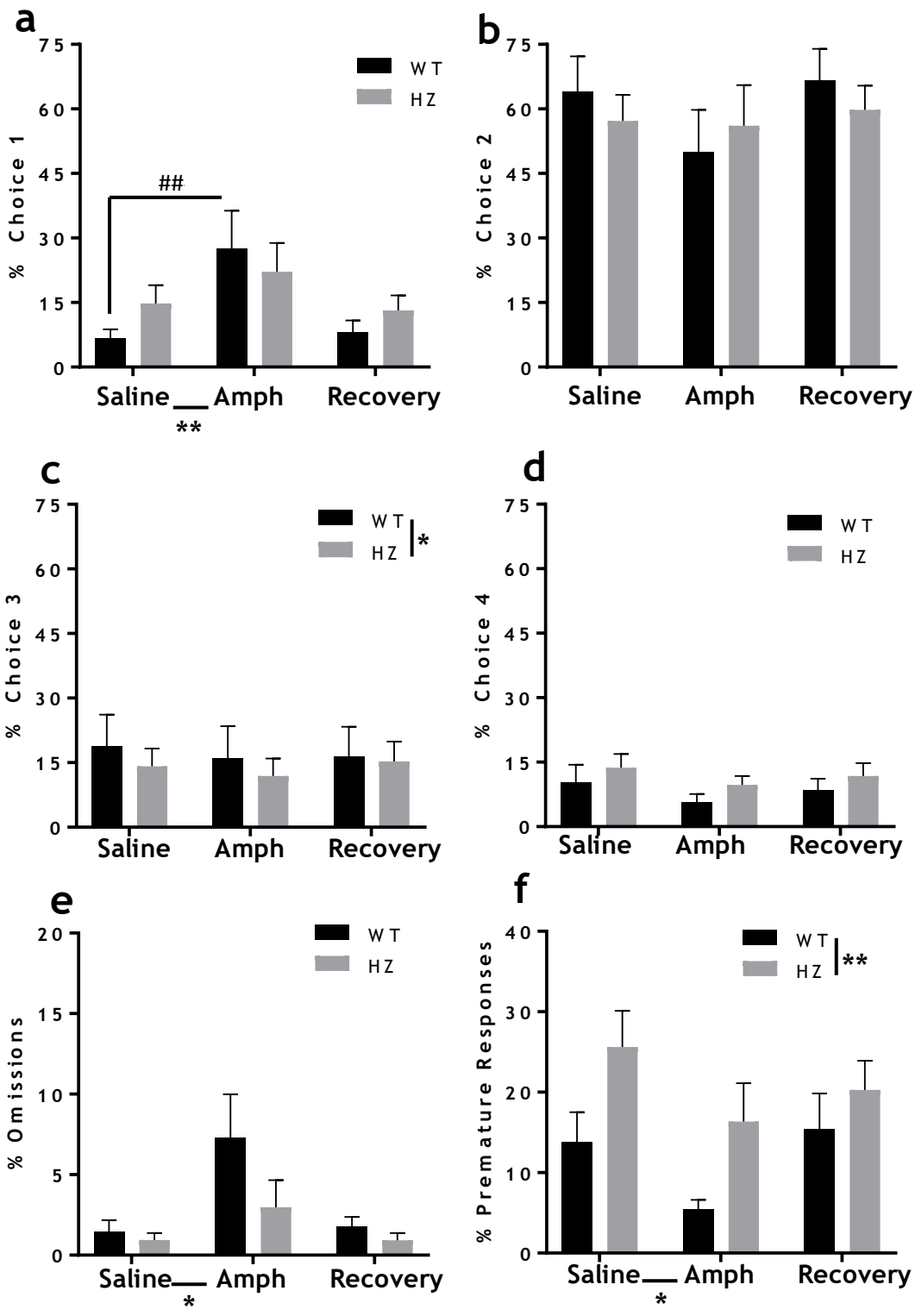

Figure 6.16. An acute dose of amphetamine modestly altered choice preference and other performance parameters in the rGT. Following 1.5mg/kg amphetamine administration, preference for Choice 1 was increased (**a**), but Choice 2 (**b**), 3 (**c**), and 4 (**d**), were not significantly altered. % omissions were increased (**e**) and premature responses decreased (**f**) for all mice. Data represent the mean; error bars represent the SEM. Data was analysed by a three-way repeated measures ANOVA with treatment (saline or amphetamine) as a within subjects factor, genotype and sex as a between

subjects factor and each individual mouse nested within genotype and sex. Tukey's post hoc analyses were carried out where appropriate. Lines linking groups represent significant differences between those groups: *p<0.05, **p<0.01 (ANOVA); ##p<0.01 (Tukey's). Recovery is included on graph but not in analysis. Three-way ANOVA (with treatment (saline, amphetamine, recovery) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex) were carried out on all the data before proceeding with comparison between saline and amphetamine in order to establish that proper recovery had occurred. According to Tukey's post hoc analyses, there were no significant differences between saline treatment and recovery in any of the parameters. Therefore, the data from the saline group had no detectable carry-over effects from amphetamine on the second day of drug administration so saline was then only compared with amphetamine. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

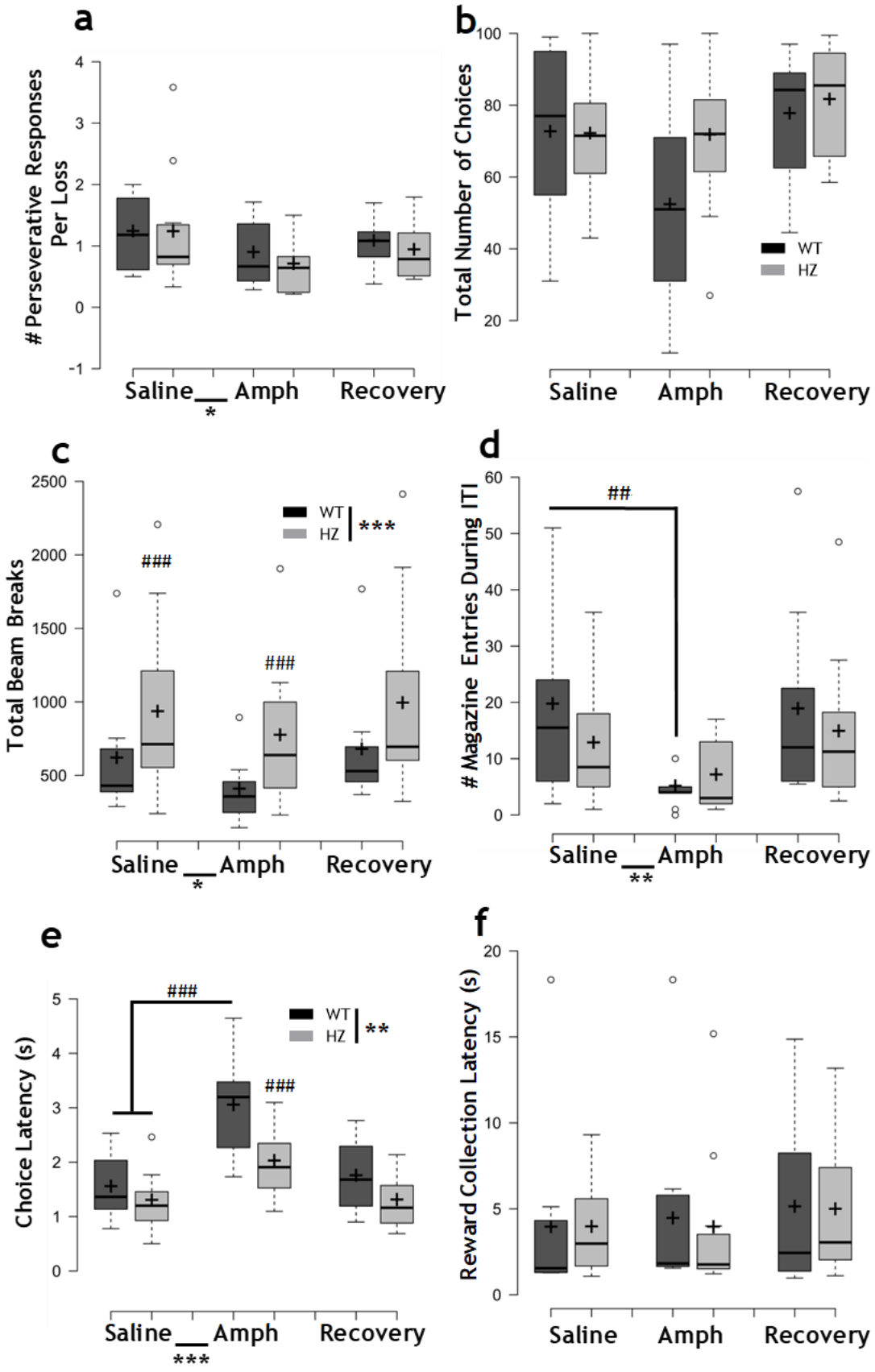

Figure 6.17. An acute dose of amphetamine altered other performance / motivational parameters in the rGT. Following 1.5mg/kg amphetamine administration, perseverative responses (**a**), total number of beam breaks (**c**) and

magazine entries during the ITI (**d**) decreased, whereas choice latency (**e**) increased and total number of choices (**b**) and reward collection latency (**f**) remained the same. WT mice were particularly affected by amphetamine for choice latency (**e**) and magazine entries during the ITI (**f**). Data was analysed by a three-way repeated measures ANOVA with treatment (saline or amphetamine) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex. Box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles; crosses represent sample means. Tukey's post hoc analyses were carried out throughout where appropriate. Lines linking groups represent significant differences between those groups: **p<0.01, ***p<0.0001 (ANOVA, relative to corresponding WT group where not specifically indicated); ##p<0.01, ###p<0.001 (Tukey's, relative to corresponding WT group where not specifically indicated). Recovery is included on graph but not in analysis. Three-way ANOVA (with treatment (saline, amphetamine, recovery) as a within subjects factor, genotype and sex as a between subjects factor and each individual mouse nested within genotype and sex) were carried out on all the data before proceeding with comparison between saline and amphetamine in order to establish that proper recovery had occurred. According to Tukey's post hoc analyses, there were no significant differences between saline treatment and recovery in any of the parameters. Therefore, the data from the saline group had no detectable carry-over effects from amphetamine on the second day of drug administration so saline was then only compared with amphetamine. *Map2k7*$^{+/-}$: n=12 (6M, 6F), WT: n= 10 (5M, 5F).

6.5 Discussion

In this chapter it is shown that mice are able to learn the different contingencies of reward and punishment in the touchscreen rGT and that they exhibited a consistent pattern of optimal responding: they avoided high-risk high-reward options more than the advantageous options of frequent, small rewards. Importantly, they chose the most advantageous option the majority of the time and the most disadvantageous option the least. To the best of our knowledge, similar versions of the rGT have been carried out using mice (for example, van Enkhuizen *et al.*, 2013) and rats (for example, Zeeb *et al.*, 2009) in the 9-hole operant box, but have not been published using mice in the touchscreen before, so this result validates the touchscreen equipment for use with this task. When an element of reversal learning was introduced to the task by switching subtly different contingencies (Manipulation 1), mice can notice, track the change and alter their pattern of responding accordingly, further validating the rGT as a reliable, translational task. We have also shown that mice take a longer time to switch responding when the punishment length is made less extreme. *Map2k7*$^{+/-}$ mice are particularly sensitive to this alteration and take significantly longer than WTs to alter their response, possibly reflecting a deficit in cognitive flexibility, or altered reward/punishment valuation based on prior knowledge, a trait seen in patients with schizophrenia (Pantelis *et al.*, 1999). Finally, administration of amphetamine altered choice preference in the rGT by increasing % responding for Choice 1 (the least risky). Administration of amphetamine also altered other task performance measurements (% omissions, % premature responses, perseverative responses, magazine entries during the ITI, total beam breaks and choice response latency), and also caused a modest differential effect between *Map2k7*$^{+/-}$ and WT mice with respect to choice preference.

6.5.1 Learning the rGT

As part of training, the mice were required to touch a square on a pseudorandom part of the screen at every trial for a strawberry milkshake reward. For this section of training, mice were moved onto the next sub-stage when they reached criteria for that stage (defined as >80% accuracy, i.e. touching the lit square, and <20% omissions) for two consecutive sessions. *Map2k7*$^{+/-}$ mice progressed through this part of training more quickly than WT mice by taking fewer sessions and trials on average.

Following initial habituation and training, mice were subjected to four sessions of "Forced Choice" where a single option appeared at each trial and mice were forced to pick it. This was to ensure they learned that each square was associated with a different reward/punishment contingency, and also so they were given equal exposure to each of the contingencies, and is similar to verbal instruction to participants in the IGT that some decks are more advantageous than others). Then, the mice were subjected to "Free Choice", where they could pick whichever square they liked; all mice reached Baseline performance (stable choice preference) of the Free Choice rGT over the course of 13 sessions. Once stable responding was established, *Map2k7*$^{+/-}$ mice showed slightly enhanced ability to perform the task: they had a significant increase in preference for Choice 2 (the most advantageous option) and a decrease in preference for Choice 4 (the least advantageous option), compared to WT mice. Although patients with schizophrenia do not appear to show better performance in the IGT, they have frequently been seen to perform as well as healthy controls (Evans *et al.*, 2005; Rodríguez-Sánchez *et al.*, 2005). As the significant difference in the current experiment was very modest (difference of +4.08 ± 0.14 responses for Choice 2 and -2.89 ± 0.64% responses for Choice 4), it is likely not a cause for concern regarding the validity of any further findings, or large enough to suggest that they are profoundly "less risky" than WT mice. On the whole, all mice showed a choice preference remarkably in line with the total possible amounts of strawberry milkshake available. Choice 1 (295 possible rewards) was picked 25.5 ± 4.1% of the time, Choice 2 (411 possible rewards) was picked 56.9 ± 5.9% of the time, Choice 3 (135 possible rewards) was picked 11.1 ± 2.3% of the time and Choice 4 (99 possible rewards) was picked 6.2 ± 1.1% of the time. This is consistent with the choice preferences of pair-housed mice (the same set up as in this experiment) in Zeeb *et al.* (2013), the study that the reward/punishment contingencies for the current experiment were based on.

6.5.2 Motivation Performance

Once performing at stable choice preference, there were some aspects of general behaviour whilst carrying out the task that were different between *Map2k7*$^{+/-}$ and WT mice. There are infra-red beams that cross the front (by the screen) and back (by the reward magazine) of the touchscreen arena. In total, *Map2k7*$^{+/-}$ mice made more beam breaks per session than WT mice, suggesting they were hyperactive

(at least in the horizontal plane) throughout the task. This hyperactivity in *Map2k7*$^{+/-}$ mice has been demonstrated throughout other operant experiments, too (for example, in the open field (**Chapter 4**), and in other operant-based experiments (the 5-CSRTT in **Chapter 5**, PAL and PD **(**data not shown), also see **Discussion Section 8.3.1**). Additionally, *Map2k7*$^{+/-}$ mice collected the reward just as quickly as WT mice and were quicker to make a correct response. They also made more choices per session, which may partly reflect their slight hyperactivity (as shown by increased beam breaks across the session), but also suggests they were highly motivated to perform the task. However, *Map2k7*$^{+/-}$ mice made slightly less entries into the magazine throughout the ITI period and less perseverative responses per loss (which is different to what was seen in the 5-CSRTT in **Chapter 5**, discussed in **Section 8.3.1)**, suggesting either that they had less interest in the reward than WT mice, or that they were more aware of the demands of the task and therefore made less unnecessary movements than WT mice. The fact that *Map2k7*$^{+/-}$ mice collected the reward just as quickly as WT mice and were quicker to make a correct response suggests that it may be due to the latter.

6.5.3 Performance Following Manipulation 1

Manipulation 1 consisted of switching the two more subtly different options in the task – Choice 1 with Choice 3. Whilst Choice 1 was an advantageous option and Choice 3 disadvantageous, neither were the "best" or "worst" options in the amount of reward available per unit time. % Choice for 1 and 3 was reversed, as is expected, and mice responded quickly, reaching stable performance and altering their responding accordingly (i.e. switching % Choice 1 for 3 and vice versa) once again after six sessions. Both *Map2k7*$^{+/-}$ and WT mice learned the switch to the same extent, with *Map2k7*$^{+/-}$ mice still performing slightly but significantly better once stable performance had been re-attained (less % Choice 4 and more % Choice 2). This ability of mice to notice a subtle change in reward-punishment contingencies is quite remarkable and informs us that the task is being carried out by the mice exactly how it was intended, highlighting the tasks' validity: the mice are clearly able to evaluate small differences in either the amount of reward they are receiving, the probability that they get rewarded/punished, the extent of the punishment when it occurs, or a

combination of all three, and then alter behaviour to maintain optimal performance.

6.5.4 Performance Following Manipulation 2

Manipulation 2 consisted of switching the location of Choices 2 and 4 (the most and least optimal options), as well as decreasing the punishment severity of Choices 3 and 4 so that punishment length increased linearly with reward amounts rather than exponentially (**Fig. 6.4, Table 6.1**). Again, in the sessions immediately following Manipulation 2, mice completely swapped their % Choices of the two that had been switched. WT mice were responding 54.64 ± 9.37% to Choice 2 before, then 9.97 ± 2.39% after the manipulation and *Map2k7*$^{+/-}$ mice were responding 58.72 ± 8.52% to Choice 2 before and 5.70 ± 1.85% after. For Choice 4, WT mice were responding 7.77 ± 1.92% before and 49.21 ± 8.72% afterwards; *Map2k7*$^{+/-}$ mice were responding 4.89 ± 1.29% before and 55.4 ± 9.05% immediately afterwards. All mice took considerably longer to reach stable choice preference following this switch compared with Manipulation 1, and *Map2k7*$^{+/-}$ mice took significantly longer than WTs to do this (*Map2k7*$^{+/-}$ mice took 30.25 ± 4.17 sessions compared to WTs, who took 12.1 ± 4.32 sessions). The overall increase in the number of sessions until re-achieving a stable choice pattern is most likely due to the alteration of the punishment period, because it took the mice just 6 sessions following Manipulation 1, even when the switch here was more subtly different in the amount of reward available over time. By decreasing the punishment lengths for Choices 3 and 4, the difference between the most and least optimal options is much less, so that Choices 3 and 4 are less distinguishably "worse" and therefore mice took longer to notice and alter their preference. In fact, using the same calculations as before, the maximum number of rewards for each choice after Manipulation 2 is 295, 411, 216 and 168 for Choices 1, 2, 3 and 4, respectively, as opposed to 295, 411, 135 and 99. The amount of reward able to be achieved per unit time for the least optimal choices are now more similar to each other and would therefore make decisions more difficult. Additionally, if preference was based solely on either quantity of reward obtained per choice, or probability of reward, or both together, it could be expected that mice would switch responding to the same extent as in Manipulation 1, because the difference in the number of strawberry milkshake rewards obtained between the choices is 2 for both Manipulation 1 and 2, and the probability of reward remains the same for each

choice. It could even be expected that the mice would switch responding more rapidly than before because this is the second time they have encountered a switch, and Baseline performance showed more extreme differences in choice preference between 2 and 4 than 1 and 3, so they would come across the switch more frequently. In other words, because, at stable performance, they select Choice 2 most (about 55% of the time) and Choice 4 about 8% of the time, they would encounter the switch more frequently than with Manipulation 1, where they picked Choices 1 and 3 about 25 and 12%, respectively. However, the mice did not take the same amount of time, or less, to switch their responding. WT mice took, on average, ~5 extra days to learn Manipulation 2 than Manipulation 1 whereas *Map2k7*$^{+/-}$ mice took ~24 extra days. This shows that punishment length, or possible reward achieved over time (which is directly affected by punishment length) has a huge impact on their decision-making, perhaps even more so than quantity or immediate probability of reward, and that this effect is stronger in *Map2k7*$^{+/-}$ than WT mice.

Interestingly, even though mice were receiving negative feedback in the form of increased probability and length of punishment when selecting Choice 4 (compared to what they had previously learned as Choice 2), *Map2k7*$^{+/-}$ mice in particular continued to persevere with the now least advantageous option, and took significantly longer than WT mice to switch responding preference. They appeared to show over-reliance on the option that they had previously established to be most favourable and were more inflexible in their decision-making than WT mice. In fact, *Map2k7*$^{+/-}$ mice behaved in a very similar way to that seen in patients with schizophrenia in the IGT study by Turnbull *et al.* (2006) (see **Section 6.1**), who also altered task contingencies.

There are several explanations for why subjects may show impairment in cognitive flexibility. It may be a consequence of increased perseverative behaviour (Turnbull *et al.*, 2006), or reduced working memory capacity (and therefore the individual would have problems using the outcomes from past choice to direct future behaviour; (Dunn *et al.*, 2006). It could also be due to lack of attention, lack of motivation, or altered sensitivity to reward and punishing outcomes (Dunn *et al.*, 2006). Alternatively, it may be explained by habit-induced rigidity, in which the subject continues to behave in a way that is very well-consolidated and

therefore fails to notice negative reinforcement; (Paglieri *et al.*, 2014; Waltz, 2017).

These possible reasons for cognitive inflexibility are challenging to distinguish between behaviourally and are not likely to be mutually exclusive, nor the extent of the explanations. For example, it is inevitable that emotional factors and actively planning future actions are involved in the IGT, but it is hard to establish whether these processes occur in the rGT in mice as they do in humans. However, of these potential explanations, in the current study it is unlikely to be due to working memory as, although not directly studied in this thesis, *Map2k7*$^{+/-}$ mice have not shown deficits in working memory compared to their WT littermates when learning any tasks in the operant 9-hole chamber or touchscreen. It is possible that there are elements of *Map2k7*$^{+/-}$ mice's attentional deficit (**Chapter 5**) that have influenced inflexible behaviour in this task, but is unlikely to be the sole explanation because % omissions (which were significantly increased in the attentional task) are not increased here, and *Map2k7*$^{+/-}$ mice switched responding just as quickly as WT mice following Manipulation 1. Similarly, *Map2k7*$^{+/-}$ mice do not show a lack of motivation (following Manipulation 2, their % omissions and total number of choices are similar to WT mice; the number of beam breaks are increased, and they are faster to make a choice). They are, however, slower at collecting reward throughout the course of Manipulation 2. This is surprising, because all other performance measurements throughout Manipulation 2 suggest that *Map2k7*$^{+/-}$ mice are very motivated to carry out the task. Again, *Map2k7*$^{+/-}$ mice have not shown an increase in reward collection latency in other operant tasks (explored further in the main **Discussion**, **Section 8.3.1**). It appears that they have lost motivation for taking part in the task in the first place, which has perhaps surfaced a lack of motivation for *amount* of reward that was not observable before.

The other possible explanations for cognitive inflexibility mentioned (increased perseverative behaviour, altered sensitivity to reward or punishing outcomes and habit-induced rigidity) are challenging to distinguish between with the current data and will require further, more specific, behavioural study in order to decipher them. However, *Map2k7*$^{+/-}$ mice have not shown an increase in perseverative responding in the past, and perseverative responding as recorded in this task did not show that *Map2k7*$^{+/-}$ mice are impaired compared to WT mice (in

fact, *Map2k7*$^{+/-}$ mice show less perseverative responses than WT mice at Baseline). However, "perseveration" was recorded during the time-out following any loss, so it appears the perseverative responses recorded here are showing that *Map2k7*$^{+/-}$ mice are simply more aware of when they are not receiving optimal amounts of reward than WT mice, as opposed to continuing to try and gain optimal rewards despite this. It may also be that WT mice are quicker at this manipulation because the punishment period has less salience for them as they are more focussed on obtaining a reward, so when it is decreased it has less of an effect on their drive to switch. *Map2k7*$^{+/-}$ mice are showing signs of having decreased sensitivity to reward compared to WT mice.

Based on excluding working memory and perseveration as possible factors in explaining this, it is possible that *Map2k7*$^{+/-}$ mice are showing signs of habit-induced rigidity (Waltz, 2017), and altered sensitivity to punishing outcomes (Dunn *et al.*, 2006). This may be what is causing *Map2k7*$^{+/-}$ mice to be inflexible in their abilities. When punishments are severe, *Map2k7*$^{+/-}$ mice are capable of quickly learning the best option. However, when the punishment is less severe they find it challenging, as if they are driven primarily by severity of punishment. This is supported by the fact that their reward collection latencies significantly increased throughout this period whilst showing good motivation to carry out the task in other measurements and may reflect aberrant salience attribution by the *Map2k7*$^{+/-}$ mice, a feature which patients with schizophrenia exhibit (Howes & Nour, 2016).

In any case, it is interesting that *Map2k7*$^{+/-}$ mice did not display cognitive inflexibility when carrying out a task designed to measure exactly this: Pairwise Discrimination and Reversal (data not shown). An explanation for this may be that *Map2k7*$^{+/-}$ mice have a subtle deficit in cognitive flexibility and this deficit only appears when the cognitive load involved is high and the reversal is significantly more complex.

6.5.5 Manipulation 2 Stable Performance

Interestingly, when their performance was once again stable for five sessions following Manipulation 2, the choice preference of the two groups of mice settled differently to what previously occurred following Manipulation 1 (and at Baseline): both groups of mice increased their preference for Choice 3 and Choice 4; WT

mice decreased their preference for Choice 1, and *Map2k7*$^{+/-}$ mice instead decreased their preference for Choice 2. The increase in % Choices 3 and 4 is logical, because they are no longer as disadvantageous as they were previously because of their decreased punishment length. The alteration in preference for Choices 1 and 2 by WT and *Map2k7*$^{+/-}$ mice is presumably in sacrifice for the increase in Choices 3 and 4, however it is interesting that both groups picked different choices to sacrifice. In this instance, *Map2k7*$^{+/-}$ mice are performing 'worse', because they have sacrificed a more optimal choice in favour of the lesser optimal choice 3 and 4. The reason for this is unclear, but it may reflect differences in reward valuation based on their previous knowledge of reward/punishment contingencies (Glimcher *et al.*, 2013). As the contingencies have been altered for the second time, this would affect the way the mice view its reliability. For instance, with pairwise discrimination and reversal, also carried out in the touchscreen equipment, it is known that mice take longer to learn the first reversal than each reversal thereafter (Dickson *et al.*, 2013). It is possible that after the second time the contingencies have been switched, they have preference for different choices because the new reward/punishment valuation based on previous knowledge of contingency patterns is different for mice whose drive to learn appears to be more punishment driven (i.e. *Map2k7*$^{+/-}$ mice) than reward driven. As the only difference that occurred with Manipulation 2 (aside from the actual choice switch) compared to Manipulation 1 was that the punishment was made less extreme, it would be reasonable to assume that this is why the mice took so long to recover, and why they settled on different choices preferences.

6.5.6 Performance Following Amphetamine Challenge

Administration of an acute dose of amphetamine altered choice preference in the rGT by increasing % responding for Choice 1, particularly for WT mice, and altering other task performance measurements, such as % omissions, % premature responses, # perseverative responses, total beam breaks, magazine entries during the ITI and choice latency. For some measurements, there was an enhanced effect in WT mice compared with *Map2k7*$^{+/-}$ mice.

Amphetamine administration increases dopamine (DA) in the midbrain via various mechanisms (reviewed in Seiden *et al.*, 1993). At small doses such as the dose we used, amphetamine is known to increase DA in the synaptic cleft by potentiating

DA vesicular release and by causing the DA transporter (DAT) to work in reverse, transporting DA from the cytoplasm into the extracellular space (Daberkow *et al.*, 2013). Patients with schizophrenia show an enhanced release of DA in response to amphetamine administration (Laruelle *et al.*, 1996). We therefore hypothesised that amphetamine administration in *Map2k7*$^{+/-}$ mice may have a differential, enhanced effect compared to WTs. However, we did not see this, and in some circumstances, saw the opposite, suggesting that dopaminergic transmission, at least in the brain areas required for carrying out this task, may partly interact with the MKK7/JNK pathway.

Amphetamine significantly increased responding for Choice 1, with signs of this increase being more pronounced in WT mice compared to *Map2k7*$^{+/-}$ mice. With the exception of one study (Young *et al.*, 2011), in the published literature, this increase in Choice 1 was always observed in the rGT following amphetamine administration (Baarendse & Winstanley, 2013; van Enkhuizen *et al.*, 2013; Silveira *et al.*, 2015; Zeeb *et al.*, 2009). An accompanying effect often seen in those studies mentioned just prior (which was not significant here) is a decrease in responding for Choice 2 such that responding for Choice 2 was switched from responding for Choice 1. This decrease in preference for Choice 2 was not quite significant in the current study ($p=0.172$), however future power analyses will enable us to decide if this is an effect that is worth pursuing further. It is possible that mice had actually switched their responding from Choice 2 to Choice 1, but the stable % Choice 2 was higher to begin with than % Choice 1, so the increase in Choice 1 is significant and the decrease in Choice 2 is insignificant because the proportion of responses changed are less. On the whole, this alteration in risk choice profile similar to previous studies carried out in rats in the 9-hole operant box further establishes the touchscreen rGT as having good cross-species, and also cross–equipment, translational validity.

The reasons behind the shift in preference for less risky options is not clear. Zeeb *et al.* (2009) suggest that amphetamine induces a state of over-weighting the severity of punishment so the mice perceive the punishment as worse than previously. The authors suggest that amphetamine increases the activity of the internal pacemaker, causing the punishment period lengths to be perceived as longer than they are and therefore biasing preference towards Choice 1 (Zeeb *et al.*, 2009). If this is true, this suggests that animals under the influence of

amphetamine are affected by punishment length /probability over reward amount (Zeeb *et al.*, 2009), which may be another explanation as to why the accompanying decrease in % Choice 2 was not seen here: as the punishment period for Choices 2 and 3 are less than in comparison to previous studies that observed an increase in % Choice 1 and a decrease in % Choice 2 (Van Enkhuizen *et al.*, 2013), the whole dynamic of % Choices is altered.

However, another possibility is that the shift is caused by a difference in reward valuation perceived by the mice because of alterations of dopaminergic firing patterns induced by the amphetamine. Transient, phasic bursts of dopaminergic activity occur tonically and spontaneously in the brain but also occur in a time-locked fashion in response to reward prediction from learned cues, as well as for unexpected rewards (Day *et al.*, 2007; Roitman *et al.*, 2008). Daberkow *et al.* (2013) have shown that amphetamine increased the amplitude, duration and frequency of the tonic, naturally-occurring, phasic increases in extracellular dopamine. Not only this, but they showed that low-dose amphetamine augmented the dopamine phasic response to reward prediction cues. It is feasible that the mice in the current study, after a low dose of amphetamine, experience an enhanced dopaminergic response to obtaining a reward they were expecting and therefore bias their responding to Choice 1: if they are experiencing enhanced enjoyment from a single reward delivery, they may not be as motivated to try and achieve a larger amount of reward at the expense of more frequent punishments. This may also be different between the two groups of mice as *Map2k7*$^{+/-}$ mice showed signs of having a reduced behavioural response to amphetamine than WT mice. Future experiments could look into the phasic dopaminergic firing in these mice whilst carrying out the rGT to observe whether this is the case.

Amphetamine caused changes in some other performance parameters: magazine entries during the ITI and choice latency, a differential effect in the two groups of mice such that amphetamine had a stronger effect on WT than *Map2k7*$^{+/-}$ mice. However, these two performance parameters are not normally altered by amphetamine in the rGT in mice or rats (Van Enkhuizen *et al.,* 2013, Zeeb *et al.*, 2009), so these results are difficult to interpret accurately as it may be that the differential genotype effect may be artificially induced by anomalous control results.

6.5.7 Conclusions

To summarise, we have shown that mice are capable of learning the reward-punishment contingencies in the touchscreen rGT in a comparable way to humans. They show a pattern of responding that reflects the number of rewards per unit time, and are able to notice the differences when contingencies are switched and alter their responding accordingly. We also show that *Map2k7*$^{+/-}$ mice show signs of impairment in the length of time to adjust to negative feedback, in a similar way to patients with schizophrenia: they appear inflexible in their adaptive learning and appear to have altered sensitivity to reward/punishment (Turnbull *et al.*, 2006; Waltz, 2017; Dunn *et al.*, 2006). Finally, amphetamine had a similar effect on the pattern of choice preference as seen in previous studies in rats and there was a modest differential genotype effect. Overall, the current study shows MKK7 and/or other signalling components in its pathway have a role in risky, reward-based decision-making and further highlights the use of *Map2k7*$^{+/-}$ mice for dissecting the cognitive deficits of schizophrenia that could be targeted by novel compounds.

Chapter 7 Investigation of a gene x environment risk factor model using maternal immune activation

7.1 Introduction

There is a substantial body of research suggesting environmental risk factors that act during the pre-, peri- and early postnatal period are an important factor involved in the pathogenesis of schizophrenia in adult offspring (Brown & Derkits, 2010; Murray & Lewis, 1987). Stressful events, such as psychological trauma, maternal malnutrition, gestational infection and other obstetric complications cause physiological changes in the developing fetal environment, disturbing the normal course of brain development and inducing structural and functional brain abnormalities which emerge later, in adult life (Meyer *et al.*, 2009; Rees & Inder, 2005). In particular, maternal infection and the associated inflammatory response in the mother and developing fetal brain have been a significant focus of investigation. Initially, these were epidemiological studies that had limitations, were mainly retrospective in nature, and did not confirm causality (Brown & Susser, 2002; Meyer *et al.,* 2009). Increasingly prospective study designs led to human serologic evidence (Brown *et al.*, 2004) and investigations into the effect of maternal immune activation (MIA) on offspring in preclinical rodent models (reviewed in Kneeland & Fatemi, 2013), which provided, and continue to provide, insight into the potential pathogenic mechanisms involved in prenatal infection and the neurodevelopmental hypothesis of schizophrenia (first proposed by Weinberger, 1986; Keshavan & Hogarty, 1999).

Rodent maternal exposure to human influenza virus (e.g. Fatemi *et al.*, 2002), the bacterial endotoxin lipopolysaccharide (LPS) (Urakubo *et al.,* 2001), select inflammatory cytokines (Smith *et al.*, 2007) or the viral mimetic polyriboinosinic-polyribocytidylic acid (Poly I:C; Reisinger *et al.*, 2015) have been widely studied, with subsequent examination of molecular, physiological, structural and behavioural changes in the offspring (Reviewed in Meyer *et al.*, 2006; Meyer *et al.,* 2005; Patterson, 2009). MIA using Poly I:C, a synthetic, commercially available analog of double-stranded RNA is particularly well-studied over alternative methods of immune activation. This is partially because of its relatively short-

lived, well-characterised and specific immune response profile, so the point of impact is precise and cellular mechanisms can be identified relatively easily (Reisinger *et al.*, 2015). Viruses create double stranded RNA (dsRNA) as part of replication or as a by-product, which, along with viral mimetics such as Poly I:C, are recognised by toll-like receptor 3 (TLR3; a pattern recognition receptor) as "foreign" (Alexopoulou *et al.*, 2001). Once Poly I:C has been recognised by TLR3, specific intracellular adaptor proteins are recruited and a strong inflammatory response is initiated that brings cells to the site of infection in order to help kill the invading pathogen (Zhang *et al.*, 2013). This has been shown to occur via the activation of MAPKs, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and interferon regulatory factors (IRFs) (Kawai & Akira, 2008; Park *et al.*, 2006) and subsequently upregulates genes coding for pro- and anti-inflammatory mediators such as cytokines, chemokines and colony stimulating factors (CSFs) (Arrode-Brusés & Brusés, 2012b; Harvey & Boksa, 2012).

Additionally, the signalling cascade downstream of Poly I:C's recognition by TLR3 interacts with the MKK7 pathway (see **Fig. 7.1**). When Poly I:C is recognised by TLR3, whose toll/interleukin 1 resistance/receptor protein (TIR) domain exclusively recruits the adaptor protein TIR domain-containing adaptor proteins inducing IFNß (TRIF) (Oshiumi *et al.*, 2003), the signalling pathway branches off and either activates IRF3, NF-κB or AP-1 (Kawasaki & Kawai, 2014; Reisinger *et al.*, 2015; Zhang *et al.*, 2013). The pathway that is reliant on JNK/MKK7, however, continues by TRIF subsequently interacting with TNF receptor-associated factor 6 (TRAF6), which recruits receptor interacting protein 1 (RIP1) and, in turn, activates the MKKK7 (also known as TAK1) complex, which consists of MKKK7, TAB2 and TAB3 (together referred to as TRIKA2; (Jiang *et al.*, 2003; Kawasaki & Kawai, 2014). This complex goes on to activate MKK7 and then JNK, which activates the AP-1 family of transcription factors that consequently translocate to the nucleus in order to have an effect on gene expression of cytokines, chemokines and colony stimulating factors, including IFN-ß, TNF-α, IL-12, IL-13, IL-15, IL-7, IL-1ß, IL-6, CXCL1, CXCL9, CXCL10, CCL5, IL-8, CCL2, CCL3, CCL5, GM-CSF and VEGF in maternal plasma and/or fetal brain (**Fig. 7.1**; Arrode-Brusés & Brusés, 2012; Park *et al.*, 2006; Smith *et al.*, 2007). In fact, JNK activation (by MKK4/7) is required for Poly I:C – TLR3 mediated increase in CXCL10 and IL-8 expression (Park *et al.*, 2006) and perhaps other immune molecules that were not measured. Thus, the

experiment described in this chapter is extremely appropriate when considering a potential gene x environment interaction model with mice deficient in the *Map2k7* gene.

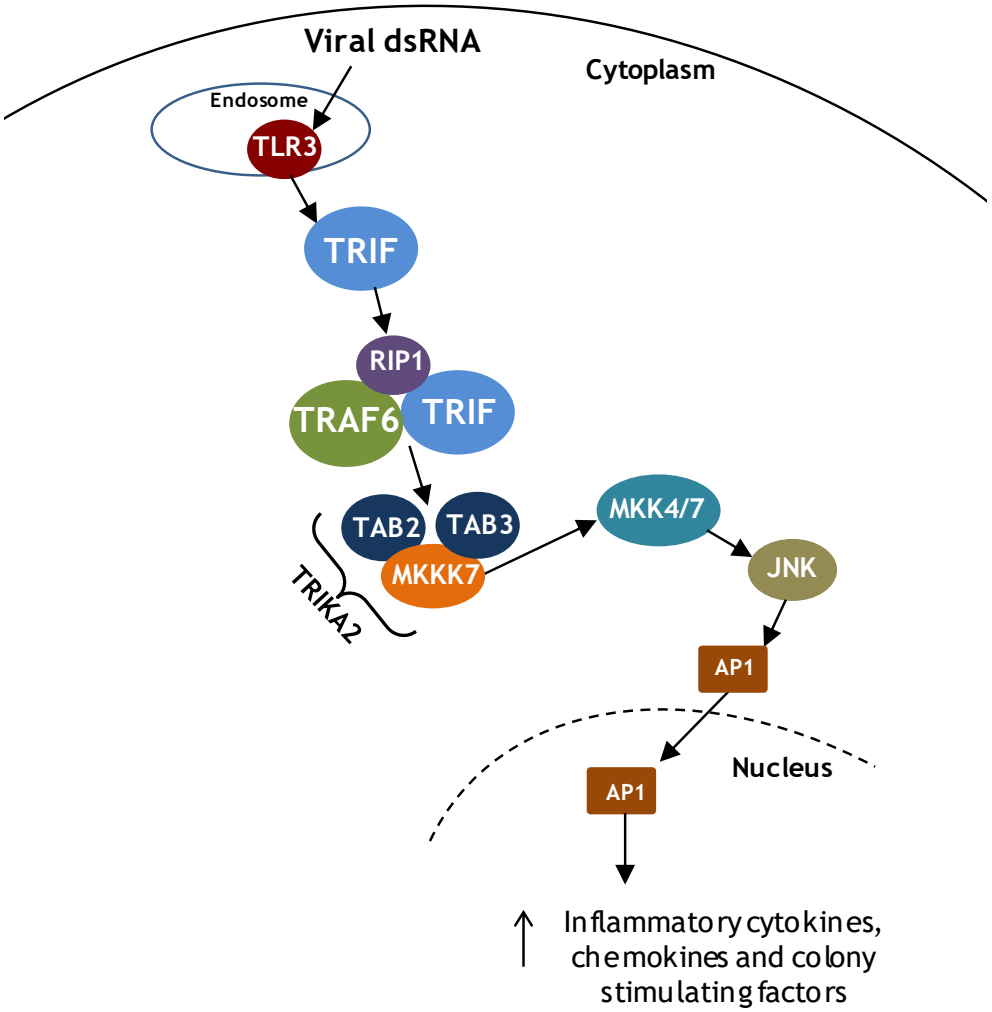

Figure 7.1 An overview of Poly I:C's ability to interact with the MKK7 pathway. TLR3 is localised to endosomes within cells. Recognition of Poly I:C or other double stranded RNA initiates an inflammatory response partially via MKK7/JNK. Not shown in this diagram are other pathways that lead to activation of the NF-κB and IRF3, which have further effects on other immune molecules. JNK activates AP1, which translocates to the nucleus and increases the expression of inflammatory cytokines, chemokines and colony stimulating factors (figure made using information from Arrode-Brusés & Brusés, 2012; Kawasaki & Kawai, 2014; Park *et al.*, 2006; Reisinger *et al.*, 2015; Smith *et al.*, 2007; Zhang *et al.*, 2013). TLR3 = Toll-like receptor 3; TRIF = TIR domain-containing adaptor proteins inducing IFNbeta; TIR = Toll/interleukin 1 resistance/receptor protein; TRAFs = TNF (tumor necrosis alpha) receptor-associated factors; AP1 = Adaptor protein 1; RIP1 = Receptor interacting protein 1; TAK1 aka MKKK7 (mitogen activated protein kinase kinase

kinase 7); TAB = TAK1 binding protein (aka MKKK7 binding protein); TRIKA2 = TRAF6-regulated IKK activator 2; IKK – IκB Kinase.

Poly I:C administration to gestating rodents has repeatedly been shown to induce molecular, structural, physiological and behavioural changes related to schizophrenia in adult offspring (Da Silveira *et al.*, 2017; reviewed in Patterson, 2009). Specifically, molecular changes in the offspring include: alterations in glutamic acid decarboxylase-67 (GAD_{67}) expression, altered microglial staining, smaller, denser neurons in the hippocampus, altered dopamine metabolism in the striatum, reduced parvalbumin positive cells in the PFC, reduced expression of dopamine D1 receptors in the PFC and NMDAR subunit GluN1 in the hippocampus (Cassella *et al.*, 2016; Meyer *et al.,* 2009; Meyer *et al.,* 2008; Patterson, 2009). Structural changes in the hippocampus have also been observed (Zuckerman & Weiner, 2003), as have enlarged ventricles (Piontkewitz *et al.*, 2011). Physiological deficits include reduced frequency and amplitude of mEPSCs with an increased sensitivity to dopamine when a pregnant mouse was injected on embryonic day 12 (E12) with 20mg/kg Poly I:C (Smith *et al.,* 2008). Many behavioural changes have been reported, including deficits in: PPI (Ozawa *et al.*, 2006), latent inhibition (Zuckerman *et al.*, 2003), attentional set shifting (Zhang *et al.,* 2012), social behaviour (Bitanihirwe *et al.*, 2010), and spatial learning (Meyer *et al.*, 2006). Offspring of Poly I:C exposed dams also experience alterations in glucose preference (Silveira *et al.*, 2017), increased anxiety (Meyer *et al.*, 2008) and enhanced responses to psychotomimetics such as ketamine (Silveira *et al.*, 2017) and amphetamine (Vorhees *et al.*, 2015).

These findings have initiated research into a whole new level of animal modelling: combining models that confer genetic risk for schizophrenia with maternal infection and examining the effect this has on offspring. Several studies have investigated this so far: Abazyan *et al.* (2010) combined a mutation in the DISC1 gene with prenatal infection of Poly I:C; Ibi *et al.* (2010) also used the DISC1 model but exposed the offspring to Poly I:C neonatally; O'Leary *et al.* (2014) combined mice with a genetic deletion in the Neuregulin (NRG1) gene with Poly I:C exposure, and Vuillermot *et al.* (2012) administered Poly I:C to gestating mice with a deletion in nuclear receptor related 1 (Nurr1). These studies highlighted the complexity of gene x environmental interactions as some results produced behavioural differences that are not observable with either genetic alteration or

MIA alone; some showed synergistic effects, and some showed additional behavioural deficits when MIA is combined with genetic disruption (Moran *et al.*, 2016). Creating a gene x environment risk factor model with *Map2k7*$^{+/-}$ mice would be highly relevant, especially because of *Map2k7*'s potential ability to interact with the downstream pathway of Poly I:C, and its role in the cellular stress response. However, carrying out an experiment like this would require large numbers of animals and would take a considerable period of time with no reassurance that dose and timings are correct. Firstly, it is important to verify that the fine details of the experiment are right, and to investigate at the molecular level whether *Map2k7* heterozygosity does indeed interact with the response to Poly I:C exposure.

7.2 Aims

This chapter aims to explore the possibility of *Map2k7*$^{+/-}$ mice being an important gene x environment MIA model relevant to neurodevelopmental disorders such as schizophrenia. 20mg/kg Poly I:C will be exposed to gestating dams at E12.5 and the immune response profile determined in maternal plasma and brains of the developing embryos. It is hypothesised that, as the mechanism of Poly I:C administration has been shown to interact with the MKK7 pathway and *Map2k7*$^{+/-}$ mice have decreased MKK7 in the brain, the immune response profile following exposure to Poly I:C may be altered in *Map2k7*$^{+/-}$ mice compared to WTs when either: it is the mother that carries genetic risk for schizophrenia, the developing embryo that carries genetic risk, or both. If any of these possibilities are the case, it will be interesting to study the *Map2k7*$^{+/-}$ mice further as a model of relevance to schizophrenia that carries both genetic and environmental risk, as this provides infinitely more construct validity of the disorder than either alone.

7.3 Materials and Methods

7.3.1 Mice

8 WT male mice, 8 WT female mice, 8 *Map2k7*$^{+/-}$ male mice and 8 *Map2k7*$^{+/-}$ female mice were used in the experiment. Mice were time mated according to the combinations outlined in **Table 7.1**. Mouse pairs were put together at 5pm and separated in the morning the next day. If they had conceived, this was taken as embryonic day 0.5. Female mice were weighed and monitored for 12 days and any pregnant mice continued in the experiment; any females who had not conceived were put together with a different male mouse and the process was repeated until the combinations in **Table 7.1** had been achieved.

	Female WT	Female *Map2k7*$^{+/-}$
Male WT		4 x Saline
Male WT		4 x Poly I:C
Male *Map2k7*$^{+/-}$	4 x Saline	
Male *Map2k7*$^{+/-}$	4 x Poly I:C	

Table 7.1 Mating combinations. 4 pairs in each group were successfully time mated, totalling 16 pairs.

Female mice weighed 22.1 ± 0.34g on average at the start of the experiment and 28.7 ± 0.77g on average when 12.5 days pregnant. All mice were aged 12.23 ± 0.47 weeks at the point of conception. Mice were singularly housed (when not paired) in a temperature and humidity-controlled room with a 12-hour light/dark cycle (lights on at 07:00). All injections and dissections occurred between 08:00 and 18:00 and in accordance with the Animals (Scientific Procedures) Act 1986.

7.3.2 Drug Administration

Pregnant dams were weighed and given either 20mg/kg at 2ml/kg Poly I:C (Invivogen, LMW) or 2ml/kg saline on embryonic day 12.5 (E12.5). This dose has previously been shown to induce long-lasting behavioural and pharmacological changes in mouse offspring (Shi *et al.,* 2003) and was chosen because it is the optimal dose that causes MIA (Garay *et al.*, 2013). All injections were given subcutaneously to avoid accidental injection into an embryo. With respect to brain development (Clancy *et al.*, 2001) and brain gene expression (Liscovitch & Chechik, 2013), embryonic day 12.5 is equivalent to ~54th day (7.8th week) of gestation for humans. Therefore, embryonic day 12.5 is the murine equivalent of

three-quarters of the way through trimester 1 in humans, a period where the developing nervous system is particularly vulnerable to maternal infection and most associated with increased incidence of schizophrenia (Brown *et al.*, 2004).

7.3.3 Dissection Procedure

6 hours following the Poly I:C or saline injection, the pregnant dam was injected with a lethal dose (0.1ml) of Pentobarbital sodium (Euthatal, Merial Animal Health Ltd.) and trunk blood was collected via cardiac puncture into an EDTA-coated syringe. The blood was injected into an EDTA-coated 1.5ml Eppendorf tube containing an additional 80µl EDTA and shaken to mix the blood with the EDTA. If more than 0.8ml of blood was collected, 10µl EDTA per 100µl extra blood was added to the tube to ensure sufficient prevention of blood clotting. The Eppendorf containing the maternal blood and EDTA was spun at 10,000g at 4°C for 10 minutes. The supernatant (plasma) was then transferred into a new Eppendorf and frozen at -80°C until ELISA or Luminex were carried out.

While the blood was spinning, the embryos were carefully removed from the mother and placed on a ceramic tile on ice. The brain and a small amount of tissue (for genotyping) were taken from each embryo, placed in separate Eppendorfs and kept at -80°C until required. The ceramic tile and dissection tools were cleaned with ethanol between each embryo to avoid contamination.

The embryos were individually genotyped according to the **Genotyping** outlined in **Section 2.2**.

7.3.4 Protein Extraction for ELISA and Luminex

Embryonic brain samples were pooled for genotype for each group so that from one litter, all WT embryo's brain tissue were placed in a 1.5ml Eppendorf and all the *Map2k7*$^{+/-}$ embryo's brain tissue were placed in a separate 1.5ml Eppendorf. Pooled embryonic brain tissue was homogenised manually in 275µl lysis buffer (1 x PBS with 0.1% w/v Triton X-100 (Sigma), 5µM EDTA (GIBCO) and 1% w/v proteinase inhibitors (Sigma, P8340) and then spun at 8,000g for 10 minutes at 4°C.

Supernatant was collected and total protein was quantified straight away using the BCA Assay as outlined below. The supernatant containing the protein was then halved (~110µl each) into two separate 0.5ml Eppendorfs, one for ELISA and one

for Luminex, so that multiple freeze-thaw cycles did not occur as this can affect protein composition. The samples were stored at -20°C for 1 or 2 days until ELISA or Luminex were carried out.

7.3.5 BCA Assay

Embryonic brain protein concentrations were determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions provided (Merck) using bovine serum albumin (BSA) as a standard. Standards were diluted as appropriate in lysis buffer (see *Protein Extraction* above for lysis buffer details). The highest standard was 1000µg/ml and was serially diluted to give final known concentrations of 1000, 500, 250, 125 and 25µg/ml, and a 0µg/ml blank of lysis buffer was also added. Samples were diluted 1:250 in lysis buffer and 25µl of BSA standards or samples were added in duplicate to a 96-well plate. 200µl of BCA working reagent (BCA solution and 4% Cupric sulphate in a ratio of 50:1) was added to each well, then the plate was covered from light and incubated at 37°C for 40 minutes. The optical density at wavelength 590nm was subtracted from the optical density at 540nm to correct for optical imperfections in the plate, duplicate readings were averaged, and then blank readings were subtracted from all other standard and protein samples to give corrected optical density. The samples were interpolated from the standard curve and multiplied by the dilution factor to give total protein concentrations of samples.

7.3.6 ELISA

CCL5 levels in maternal plasma and embryonic brain tissue supernatant were measured by an enzyme-linked immunosorbent assay (ELISA; Mouse/Rat CCL5/RANTES Quantikine® ELISA, R&D Systems: MMR00) according to the manufacturer's instructions provided. This ELISA utilised a traditional quantitative sandwich technique: primary antibody – sample – secondary antibody – substrate (detection). 50µl of assay diluent (provided) and 50µl of the diluted standards and samples (undiluted) were loaded in duplicate into the wells onto which a monoclonal antibody for CCL5 had been pre-coated. The highest standard was 500pg/ml and was serially diluted in calibrator diluent (provided) to give final known concentrations of 500, 250, 125, 62.5, 31.3, 15.6 and 7.8pg/ml, and a 0pg/ml blank of lysis buffer was also added. Any CCL5 present in standards and samples bound to the antibody throughout a two-hour incubation at room temperature. The standards and samples were then washed five times with the

wash buffer provided, then the conjugate polyclonal antibody for CCL5 was added to the wells and incubated at room temperature for 2 hours. Another 5 wash steps were carried out and the substrate was added, which produced a colour reaction (blue) that was stopped after 40 minutes (and turned yellow). The optical density was then read by a plate reader (Multiskan Spectrum, Thermo Fisher, using SkanIt™ Software): absorbance readings at 540nm were subtracted from readings at 450nm to correct for optical imperfections in the plate. The duplicate readings were averaged, and the blank readings were subtracted from all other standards and samples in order to give corrected optical density. The sample readings were then interpolated from the standard curve and multiplied by the dilution factor (2, as it was necessary to include 1:1 assay diluent to sample) to give the concentration of CCL5 in maternal plasma and embryonic brain. Embryonic brain concentrations were then normalised to total protein as established by the BCA assay using the calculation: (concentration of CCL5 in pg/ml / concentration of total protein in mg/ml) to give normalised CCL5 concentration in pg/mg protein.

7.3.7 Luminex Assay

The concentration of 20 cytokines, chemokines and colony stimulating factors were simultaneously determined in maternal plasma and embryonic brain tissue supernatant using a mouse cytokine magnetic 20-plex assay according to the manufacturer's instructions (Invitrogen: LMC0006M). The protein molecules measured and the concentration of the corresponding reconstituted standards are outlined in **Table 7.2**. These three groups of immune molecules are involved in the first steps following viral infection and initiate the movement, transcription and release of various immune cells, therefore giving a broad overview of the maternal and embryonic reaction to such an infection.

Cytokines				Chemokines		CSFs	
GM-CSF	13,600	IL-10	32,600	CXCL10	14,500	VEGF	4,400
IFN-γ	12,000	IL-12	5,450	CXCL1	50,500	FGF basic	25,700
IL-1α	19,100	IL-13	18,200	CCL2	15,800		
IL-1ß	16,700	IL-17	5,250	CXCL9	6,200		
IL-2	5,050	IL-5	15,100	CCL3	28,600		
IL-4	32,200	IL-6	21,200				
TNF-α	17,000						

Table 7.2. Cytokines, chemokines and colony stimulating factors measured using the Luminex assay and the concentrations of their reconstituted standard in pg/ml. CSFs = colony stimulating factors.

Two identical plates were required so that all samples could be analysed in duplicate, and were run in parallel. First, beads of defined spectral properties that are conjugated to protein specific antibodies for each of the proteins in **Table 7.2** were added to the wells. Antibody beads were vortexed for 30 seconds then sonicated for 30 seconds immediately before they were added into each well (25µl of antibody bead solution per well). Wells were then washed twice with 200µl of 1x wash solution using the magnetic separator method: the wash solution was added, the plate was adhered to the hand-held magnet, left to settle for 1.5 minutes, then inverted and tapped on paper towels several times. The plate was then removed from the magnet, 200µl of wash solution was re-added and the process repeated. Next, 50µl of incubation buffer was added into each well followed by the diluted standards in duplicate (100µl each). The highest standard was a different concentration for each antibody (see **Table 7.2**) and were serially diluted 1:3 6 times with 50% assay diluent (provided) and 50% lysis buffer, plus the undiluted standard and lysis buffer blank, to give 8 standards. The standard curve for each antibody are given in **Appendix 2**. Samples were diluted 1:1 with assay diluent and 100µl of this was added per well, along with 50µl incubation buffer. The plate was then protected from light and incubated on an orbital shaker at 600 rpm overnight at 4°C.

The following morning, wells were washed twice in wash solution followed by the addition of 100µl 1x biotinylated detector antibody solution. The plate was then protected from light and incubated on an orbital shaker at 600 rpm for 1 hour at room temperature. Wells were then washed twice in wash solution and 100µl of 1x streptavidin-RPE was added to each well, followed by shaking at 600 rpm for 30 minutes at room temperature, protected from light. Liquid was then removed and each well washed 3 times in wash solution. Finally, 125µl of wash solution was added to each well and the plate was shaken for 3 minutes on an orbital shaker at 600 rpm at room temperature. The plates were then read on a recently calibrated and validated Bio-Plex® 200 MAGPIX multiplex reader (Bio-Rad, CA) using Bio-Plex Manager™ 5.0 software (Bio-Rad, CA). The appropriate bead region was assigned to each analyte, a detection target of 100 beads per region and the recommended doublet discriminator (DD) gates of 7,800 – 20,000 were used, and the median fluorescent intensity (MFI) was collected. The coefficient of variation (% CV) of duplicate wells was checked; a plate was acceptable if the mean

CV<15%, and if not more than 20% of duplicates have CV>25%. The mean CV for each plate was 5.25% and 6.74% and the percentage of duplicates which have a CV >25% was 1.35% and 3.12% for each plate, respectively, which was well within this range. Any points from the standard curve that had a % CV >25% and accuracy outside of 70-120% of expected were excluded. % accuracy was calculated by the formula: (observed value/expected value)*100. The analysis software was then used to fit a curve to this set of reliable standards data using 5-parameter logistic regression with default automated weighting (all fitted to ≥ 6 points; see **Appendix 2**). Lower and upper limits of quantification (LLOQ and ULOQ) were calculated as the highest and lowest measured reliable standards for each standard curve after assessment as above. Concentration values that fall outside of this curve range were not included in analysis because they are extrapolated values, unless they were out of range because of a group difference. For example, mice that had received Poly I:C were likely to have cytokine levels within range but mice that had saline were not. In this case, the cytokine levels out of range (OOR; too small) were given a value of 0. Embryonic brain concentrations were then normalised to total protein as established by the BCA assay using the calculation: (concentration of immune molecule in pg/ml)/(concentration of total protein in mg/ml) to give normalised immune molecule concentration in pg/mg protein.

7.3.8 Statistical Analysis

All statistical analyses were carried out using Minitab® 17 Statistical Software. For maternal plasma, each cytokine was analysed separately by a two-way ANOVA with maternal genotype (WT or *Map2k7*$^{+/-}$) and drug (saline or Poly I:C) as between subjects factors. For embryonic brain, each cytokine was analysed separately by a three-way ANOVA with maternal genotype (WT or *Map2k7*$^{+/-}$), embryonic genotype (WT or *Map2k7*$^{+/-}$) and drug (saline or Poly I:C) as between subjects factor and each litter nested within maternal genotype and drug. 2-way pairwise comparisons were made between factors using Tukey's method. Bar graphs were created using GraphPad Prism 7. Data are presented as mean ± standard error of the mean (SEM) and results were considered significant if $p<0.05$.

Several of the cytokine levels measured contained a large number of zero's because values that were completely OOR (too small) or extrapolated from the lower part of the standard curve within one experimental group were replaced

with a zero. Therefore, a large amount of data in this chapter were violating the assumptions of an ANOVA: equal variances and a normal distribution. Using Levene's method to test for equal variances and the Anderson-Darling test for normality showed that the variances were unequal and the data was not normally distributed for several cytokines; however, data within each drug group (Saline or Poly I:C) had equal variances when tested separately. Additionally, visual inspection of the graphs from data which had shown a significant difference were noticeably very different. On this basis, and because there were equal numbers of genotypes in each of the treatment groups and genotype data grouped separately are normally distributed, it was decided that an ANOVA remained the most suitable statistical test to use.

7.4 Results

Female WT and *Map2k7*$^{+/-}$ mice that were in their 12.5th day of pregnancy were subcutaneously injected with either Poly I:C or saline. Maternal plasma and embryonic brain tissue were taken 6 hours later and embryonic brains were pooled for genotype from each litter. The concentrations of 21 different cytokines, chemokines and colony stimulating factors were then analysed in maternal plasma and embryonic brain supernatant. For the maternal plasma measurements, 14 of the 21 immune molecules analysed were within a detectable range and met criteria for inclusion in analyses; for the embryonic brain cytokine measurements, 5 were detectable. This is most likely due to the fact that the levels of immune molecules are naturally a lot lower in embryonic brain tissue than maternal blood plasma (Garay *et al.*, 2013) and/or that they are not present in embryonic brain at detectable levels at this stage of development (Arrode-Brusés & Brusés, 2012b).

7.4.1 Maternal plasma from mice injected with Poly I:C had increased levels of most immune molecules measured compared to those injected with saline

Of the 21 cytokines tested, 14 were detectable in maternal plasma. 12 of these were elevated following Poly I:C administration compared to saline: CCL5, IL-1ß, CXCL10, CCL2, IL-6, CXCL9, IL-10, IL-5, TNF-α, CXCL1, IL-2 and IL-12 (effect of drug; see **Table 7.4**, **Fig. 7.2**). FGF Basic and VEGF, the only two colony stimulating factors measured, were not elevated following Poly I:C (**Fig. 7.2**). Data and p-values for all immune molecules are shown in **Table 7.3** and **7.4**, respectively.

7.4.2 Map2k7$^{+/-}$ *mice showed an enhanced cytokine response in maternal plasma to Poly I:C*

The levels of VEGF, IL-10, TNF-α, CXCL1, IL-2, and IL-12 were higher in *Map2k7*$^{+/-}$ mice than WT mice overall (significant overall effect of genotype; see **Table 7.4**, **Fig. 7.2**). For the cytokines TNF-α, IL-2, IL-10 and IL-12, this was particularly striking, as post hoc analyses revealed they were significantly more elevated in *Map2k7*$^{+/-}$ mice than they were in WT mice following Poly I:C administration (significant genotype x drug interaction; see **Table 7.4**, **Fig. 7.2**). Data and p-

values for all immune molecules in maternal plasma are shown in **Table 7.3** and **7.4**, respectively.

Cytokine/Chemokine	WT with Saline (pg/ml)	HZ with Saline (pg/ml)	WT with Poly I:C (pg/ml)	HZ with Poly I:C (pg/ml)
CCL5	202.98 ± 51.62	202.57 ± 41.15	1907.11 ± 21.87	1844.67 ± 11.80
GM-CSF	<OOR	<OOR	<OOR	<OOR
IFN-γ	<OOR	<OOR	<OOR	<OOR
IL-1α	<OOR	<OOR	<OOR	<OOR
IL-1ß	45.16 ± 16.04	65.07 ± 27.99	151.78 ± 16.66	261.19 ± 83.27
IL-2	0.00 ± 0.00	5.80 ± 3.37	22.33 ± 3.85	50.42 ± 9.48
IL-4	<OOR	<OOR	<OOR	<OOR
IL-5	154.93 ± 29.26	224.58 ± 46.25	507.73 ± 14.50	486.97 ± 63.11
IL-6	0.00 ± 0.00	109.29 ± 109.29	514.55 ± 41.50	650.43 ± 91.93
IL-10	73.15 ± 60.85	117.01 ± 54.62	164.85 ± 46.99	447.26 ± 44.59
IL-12	115.65 ± 27.26	72.56 ± 29.72	713.84 ± 113.16	1198.62 ± 171.63
IL-13	<OOR	<OOR	<OOR	<OOR
IL-17	<OOR	<OOR	<OOR	<OOR
TNF-α	10.49 ± 1.51	13.11 ± 1.64	117.92 ± 14.23	172.66 ± 17.37
CXCL10	35.56 ± 4.56	49.04 ± 3.52	9638.78 ± 1932.09	11413.58 ± 2035.53
CXCL1	356.44 ± 139.31	630.33 ± 121.37	5040.50 ± 553.58	7068.87 ± 880.44
CCL2	107.45 ± 24.43	131.46 ± 24.06	16452.15 ± 5258.73	13278.52 ± 3323.51
CXCL9	157.31 ± 40.27	463.66 ± 159.82	9527.16 ± 783.88	17360.52 ± 7296.51
CCL3	<OOR	<OOR	<OOR	<OOR
VEGF	20.77 ± 2.72	24.49 ± 3.50	19.62 ± 0.82	34.82 ± 6.11
FGF basic	410.77 ± 38.58	3048.48 ± 2252.39	859.62 ± 588.23	699.75 ± 236.63

Table 7.3. Immune molecule levels in maternal plasma. Data shown as mean ± SEM.

Cytokine/Chemokine	WT vs. HZ (Effect of Genotype)	Poly I:C vs. Saline (Effect of Treatment)	WT with Poly I:C vs. HZ with Poly I:C (Genotype x Treatment interaction)
CCL5	0.391, ns	<0.0001, $F_{(1,12)}$=2249.70	0.397, ns
GM-CSF	NA	NA	NA
IFN-γ	NA	NA	NA
IL-1α	NA	NA	NA
IL-1β	0.180, ns	0.006, $F_{(1,12)}$=11.11	0.344, ns
IL-2	0.008, $F_{(1,12)}$=9.88	<0.0001, $F_{(1,12)}$=38.57	0.061, ns *
IL-4	NA	NA	NA
IL-5	0.575, ns	<0.0001, $F_{(1,12)}$=52.64	0.307, ns
IL-6	0.125, ns	<0.0001, $F_{(1,12)}$=50.39	0.861, ns
IL-10	0.009, $F_{(1,12)}$=9.78	0.002, $F_{(1,12)}$=16.36	0.041, $F_{(1,12)}$=5.23
IL-12	0.057, $F_{(1,12)}$=4.44	<0.0001, $F_{(1,12)}$=67.74	0.027, $F_{(1,12)}$=6.35
IL-13	NA	NA	NA
IL-17	NA	NA	NA
TNF-α	0.026, $F_{(1,12)}$=6.46	<0.0001, $F_{(1,12)}$=139.97	0.040, $F_{(1,12)}$=5.33
CXCL10	0.536, ns	<0.0001, $F_{(1,12)}$=55.82	0.542, ns
CXCL1	0.050, $F_{(1,12)}$=4.75	<0.0001, $F_{(1,12)}$=110.88	0.123, ns
CCL2	0.622, ns	<0.0001, $F_{(1,12)}$=22.47	0.617, ns
CXCL9	0.289, ns	0.004, $F_{(1,12)}$=12.80	0.325, ns
CCL3	NA	NA	NA
VEGF	0.029, $F_{(1,12)}$=6.19	0.251, ns	0.157, ns
FGF basic	0.311, ns	0.433, ns	0.255, ns

Table 7.4. p-values from statistical tests showing significance between experimental groups of immune molecule levels in maternal plasma.

Table 7.3 and 7.4. Immune molecule levels in maternal plasma (**Table 7.3**) of WT and *Map2k7*$^{+/-}$ mice that had either received Poly I:C or Saline 6 hours prior to collection of plasma, and the p-values showing significance (**Table 7.4**). Data collected using either an ELISA (for CCL5) or Luminex Multiplex Panel (for all other cytokines/chemokines/colony stimulating factors). Concentrations are expressed as the mean ± SEM pg/ml. <OOR = Out of range (too small). Data were analysed by 2-way ANOVAs with maternal genotype and drug as between subjects factors, and Tukey's post hoc. All significant differences indicate **elevated** cytokine levels for *Map2k7*$^{+/-}$ mice compared to WT, for Poly I:C treated compared to Saline treated and for *Map2k7*$^{+/-}$ mothers that received Poly I:C compared to WT mothers that received Poly I:C. N= 4 per group (16 total).

* Although the interaction term was not significant, Tukey's post hoc analysis showed that there was a significant difference in IL-2 levels between *Map2k7*$^{+/-}$ mothers that received Poly I:C and WT mothers that received Poly I:C (see **Fig. 7.2**).

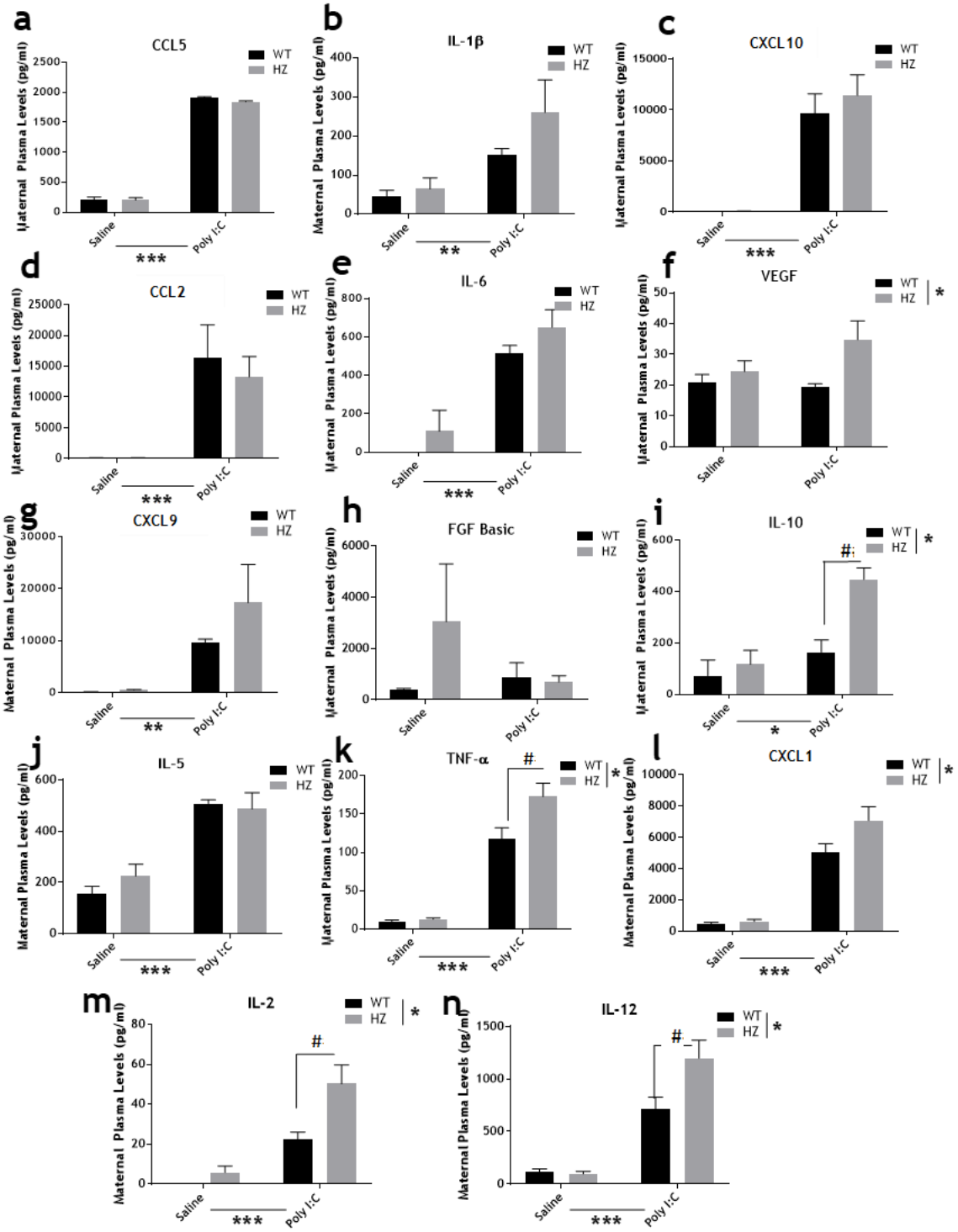

Figure 7.2 Cytokine levels in the maternal plasma as measured by a) ELISA and b-n) Luminex multiplex panel. All cytokines/chemokines were elevated in plasma of the mothers that received Poly I:C compared to those that received saline (note that 7 cytokines are not included in analysis because their levels were out of range (too small). FGF Basic and VEGF, the two colony stimulating factors measured, were not elevated in response to Poly I:C. IL-10, TNF-α, IL-2 and IL-12 were significantly more elevated in *Map2k7*$^{+/-}$ mice than they were in WT mice following Poly I:C administration compared to saline. Data analysed by a two-way ANOVA with maternal genotype (WT or *Map2k7*$^{+/-}$) and drug (saline or Poly I:C) as between subjects factor.

2-way pairwise comparisons were made between factors using Tukey's method. Lines between drug groups and between genotype groups indicate overall significant difference between those groups: *p<0.05, **P<0.01, ***p<0.001 (ANOVA); #p<0.05 (Tukey's). *Map2k7*$^{+/-}$: n=8 (4 saline, 4 Poly I:C), WT: n= 8 (4 saline, 4 Poly I:C).

7.4.3 CCL5 and CXCL10 were elevated in embryonic brain following Poly I:C

Of the immune molecules analysed in embryonic brain, 5 were within a detectable range and met criteria for inclusion in analyses: CCL5, CXCL10, CCL2, VEGF and FGF Basic. CCL5 and CXCL10 levels were significantly increased in the brains of embryos whose mother had been exposed to Poly I:C compared to the brains of embryos whose mother had been given saline (significant effect of drug; see **Table 7.7**). CCL2, VEGF and FGF Basic were not elevated in response to Poly I:C.

7.4.4 Map2k7$^{+/-}$ *embryos from WT mothers had a differential CCL5 brain response to Poly I:C than* Map2k7$^{+/-}$ *embryos from* Map2k7$^{+/-}$ *mothers*

Although modest, CCL5 levels were higher in embryos from maternal *Map2k7*$^{+/-}$ mice than those from maternal WT mice overall (significant overall effect of maternal genotype; see **Table 7.6**, **Fig. 7.3a**), which showed signs of being driven by an increase in CCL5 in *Map2k7*$^{+/-}$ embryonic brain as opposed to an increase in CCL5 in WT embryonic brain. Although there is no significant difference between CCL5 levels in WT and *Map2k7*$^{+/-}$ embryonic brain that had received Poly I:C via their mothers, there was a significant increase in *Map2k7*$^{+/-}$ embryonic brain CCL5 when they had received Poly I:C via *Map2k7*$^{+/-}$ mothers compared to *Map2k7*$^{+/-}$ embryonic brain CCL5 when they had received Poly I:C via WT mothers. On the other hand, there was no significant increase in CCL5 in WT embryonic brain when they received Poly I:C from *Map2k7*$^{+/-}$ mothers, compared to WT embryonic brain when they received Poly I:C from WT mothers (see **Table 7.7**, **Fig. 7.3**). Maternal and embryonic genotype, and drug (saline or Poly I:C) did not have an effect on CCL2, VEGF and FGF Basic levels. Data and p-values for all immune molecules in embryonic brain are shown in **Table 7.5, 7.6** and **7.7**.

Cytokine/Chemokine	WT Embryo from Maternal WT with Saline (pg/mg)	WT Embryo from Maternal HZ with Saline (pg/mg)	HZ Embryo from Maternal WT with Saline (pg/mg)	HZ Embryo from Maternal HZ with Saline (pg/mg)
CCL5	0.05 ± 0.03	0.27 ± 0.18	0.03 ± 0.03	0.08 ± 0.08
CXCL10	0.38 ± 0.21	1.47 ± 0.51	0.26 ± 0.26	1.41 ± 0.71
CCL2	4.74 ± 0.61	3.50 ± 0.68	4.81 ± 1.30	4.17 ± 1.08
VEGF	1.39 ± 0.21	1.20 ± 0.24	1.27 ± 0.41	1.52 ± 0.39
FGF basic	45.37 ± 9.17	54.95 ± 10.73	67.39 ± 21.24	76.52 ± 23.93

Table 7.5. Immune molecule levels in embryonic brain with maternal exposure to saline. Data shown as mean ± SEM.

Cytokine/Chemokine	WT Embryo from Maternal WT with Poly I:C (pg/mg)	WT Embryo from Maternal HZ with Poly I:C (pg/mg)	HZ Embryo from Maternal WT with Poly I:C (pg/mg)	HZ Embryo from Maternal HZ with Poly I:C (pg/mg)
CCL5	1.30 ± 0.26	1.40 ± 0.54	0.64 ± 0.31	2.06 ± 0.69
CXCL10	3.88 ± 0.66	2.91 ± 0.47	2.84 ± 0.44	6.64 ± 2.16
CCL2	4.44 ± 0.94	3.38 ± 0.62	3.65 ± 0.67	6.81 ± 2.06
VEGF	1.27 ± 0.19	1.08 ± 0.22	1.36 ± 0.36	2.28 ± 0.96
FGF basic	48.81 ± 11.92	37.91 ± 8.64	31.79 ± 3.48	72.77 ± 15.41

Table 7.6. Immune molecule levels in embryonic brain with maternal exposure to Poly I:C. Data shown as mean ± SEM.

Cytokine/Chemokine	Embryonic WT vs. HZ	Maternal WT vs. HZ	Poly I:C vs. Saline	*(Embryonic Genotype x Drug x Maternal Genotype interaction)
CCL5	0.771, ns	0.031, $F_{(1,12)}$=5.95	<0.0001, $F_{(1,12)}$=46.20	0.063, ns **
CXCL10	0.366, ns	0.084, ns	<0.0001, $F_{(1,12)}$=22.58	0.105, ns
CCL2	0.292, ns	0.944, ns	0.738, ns	0.262, ns
VEGF	0.278, ns	0.565, ns	0.651, ns	0.616, ns
FGF basic	0.123, ns	0.213, ns	0.178, ns	0.183, ns

Table 7.7. p-values from statistical tests showing significance between experimental groups of immune molecule levels in maternal plasma.

Table 7.5, 7.6 and 7.7. Cytokine and chemokine levels (**Table 7.4** and **7.5**) in embryonic brains of WT and *Map2k7*$^{+/-}$ mice that had either received Poly I:C or Saline via their WT or *Map2k7*$^{+/-}$ mother 6 hours prior to collection of tissue, and the p-values showing significance (**Table 7.6**). Data collected using either an ELISA (for CCL5) or Luminex Multiplex Panel (for all other cytokines/chemokines/colony stimulating factors). Concentrations are expressed as the mean ± SEM pg/ml. Data were analysed by 3-way ANOVA with Embryonic Genotype, Maternal Genotype and Drug as between subjects factors, and Tukey's post hoc. n = 4 WT and 4 *Map2k7*$^{+/-}$ pooled embryonic brain samples for each: Maternal WT Saline, Maternal WT Poly I:C, Maternal *Map2k7*$^{+/-}$ Saline, Maternal *Map2k7*$^{+/-}$ Poly I:C. For clarity, only interaction terms in the model that produced a significant result in at least one cytokine are shown; all other interaction terms were non-significant. All cytokines not shown here but shown in **Table 7.2** were OOR (too small).

*Embryonic HZ produced by a WT Mother with Poly I:C vs. Embryonic HZ produced by a HZ Mother with Poly I:C. ** Although the interaction term was not significant, Tukey's Post Hoc analysis showed that a *Map2k7*$^{+/-}$ embryo from a *Map2k7*$^{+/-}$ mother that had received Poly I:C had increased levels of CCL5 compared to a *Map2k7*$^{+/-}$ embryo from a WT mother who had Poly I:C.

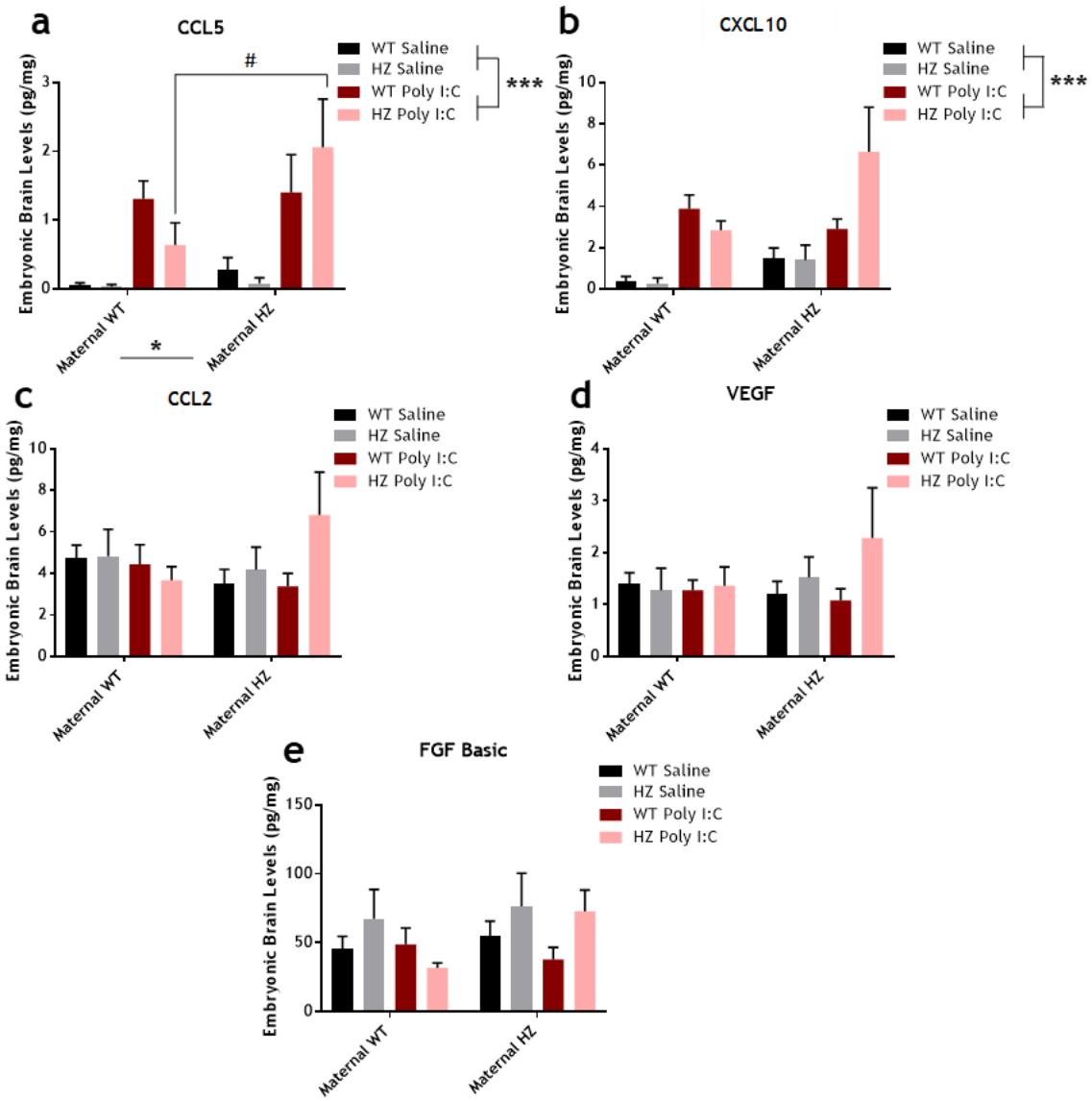

Figure 7.3 Cytokine levels in the embryonic brain as measured by a) ELISA and b, c, d, e) Luminex Multiplex panel. Overall, CCL5 (a) and CXCL10 (b) levels were significantly increased in the brains of embryos whose mother had been exposed to Poly I:C, compared to embryonic brain levels whose mother had received saline. For CCL5 (a), this increase in Poly I:C in embryonic brain was attenuated in the *Map2k7*$^{+/-}$ embryos produced by a WT, compared to *Map2k7*$^{+/-}$ embryos produced by a *Map2k7*$^{+/-}$mouse. CCL2 (c), VEGF (d) and FGF Basic (e) levels were unaffected by whether the mother had received Poly I:C or saline, or whether the mother was a WT or a *Map2k7*$^{+/-}$ mouse. All other cytokines measured in the Luminex assay were OOR (too small; data not shown). All embryos were genotyped and WT and *Map2k7*$^{+/-}$ embryos from each litter (n = 3-10) were pooled together for analysis. Data normalised to total protein levels as determined by BCA assay and analysed by a three-way ANOVA with embryonic genotype, maternal genotype and drug as between subjects factors with maternal genotype and treatment nested within litter. 2- and 3-way pairwise comparisons were made between all factors using Tukey's method. N = 4 WT and 4 *Map2k7*$^{+/-}$ embryonic brain samples for each: Maternal WT Saline, Maternal WT Poly I:C, Maternal *Map2k7*$^{+/-}$ Saline, Maternal *Map2k7*$^{+/-}$ Poly I:C. Lines between drug groups and between genotype groups indicate overall significant difference between those groups. *p<0.05, ***p<0.001 (ANOVA);

#p<0.05 (Tukey's). *n* = 4 WT and 4 *Map2k7*$^{+/-}$ pooled embryonic brain samples for each: Maternal WT Saline, Maternal WT Poly I:C, Maternal *Map2k7*$^{+/-}$ Saline, Maternal *Map2k7*$^{+/-}$ Poly I:C.

7.5 Discussion

In this chapter, it has been shown that the immune response of *Map2k7*$^{+/-}$ mice differs from that of WTs following maternal exposure to Poly I:C. 12 cytokines were increased in maternal plasma following Poly I:C exposure: IL-2, IL-5, IL-6, IL-10, IL-12, TNF-α, IL-1ß, CCL2, CCL5, CXCL1, CXCL9 and CXCL10. 4 of these: IL-2, IL-10, IL-12 and TNF-α, were all increased to a significantly higher extent in maternal plasma of *Map2k7*$^{+/-}$ mice compared to WT mice, suggesting that *Map2k7*$^{+/-}$ mice have a less well-controlled/regulated immune response to viral infection. Furthermore, CCL5 and CXCL10 were increased in the embryonic brain of the embryos whose mother had been exposed to Poly I:C. Although CCL5 levels increased following Poly I:C in maternal serum to the same extent in *Map2k7*$^{+/-}$ and WT mice, *Map2k7*$^{+/-}$ embryos from *Map2k7*$^{+/-}$ mothers had significantly increased CCL5 compared to *Map2k7*$^{+/-}$ embryos from WT mothers. On the other hand, WT embryos from *Map2k7*$^{+/-}$ mothers did not have significantly increased CCL5 levels compared to WT embryos from WT mothers in response to Poly I:C. This indicates that the immune response in the developing brains of *Map2k7*$^{+/-}$ mice functions differently to some aspects of viral infection to WTs, and may provide important information if they are studied further as a gene x environment interaction model with relevance to schizophrenia, and possibly other neurodevelopmental disorders.

7.5.1 Cytokines activated

MIA with Poly I:C in gestating rodents has been carried out many times (reviewed in Meyer, 2014; Reisinger *et al.*, 2015; Scola & Duong, 2017). The immune molecule profile has been well documented in C57Bl/6 mice; a similar dose (20mg/kg) of Poly I:C increased levels of IL-6, IL-12, IL-10, IL-13, IL-15, TNF-α, IFN-γ, CCL2, CCL3, CCL5, CXCL1, CXCL9, CXCL10, VEGF AND GM-CSF in maternal serum 6 hours following exposure at E16 (Arrode-Brusés & Brusés, 2012b). Additionally, maternal 20mg/kg Poly I:C exposure at E12.5 induced chronic alterations in immune molecules in the fetal brain: IL-1ß, IL-10, IL-12 and GM-CSF are increased in the frontal cortex at birth; IL-12 and CCL5 were increased, and CCL3 and GM-CSF were decreased in offspring blood at birth, and IL-6, IL-10 and IL1ß were increased at adulthood in the frontal cortex and/or blood of offspring serum compared to controls (Garay *et al.,* 2013). These results show a viral

mimetic challenge at a similar dose and point in embryonic development to that used in the current experiment has the ability to make long-lasting changes in immune molecule levels in the frontal cortex, an area of well-known disruption in schizophrenia (Callicott *et al.*, 2003; Pratt *et al.*, 2008). The study by Garay *et al.* (2013) also highlights the complexity of MIA mechanisms: even within the same mouse at the same point in development it is possible for the same cytokines to be increased in the blood but decreased in the brain (e.g. GM-CSF; Garay *et al.*, 2013).

In the current study, our findings matched that of Arrode-Brusés & Brusés (2012) for the majority of immune molecules in maternal plasma (increased levels of CCL2, CCL5, CXCL1, CXCL9, CXCL10, IL-6, IL-10, IL-12 and TNF-α). We also found an increase in IL-1ß, IL-5 and IL-2, which they did not test for, and we did not find an increase in VEGF where Arrode-Brusés & Brusés (2012) did. Also, the authors found IL-1ß, CCL2, CXCL9, CXCL10 and VEGF to be increased in embryonic brain following maternal exposure to Poly I:C (Arrode-Brusés & Brusés, 2012). Of the cytokines that were in a detectable range in the embryonic brain, we found an overall increase only in CXCL10 and CCL5 but not in VEGF in response to Poly I:C exposure. Other studies that used 20mg/kg Poly I:C could either not detect CCL5 or it was not contained within the set of cytokines that they measured. Interestingly, in the current study, CCL2 was increased following Poly I:C in maternal plasma and was detectable in embryonic brain but did not show an increase following maternal Poly I:C, in contrast to Arrode-Bruses & Bruses (2012), who found CCL2 to be increased in fetal brain 6 hours following Poly I:C exposure. Overall, however, our findings are closely matched and show that the levels of a broad spectrum of immune molecules are increased following administration of a viral mimetic. In fact, many of the cytokines altered in the offspring brain and maternal serum are also elevated in the blood of patients with schizophrenia, including IL-1ß, IL-2, IL-6, IL-12 and TNF-α (Crespo-Facorro *et al.*, 2008; Kim *et al.*, 2009; Theodoropoulou *et al.*, 2001)

The differences seen in cytokine upregulation between the current study and Arrode-Brusés & Brusés (2012) may be because they exposed Poly I:C to pregnant mice on embryonic day 16 as opposed to our exposure on E12.5. This may represent a different level of immune reactivity in the mother and a more (or less) permeable period for the placenta and/or blood-brain barrier in the embryo at

the different periods of gestation. There are substantial fluctuations in the functioning of the maternal host's immune system as pregnancy progresses, including in the placenta, the barrier between the mother and baby (Mor & Cardenas, 2010). Although this may at least partially explain the differences, E12.5 is relevant to the aims of the current study. Poly I:C was exposed at the murine equivalent of three quarters of the way through trimester 1 with respect to brain development (Clancy *et al.*, 2001) and brain gene expression (Liscovitch & Chechik, 2013). This is a period where the developing nervous system is particularly vulnerable to maternal infection and most associated with increased incidence of schizophrenia following maternal infection (Brown *et al.*, 2004). Neurogenesis and gliogenesis are well underway at this point, and neuronal migration and formation of blood and immune cells begins (Estes & Mcallister, 2016; Knuesel *et al.*, 2014). Hence, this is a crucial and vulnerable point in neurodevelopment because the exposed developing nervous system will incorporate environmental information and make permanent changes that influence the mature structure and function of the brain. On the other hand, environmental influences later in development will compensate to accommodate for changes, but these may not be permanent (Andersen, 2003) and therefore are not as relevant when considering a potential MIA model of neurodevelopmental disorders such as schizophrenia. Additionally, mice that have a full knockout of *Map2k7* die between embryonic day 11.5 and 13.5, showing the importance of this gene at the developmental age at which the mice were exposed to Poly I:C in this experiment (E12.5; Wada *et al.*, 2004)

7.5.2 Differential upregulation of cytokines in Map2k7$^{+/-}$ *and WT mice*

IL-10, TNF-α, IL-2 and IL-12 were significantly more elevated in maternal plasma of *Map2k7*$^{+/-}$ mice than they were in WT mice following Poly I:C administration compared to saline. Additionally, although it is a modest difference, *Map2k7*$^{+/-}$ embryos from *Map2k7*$^{+/-}$ mothers that had received Poly I:C had increased levels of CCL5 compared to *Map2k7*$^{+/-}$ embryos from WT mothers who had Poly I:C. This is interesting because CCL5 was one of the cytokines that was not increased in *Map2k7*$^{+/-}$ mothers compared to WT mothers plasma following Poly I:C. This suggests that it is the embryo's genetic risk that causes pathological immune response in *Map2k7*$^{+/-}$ mice and not the genetics of the mother. This observation is based on just one cytokine, however, so would require further experiments to

confirm this. It is unfortunate that none of the cytokines that were upregulated in maternal *Map2k7*$^{+/-}$ mice compared to WT mice following Poly I:C were detectable in the embryonic brain. It would be interesting to see the effect that increased maternal immune response would have had on embryonic brain/cytokines and whether these would have been increased, too.

Overall, these results suggest that gestating *Map2k7*$^{+/-}$ mice have a less well-controlled/regulated immune response to viral infection. Without further molecular investigation, it is difficult to know the mechanisms behind this. The signalling pathway depicted in **Figure 7.1** would suggest that as MKK7 levels are decreased from the beginning of development, following stimulation by Poly I:C, JNK and therefore AP1 activation would be decreased compared to WTs, and so the overall immune response would be decreased instead of increased; however, the opposite was observed. This may instead represent a general dysregulation rather than simple increases or decreases.

The increase in cytokine response observed in *Map2k7*$^{+/-}$ mice is not completely unexpected, as different cytokines have been shown to be increased in response to decreased MKK7/JNK signalling. For example, in response to a decrease in JNK, an increase has been observed in IL-2 (Conze *et al.*, 2002; Dong *et al.*, 2000) and TNF-α (Stewart *et al.*, 2006). A decrease of JNK1 specifically has also been shown to increase levels of TNF-α, IL-6 and IL-10 (Tran *et al.*, 2006; Zhao *et al.*, 2017). On the other hand, an increase in JNK has shown an accompanying decrease in cytokines (Mitchell & Olive, 2010). Overall, the dysregulation of the cytokine response following alterations in the MKK7/JNK pathway is complicated and warrants further investigation.

7.5.3 Pro- and anti-inflammatory cytokines/chemokines need to be balanced

The interaction between all cytokines and chemokines are very complex. In order to maintain homeostasis, some cytokines/chemokines are pro-inflammatory and some have anti-inflammatory properties. The immune system has a large role in normal development of the CNS (summarised in Ratnayake *et al.*, 2013), and an increase or decrease in inflammation in itself is not thought to cause damage, so long as the anti- and pro-inflammatory cytokines and chemokines are upregulated in a controlled manner (Meyer *et al.*, 2009). If either anti- or pro-inflammatory

cytokines are upregulated more than the other, this disrupts the intricate balance usually maintained throughout normal neurodevelopment and is what is thought to precipitate neuropathology related to schizophrenia following maternal infection. Of the immune molecules that were tested and were within range in maternal serum, two were anti-inflammatory (IL-5 and IL-10) and four were pro-inflammatory (IL-1β, TNF-α, IL-2 and IL-12). IL-6 Is considered both anti- and pro-inflammatory; five of these were chemokines that are all pro-inflammatory (CXCL1, CXCL9, CXCL10, CCL2 and CCL5), and two were colony stimulating factors (FGF Basic and VEGF). Of these, TNF-α, IL-2, IL-12 and IL-10 were increased to a significantly larger amount in *Map2k7*$^{+/-}$ mice compared to WTs, 3 of these being pro-inflammatory and 1 being anti-inflammatory. Therefore, it is conceivable for there to be an imbalance of pro- vs. anti-inflammatory cytokines in *Map2k7*$^{+/-}$ mice that have been exposed to viral infection (Meyer *et al.,* 2009). Although in the current experiment many of the immune molecules were too low in embryonic brain to make any accurate conclusions, it is possible that an imbalance in pro- vs. anti-inflammatory immune molecules in the mother will have a knock-on effect in the developing embryonic brain and may produce an altered phenotype in *Map2k7*$^{+/-}$ mice compared to WTs. Therefore, *Map2k7*$^{+/-}$ mice should be investigated further as a gene x environment risk factor model by allowing the mice to grow to adult and carrying out further molecular and behavioural tests.

7.5.4 How do immune molecules gain access to the developing embryonic brain?

The precise way the immune molecules make their way into the embryonic brain is a current subject of debate (Meyer *et al.,* 2009; Ratnayake *et al.*, 2013). It is possible for maternal cytokines to pass through the placenta and then through the blood brain barrier into the CNS of embryos (Gilmore & Jarskog, 1997); for cytokines/chemokines to be produced by the placenta and released into the fetal circulation (Meyer *et al.*, 2009) and for the developing fetus itself to produce cytokines/chemokines in response to infection (Meyer *et al.*, 2009). However, MIA-related immune activation in the developing embryo is facilitated by the Poly I:C exposure, because this produces the immune response summarised in **Section 7.1** which disrupts the integrity of the placental barrier, allowing it to let through immune components where it would not normally (Reisinger *et al.*, 2015). This then compromises the molecular, structural and functional integrity of the

developing brain, potentially resulting in permanent changes and the development of schizophrenia later in life (Reisinger *et al.*, 2015). This is supported by a study by Wang *et al.* (2004), who demonstrated that West Nile virus interacts with TLR3 and causes a peripheral inflammatory response that disrupts the blood-brain barrier and enables the virus to enter the brain. *Tlr3*$^{-/-}$ mice are more resistant to lethal West Nile virus infection than WT mice, but were similarly affected when the virus was injected directly into their brain, suggesting that the blood-brain barrier breakdown is mediated by TLR3. It is possible that a similar mechanism is occurring here: disruption of the blood-brain barrier via activation of TLR3, enabling immune molecules to pass more freely from the periphery to the brain.

7.5.5 Limitations of the study and potential future directions

This experiment was carried out in order to investigate the idea of *Map2k7*$^{+/-}$ mice being a suitable potential gene x environment model relevant to schizophrenia by studying the initial cytokine response in maternal serum and embryonic brain. Therefore, there are many experiments which can follow this. Outlined below are some examples.

7.5.5.1 Further investigation of the cytokine response

If the other cytokines tested could be detected in the Luminex assay, it would have provided so much more information. Unfortunately, this was not the case and may be because the Luminex assay measures many molecules at once. The ELISA for CCL5 appeared to work particularly well, perhaps because of the ability to adjust concentrations of samples according to that particular standard curve. It would be beneficial to carry out separate ELISAs for each immune molecule, which in practice was not possible in the current experiment due to the volume of sample which would be required. However, the Luminex is excellent for deciding which molecules require confirmation or to be pursued further by ELISA. It would be of interest to investigate those cytokines/chemokines that were differentially upregulated in *Map2k7*$^{+/-}$ vs WT mice in maternal serum in the embryonic brain in order to see what effect this has. Additionally, it would be necessary to study cytokines such as IL-6 and IL-10 further, as IL-6 is considered a key cytokine in mediating the effects of MIA on fetal brain development (Smith *et al.*, 2007) and participates in neurogenesis (Erta *et al.*, 2012). IL-6 has been shown to be required for the neuropathology and behavioural changes following MIA in offspring, because injection of IL-6 produces these changes, and blocking IL-6

following Poly I:C in pregnant dams is sufficient to prevent the changes (Smith *et al.*, 2007). IL-10, on the other hand, is strongly anti-inflammatory and Meyer *et al.* (2008) showed that when IL-10 is enhanced during prenatal development, behavioural and pharmacological abnormalities in the offspring were prevented, and enhanced levels of IL-10 without Poly I:C challenge actually caused the abnormalities to occur. The balance between IL-6 and IL-10 seems to be particularly well controlled (Kunz *et al.*, 2011; Meyer *et al.*, 2008), so both cytokines should be investigated more thoroughly in future experiments with MIA in *Map2k7*$^{+/-}$ mice, perhaps by carrying out specific ELISAs. Also, it would be particularly interesting to see the changes in IL-1β and TNF-α in embryonic brain as IL-1β can induce the conversion of rodent mesencephalic progenitor cells into a dopaminergic phenotype, and TNF-α either negatively or positively regulates the survival of fetal midbrain dopaminergic neurons depending on its concentration (Meyer *et al.,* 2009), so both cytokines are highly relevant to the pathogenesis of schizophrenia.

7.5.5.2 Sex differences

It is not possible from the current experiment to see whether the embryos were male or female. A sex determination using PCR with primers that anneal to the X-chromosome and Y-chromosome (Arrode-Brusés & Brusés, 2012) would be beneficial in order to see if there are any sex differences in cytokine release that have been missed.

7.5.5.3 Placenta differences between mice and humans

Rodents are frequently used for MIA experiments because the placenta of rodents and humans are both hemochorial and are suspected to be more similar to each other than other mammalian placentas (Schmidt *et al.*, 2015). However, there are still large differences in the placenta in both structure and function, including with immunological functions and transfer of molecules over the placental barrier (Malassiné *et al.*, 2003; Meyer *et al.*, 2009). This could mean that any findings from the current experiment may not translate reliably into humans, thus, this should be considered when translating findings.

7.5.6 Conclusions

As hypothesised in **Section 7.1,** this chapter has shown that *Map2k7*$^{+/-}$ mice have a dysregulated immune response to Poly I:C exposure maternally and suggests this

may also occur in the developing embryonic brain. This further emphasises that Poly I:C interacts with the MKK7/JNK pathway, as *Map2k7*$^{+/-}$ mice have decreased MKK7 in the PFC and HC. These results do not show clearly whether embryonic genotype is important. This experiment would benefit from an increased number of animals and further molecular investigation; however, there is enough evidence to suggest that schizophrenia-related behavioural effects on offspring should also be pursued further. This could build on current findings on behaviour in offspring of rodents following exposure to infection, as well as providing information about how the schizophrenia risk gene, *Map2k7*, in combination with Poly I:C, will affect rodents' cognitive abilities.

Chapter 8 General Discussion

8.1 Summary of aims and results

This thesis set out to examine mice heterozygous for the *Map2k7* gene as a potential mouse model of relevance to schizophrenia. To achieve this, the face, construct and predictive validity was investigated by utilising translational behavioural tests and molecular techniques. These included: -

Chapter 3: Western blotting to identify alterations in protein pathway components of the MKK7/JNK pathway.

Chapter 4: investigation of *Map2k7*$^{+/-}$ mice in tasks that are thought to be sensitive to dopaminergic dysfunction and are strongly implicated with the positive symptoms of schizophrenia.

Chapter 5: examination of the attentional aspect of cognition using a highly translational, operant-based task.

Chapter 6: assessment of cognitive decision-making abilities of mice in the rodent version of the Iowa Gambling Task utilising the touchscreen operant equipment.

Chapter 7: examination of suitability of *Map2k7*$^{+/-}$ mice for a gene x environment interaction model by administering the viral mimetic Poly I:C during a critical period of gestation, and investigation of the subsequent immune response in the mother's serum and foetal brain.

Throughout this General Discussion, results will be summarised and a further discussion given, with ideas for future directions.

8.1.1 Summary of main findings

Chapter 3 – MKK7 signalling pathway protein quantification in Map2k7$^{+/-}$ *mice*

- MKK7γ was significantly decreased in the PFC of *Map2k7*$^{+/-}$ mice compared to their WT littermates
- Protein levels of all other pathway components examined (MKK7α, JNK, MKK4, tcJun and pc-Jun) were not altered in the PFC

- Minocycline had no significant effect on protein levels in the PFC, including that of the decreased MKK7γ.

Chapter 4 – Positive symptom-related tasks

Map2k7$^{+/-}$ mice did not exhibit a deficit in sensorimotor gating

- Acute injection (5mg/kg) amphetamine just prior to PPI testing decreased % PPI overall, which occurred less obviously in female and *Map2k7*$^{+/-}$ mice
- *Map2k7*$^{+/-}$ mice were hyperactive in the open field, which presented in male *Map2k7*$^{+/-}$ mice at a younger age than it did in female *Map2k7*$^{+/-}$ mice
- Treatment of minocycline for one week prior to open field testing decreased locomotor activity levels of all female WT and *Map2k7*$^{+/-}$ mice, but did not have a significant effect on males.

Chapter 5 – The 5-choice serial reaction time task

- *Map2k7*$^{+/-}$ mice presented with deficits in aspects of attentional function
- All mice appeared to be using a temporal strategy throughout training in this experiment as evidenced using a fixed ITI, which was prevented post-training by utilising a variable ITI
- *Map2k7*$^{+/-}$ mice showed enhanced motivation as evidenced by quicker latencies to collect reward and more entries into the reward magazine than WT mice
- Some aspects of attentional deficits showed signs of being alleviated by minocycline
- Ketamine administration had a detrimental effect on 5-CSRTT performance, but did not disrupt attentional performance specifically, or exacerbate deficits seen in *Map2k7*$^{+/-}$ mice.

Chapter 6 – The rodent gambling task

- *Map2k7*$^{+/-}$ and WT mice can learn the different contingencies of reward and punishment in the touchscreen rGT, validating the touchscreen equipment for use with this task in mice for the first time
- All mice initially exhibited a consistent pattern of optimal responding: they avoided high-risk, high-reward options more than the advantageous options of frequent, small rewards, with *Map2k7*$^{+/-}$ mice showing slightly less-risky choice behaviour than WT mice

- *Map2k7*$^{+/-}$ mice were hyperactive (increased number of beam breaks) compared to WTs when carrying out the task
- In contrast to the 5-CSRTT, *Map2k7*$^{+/-}$ mice did not show enhanced motivation in this task. Instead, they showed decreased entries into the reward magazine and similar latencies to collect the reward than WT mice
- Following a switch in subtle contingencies, all mice noticed the change and altered their pattern of responding accordingly and to the same extent in *Map2k7*$^{+/-}$ and WT mice
- Following a switch in extreme contingencies plus a decrease in punishment length for the risky choices, *Map2k7*$^{+/-}$ mice showed huge difficulty in switching their responding compared to WT mice, probably reflecting a deficit in cognitive flexibility combined with altered sensitivity to punishment and/or reward
- Administration of acute amphetamine prior to rGT testing altered choice preference for the different contingencies (with a modest differential effect between *Map2k7*$^{+/-}$ and WT mice), and altered overall performance measurements.

Chapter 7 – Investigation of a gene x environment risk factor model using maternal immune activation

- Gestating *Map2k7*$^{+/-}$ mice have a less well-controlled/regulated immune response to viral infection compared to gestating WT mice as evidenced by some elevated components of the immune response, suggesting they should be pursued further as a gene x environment interaction model
- Following Poly I:C exposure during a critical period of gestation, four cytokines were upregulated to a significantly higher extent in maternal plasma of *Map2k7*$^{+/-}$ mice compared to WTs (IL-2, IL-10, IL-12 and TNF-α).
- Despite the fact CCL5 and CXCL10 levels increased following Poly I:C in maternal serum to the same extent in *Map2k7*$^{+/-}$ and WT mice, levels of CCL5 (but not CXCL10) in the developing brain of embryos were differentially upregulated following maternal Poly I:C exposure in *Map2k7*$^{+/-}$ embryos from *Map2k7*$^{+/-}$ mothers compared to *Map2k7*$^{+/-}$embryos from WT mothers

- On the other hand, WT embryos from *Map2k7*$^{+/-}$ mothers did not have significantly increased CCL5 levels compared to WT embryos from WT mothers in response to Poly I:C
- Further experiments will be required to replicate and extend these findings.

8.2 Methodological considerations

Methodological considerations are primarily discussed in the relevant chapters; however, those which apply to all chapters are discussed below.

8.2.1 How the lack of Map2k7 *may have affected development*

It is important to consider the ways that lack of *Map2k7* from the beginning of development may have affected different systems within the brain, and indeed elsewhere in the body. As *Map2k7* was disrupted peripherally as well as within the CNS, and complete lack of *Map2k7* is embryonically lethal because of liver failure, there may be aspects of *Map2k7* heterozygous mice which do not match up with patients. Although patients with schizophrenia are 1.27 times more likely to suffer from chronic liver disease than the general population, this has been attributed to abuse of alcohol and other drugs, toxic increases in liver enzymes from atypical antipsychotics, and higher prevalence of diabetes in patients, rather than the underlying genetics (Hsu *et al.*, 2014).These potential peripheral effects do not cause gross abnormalities in *Map2k7*$^{+/-}$ mice because this would have been revealed by the SHIRPA tests carried out (**Section 1.11.1**); however, it is important to be aware that some subtle peripheral effects may exist.

In **Section 3.5.2**, the potential impact on the signalling pathway resulting from lack of MKK7 protein from the beginning of development was discussed. Additionally, in **Section 4.5.1**, the potential impact of *Map2k7* heterozygosity on neurotransmitter systems was briefly discussed. Without further molecular analyses, it is impossible to say how alterations in the MKK7/JNK pathway throughout development may affect neurotransmitter systems that manifest behaviourally in some tasks but not others. As the MKK7/JNK pathway is known to interact with NMDARs (Centeno *et al.*, 2007), deficiency in MKK7 is likely to affect activity levels of the glutamatergic system. Glutamate is the most abundant neurotransmitter in the mammalian brain and interacts closely with other major neurotransmitter systems, such as the dopaminergic, cholinergic, serotoninergic

and noradrenergic systems (Carlsson *et al.*, 1999). These interactions occur in brain regions that are known to be dysfunctional in schizophrenia, including the striatum and thalamus (Carlsson *et al.*, 1999). It is conceivable that mice lacking *Map2k7* with subsequent effects on glutamatergic systems could affect multiple other neurotransmitter systems within the brain, particularly as the lack of *Map2k7* has been present throughout the entirety of development.

Section 1.5.1 describes how the GABAergic system and gamma oscillations between the PFC and hippocampus are disrupted in patients with schizophrenia. Several parts of this thesis point towards the possibility of *Map2k7*$^{+/-}$ mice also showing deficits in this system. **Chapter 3** shows alteration of MKK7 protein the PFC, and we also showed that MKK7 is decreased in the hippocampus of *Map2k7*$^{+/-}$ mice (Openshaw *et al.*, 2017). Finally, in **Chapter 5**, *Map2k7*$^{+/-}$ mice presented with various deficits in attention, a process that is made efficient by normal gamma oscillations between the PFC and the hippocampus (Fries *et al.*, 2001; Williams & Boksa, 2010).

The speculations described above have not been backed up by experimental findings, but may provide a good basis for future experiments.

8.2.2 Sex differences

Most of the studies carried out in this thesis presented with different results depending on the sex of the mouse. Females tended to be more hyperactive (**Chapter 4**), had decreased startle amplitude (**Chapter 4**), exhibited slightly altered % PPI responses following amphetamine (**Chapter 4**) and showed different performance and motivation in parameters in the touchscreen (**Chapter 6**) compared to male mice. Effects of sex were always initially investigated and were considered during statistical analyses where necessary (this was required most of the time), but nevertheless, the effects were still there. Examining every effect of sex would have been beyond the scope of this thesis, but it must be recognized and future experiments could investigate these sex differences further.

The effects of sex observed here are not novel: it is well-established that female rodents are hyperactive compared to males (reviewed in Lightfoot, 2008); there is a well-known effect of sex on decision-making in humans (reviewed in van den Bos *et al.*, 2013), startle amplitude in mice (Plappert *et al.*, 2005) and PPI in humans (Swerdlow *et al.*, 1999). The oestrous cycle in females is thought to be

the main driver of these sex differences. Some studies using male and female mice ensure that female oestrous cycles are synced by housing them together in a separate room to males and then testing at specific stages in their cycle (Kokras & Dalla, 2014). Although this would be an important consideration to make for future studies, for some experiments this is not feasible. These include those where mice are tested every day and where mice must be housed in small groups to monitor the extent of food restriction carefully (e.g. operant-based experiments).

As there were so many sex differences, the need to use both sexes when carrying out behavioural experiments is reinforced. In fact, the importance of this has been the subject of reviews, as many studies continue to use males only in their behavioural studies to minimise variation caused by the oestrous cycle in females (Orsini & Setlow, 2017; Lightfoot, 2008; Kokras & Dalla, 2014). Unless experiments are for sex-specific disorders, this is unrepresentative of the population and important findings could be missed or misinterpreted.

8.3 Further discussion

8.3.1 Operant behaviour (Chapters 5 and 6)

There were some differences in the behaviour of *Map2k7*$^{+/-}$ and WT mice in the operant-based tasks. One major difference was that in the rodent GT, the *Map2k7*$^{+/-}$ mice did not show increased motivation for reward as they did in the 5-CSRTT. In the rGT they enter the reward magazine less on average than WT mice over the course of the session, and they are similar in their latencies to collect the reward to WT mice. In the 5-CSRTT on the other hand, *Map2k7*$^{+/-}$ mice were quicker to collect the rewards and made more entries into the reward magazine over the course of the session. This suggests that the *Map2k7*$^{+/-}$ mice may have enhanced motivation as suggested in **Section 7.4.2.2**, but that it is dependent on the circumstance, as the 9-hole operant box and the touchscreen exhibit key differences. For example, there are differences in the reward delivery process between the two types of operant boxes. In the rGT, there was an explicit tone paired with reward delivery, whereas this was not true for the 5-CSRTT. Although the 9-hole operant box makes a mechanical sound when the reward is being delivered, it is not as loud or much different in timbre to other noises that may be heard at the same time. Speculatively, it may be that when reward delivery is

less obvious, the WT mice are aware but *Map2k7*$^{+/-}$ mice may not be. However, in the touchscreen, all mice can establish a very clear distinction between when a reward is given and when it is not, so *Map2k7*$^{+/-}$ and WT mice know when reward is available to the same extent in this task and therefore show similar motivation. This could be why *Map2k7*$^{+/-}$ mice visit the reward magazine more frequently and are quicker to collect the reward in the 5-CSRTT: as there is not a reward there every time they visit the reward magazine, in their experience, if they are not very quick to collect the reward, it "disappears". Additionally, the 5-CSRTT reward amount was significantly more than the rGT reward amount (20µl vs. 7µl, respectively). This was because the reward volume was optimised for satisfaction vs. satiation. In both tasks, the maximum number of trials that they could (and the vast majority of mice did) complete was 100. For the rGT, the volume of strawberry milkshake could be as much as 4 x the normal amount per trial and was therefore made to be less than in the 5-CSRTT to avoid the mice becoming satiated. This could also suggest that *Map2k7*$^{+/-}$ mice are more sensitive to the amount of reward given and may have worked faster and checked that reward had been delivered more often in the 5-CSRTT, especially because it was less obvious when a reward was there.

Additionally, *Map2k7*$^{+/-}$ mice showed evidence of hyperactivity with both direct examination in the open field and secondary measures in the 5-CSRTT (faster response latencies and increased number of beam breaks), compared to WT mice. There is strong evidence that the mesolimbic dopaminergic system disruption produces hyperactivity in rodents and that this is attributed to the positive symptoms of schizophrenia (van den Buuse, 2010), so this could be a focus for future investigations.

8.3.2 Minocycline shows signs of alleviating some deficits in Map2k7$^{+/-}$ *mice*

Throughout this thesis, minocycline was administered in an attempt to reverse deficits seen in *Map2k7*$^{+/-}$ mice on three occasions. Minocycline did not cause a significant change in MKK7/JNK pathway components in the PFC, but it did show signs of decreasing locomotor activity levels in the open field of all mice (apart from males, who were not as hyperactive as females), and some aspects of attentional deficits. All effects caused by minocycline were subtle; however, the deficits seen in *Map2k7*$^{+/-}$ mice were relatively modest in the first place. Nevertheless, it would be interesting to find out whether longer-term treatment

of minocycline would be further beneficial to *Map2k7*$^{+/-}$ mice and what the time scale of its effects are following cessation of treatment.

The effects and potential mechanism of minocycline in the individual experiments are discussed in the relevant chapters and summarised here. It is possible for minocycline to interact with the MKK7/JNK pathway as it has previously been shown to activate MKK7 in cultured neuronal cells (spoken communication with Prof. Brian Morris, unpublished data), and inhibit JNK1/2 in microglia following lipopolysaccharide stimulation (Nikodemova *et al.*, 2006). However, in **Chapter 3**, the levels of MKK7/JNK pathway components were not significantly altered by minocycline, which may be because it was acting in a cell- and/or stimulus-specific manner and might not have been strong enough to be identified via Western blotting. Alternatively, it has been shown that minocycline can act via alternative pathways; for example, by affecting a non-MKK7/JNK pathway (such as p38 MAPK) downstream of NMDA receptors (Chaves *et al.*, 2009). In a case such as this, it is unlikely that an alteration in protein levels in the MKK7/JNK pathway would be detected. Finally, one week may not be a long-enough time scale for it to alter protein levels in this way, or as much as we can detect using Western blotting. After all, the *Map2k7* heterozygosity has been present since the very beginning of development, so one week's treatment of minocycline may not be enough to induce detectable differences in individual pathway components. It is arguably more likely that minocycline is working via some other mechanism rather than directly affecting the up- or down-regulation of MKK7/JNK pathway components.

Minocycline has been shown to improve cognitive deficits in humans (Sofuoglu *et al.*, 2011) and mice (Giovanoli *et al.*, 2016; Fujita *et al.*, 2008; Levkovitz *et al.* 2007). Additionally, minocycline reduced open field locomotor activity levels in rats (Kofman *et al.*, 1990) and mice (Dansie *et al.*, 2013; Chen *et al.*, 2009; Zhang *et al.*, 2007). Despite understanding its behavioural effects, the exact and complete mechanism of action of minocycline is still unknown. Two main mechanisms have been proposed: inhibition of the proinflammatory function of microglia and/or enhancing glutamate release via NMDARs (Liu *et al.*, 2014; Lisiecka *et al.*, 2015). It is entirely conceivable that either, or a combination of both mechanisms are relevant here because of the potential for them both to interact with the MKK7/JNK pathway. The MKK7/JNK pathway is essential for the

pro-inflammatory function of microglia (Waetzig *et al.*, 2005) and in **Chapter 7**, *Map2k7*$^{+/-}$ mice showed an altered cytokine response to Poly I:C. Additionally. NMDARs are located upstream of the MKK7/JNK pathway (Centeno *et al.*, 2007), suggesting that altering the immune response and/or NMDAR activation states via minocycline have potential to affect indirect regulation of the MKK7/JNK pathway in order to produce a cognitive enhancing and locomotor (i.e. positive symptom) decreasing effect. This is backed up by a study by Zhang *et al*. (2007), who showed that administration of minocycline prior to the NMDAR antagonist dizocilpine decreased locomotor activity levels and improved PPI deficits in mice. This suggests that minocycline was acting via NMDARs in this case, and is conceivable that could be what is happening in *Map2k7*$^{+/-}$ mice, too.

Overall, gaining knowledge of the mechanism of minocycline and how it has acted to show signs of improving attention and decreasing locomotor activity will benefit from more sensitive methods of protein quantification and longer-term studies. In general, more molecular and clinical evidence on minocycline's potential as an agent to improve deficits relating to schizophrenia is needed. However, our data support the concept that this drug is effective in improving some aspects of attentional and mesolimbic dopaminergic function.

8.3.3 Is MKK7 a feasible target for novel drugs?

The gene knockout studies described in **Section 1.9.1** reveal the potential physiological/pathophysiological roles of the MKK7/JNK pathway. This pathway has been identified as being a suitable drug target for several disorders, including arthritis, cardiac hypertrophy, asthma, Parkinson's disease and Alzheimer's disease (summarised in Kumar *et al.*, 2015). JNK was originally viewed as the more obvious target, but it was soon realised that it causes toxicity easily and the inhibition of JNK1, 2 and 3 is not easy to control and does not occur in therapeutic ratios (Davies & Tournier, 2012). The MKK7/JNK pathway maintains its specificity by forming signalling modules with relevant pathway components and by their subcellular localisation (Coffey, 2014). Therefore, compounds that target MKK7 may be more attractive in order to more selectively affect output of the MKK7/JNK pathway. Specific targeting of MKK7 has been achieved by Sogabe *et al*. (2015), which shows promise in altering output of the pathway selectively whilst sparing the many other crucial processes this pathway is involved with. As the MKK7γ isoform was the only isoform of MKK7 decreased in *Map2k7*$^{+/-}$ mice identified by

Western blotting, it would be interesting to target this specifically, to see if this improves the cognitive and positive phenotypes, and the differential immune response to Poly I:C these mice possess; although so far, such a compound has not been developed.

8.4 Conclusions and future directions

This thesis has shown that *Map2k7*$^{+/-}$ mice: have decreased MKK7 in the PFC, exhibit hyperactivity, attentional deficits, deficits in reversal learning-based decision-making and show an altered cytokine response to maternal immune activation, compared to their WT littermates. Overall, they show a pattern remarkably similar to some aspects of the symptoms of schizophrenia in humans, and they have the genetic basis for a model of relevance to schizophrenia with good construct validity, arguably the most important aspect of an animal model (discussed in Nestler & Hyman, 2010). This is because *Map2k7*$^{+/-}$ mice are a model relevant to a genetic variant that significantly increases disease risk (OR = 1.9) and is also moderately common in the population, where most other SNPs are less common and do not infer such a high risk (Winchester *et al.*, 2012). Minocycline shows signs of improving deficits in *Map2k7*$^{+/-}$ mice in some tasks (the 5-CSRTT, and locomotor activity in the open field), and *Map2k7*$^{+/-}$ mice show promise for being utilised as a gene x environmental risk factor model, which will give a particularly relevant insight into the complex molecular and genetic interplay that occurs in schizophrenia in humans.

As well as behaviourally, in the future it will be necessary to investigate molecularly and morphologically the effects of the *Map2k7* deletion. These studies could focus on aspects that have been gleaned from mice with genetic manipulations in other members of the MAP kinase pathway, for example axonal growth and neuronal migration. **Chapter 7** in particular opens up many new questions and possibilities for further study. For example, investigation into the role of the placenta in mechanisms of maternal immune activation in *Map2k7*$^{+/-}$ mice is ongoing, and future experiments will look at how Poly I:C administration during gestation will affect behaviour in offspring. It is hypothesised that the offspring will exhibit exacerbated phenotypes with relevance to schizophrenia.

The mice studied throughout this thesis were based on findings from a schizophrenia genetic association study (Winchester *et al.*, 2012), and therefore

show good construct validity for schizophrenia; however, components of the MKK7/JNK pathway have also been implicated in other neuropsychiatric/neurodegenerative disorders such as Alzheimer's disease (Yarza *et al.*, 2016) and autism (de Anda *et al.*, 2012). *Map2k7*$^{+/-}$ mice show face validity for ADHD, too, with hyperactivity and deficits in attentional function. Approaching this as the RDoC initiative suggests (see **Section 1.4**), rather than attempting to replicate an entire complex disorder in one animal model, will provide relevant information that can span multiple disorders, assuming that the domains share similar underlying mechanisms across species and disorders (Young *et al.*, 2010).

Animal models are fundamental to the study of the neurobiology of neuropsychiatric disorders (McArthur, 2017). *Map2k7*$^{+/-}$ mice show utility for dissecting the cognitive deficits and positive symptoms of schizophrenia that could be targeted by novel compounds, which would be aimed at restoring the function of the MKK7/JNK pathway. Although no mouse model can recapitulate the full symptom spectrum of a human neuropsychiatric disorder, *Map2k7*$^{+/-}$ mice exhibit an interesting accumulation of phenotypic abnormalities relevant to schizophrenia and should be explored further.

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Appendix

A1: Chapter 4, Table of P Values

	p Values			
Parameter	**Genotype (WT/HZ)**	**Sex (M/F)**	**Age (Adolescent/Adult)**	**Genotype x Sex**
Distance Travelled (cm)	<0.001, $f_{(1,688)}$=33.11	<0.001, $f_{(1,688)}$=76.97	<0.001, $f_{(1,688)}$=97.98	0.337, ns
Velocity (cm/s)	<0.001, $f_{(1,688)}$=33.40	<0.001, $f_{(1,688)}$=76.55	<0.001, $f_{(1,688)}$=94.80	0.339, ns
Duration Immobile (s)	<0.001, $f_{(1,688)}$=19.74	<0.001, $f_{(1,688)}$=163.62	<0.001, $f_{(1,688)}$=377.18	0.242, ns
Duration Mobile (s)	0.032, $f_{(1,688)}$=4.59	0.096, ns	<0.001, $f_{(1,688)}$=252.16	0.192, ns
Duration Highly Mobile (s)	<0.001, $f_{(1,688)}$=42.74	<0.001, $f_{(1,688)}$=206.28	<0.001, $f_{(1,688)}$=13.09	0.875, ns

	p Values cont.		
Parameter	**Genotype x Age**	**Sex x Age**	**Genotype x Sex x Age**
Distance Travelled (cm)	0.026, $f_{(1,688)}$=5.00	<0.001, $f_{(1,688)}$=41.85	<0.001, $f_{(1,688)}$=22.50
Velocity (cm/s)	0.01, $f_{(1,688)}$=4.88	<0.001, $f_{(1,688)}$=42.41	<0.001, $f_{(1,688)}$=22.29
Duration Immobile (s)	0.479, ns	0.006, $f_{(1,688)}$=7.60	0.245, ns
Duration Mobile (s)	0.118, ns	<0.001, $f_{(1,688)}$=33.40	<0.001, $f_{(1,688)}$=20.54
Duration Highly Mobile (s)	0.397, ns	<0.001, $f_{(1,688)}$=71.72	<0.001, $f_{(1,688)}$=32.20

Table A1. P-values from Age analysis part of Open Field experiment. Data were analysed using a three-way ANOVA, with genotype and sex as between subjects factors, time bin (2 and 3; 15 minutes each; test phase only) and age as a within subjects factors and each individual mouse nested within genotype and sex.

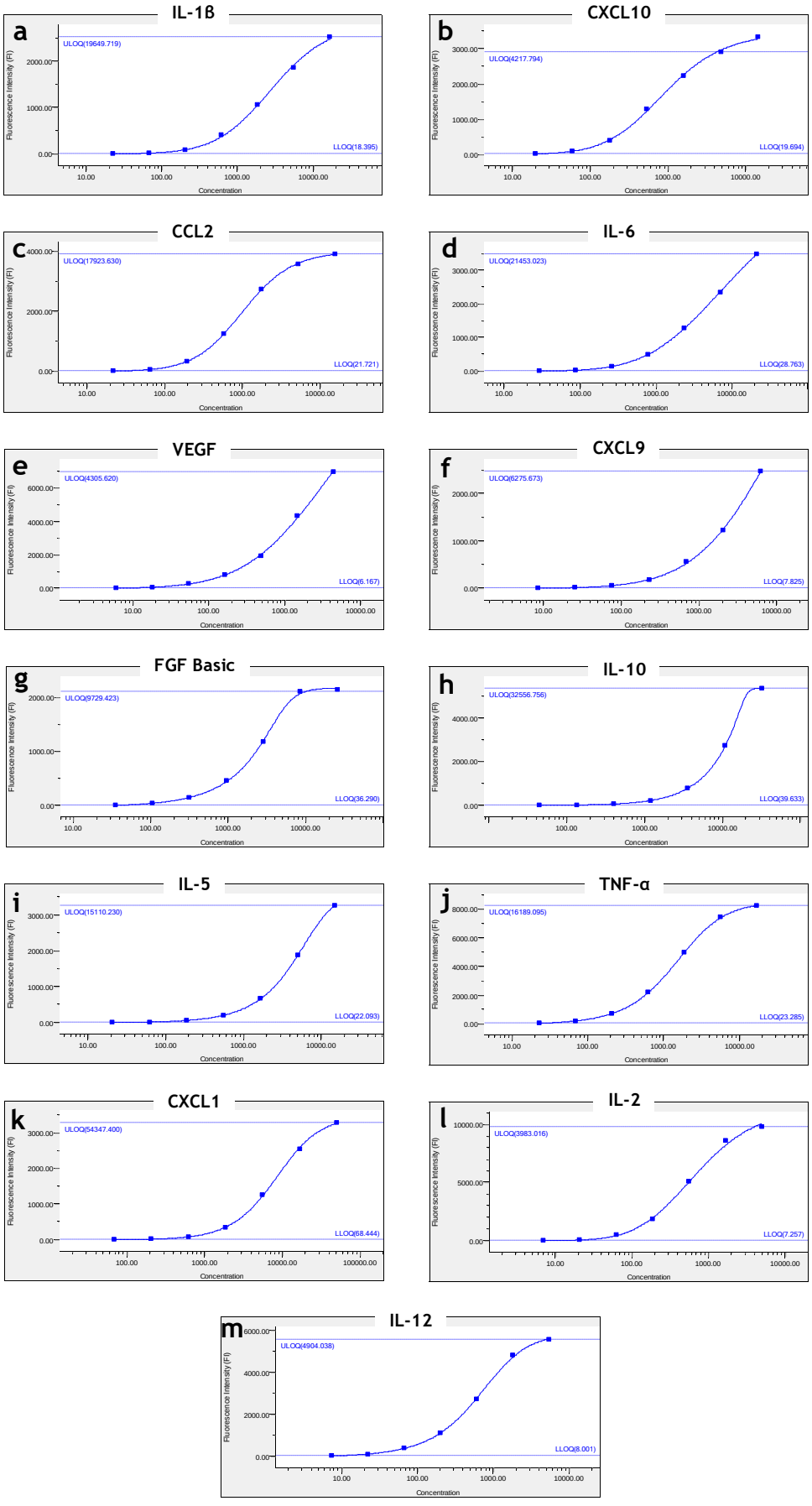
	p Values			
Parameter	**Genotype (WT/HZ)**	**Sex (M/F)**	**Treatment (Water/Mino)**	**Genotype x Sex**
Distance Travelled (cm)	<0.001, $f_{(1,314)}$=16.84	<0.001, $f_{(1,314)}$=199.26	0.001, $f_{(1,314)}$=12.21	<0.001, $f_{(1,314)}$=16.98
Velocity (cm/s)	<0.001, $f_{(1,314)}$=16.97	<0.001, $f_{(1,314)}$=200.12	0.001, $f_{(1,314)}$=12.19	<0.001, $f_{(1,314)}$=17.10
Duration Immobile (s)	0.048, $f_{(1,314)}$=3.93	<0.001, $f_{(1,314)}$=210.51	0.102, ns	0.634, ns
Duration Mobile (s)	<0.001, $f_{(1,314)}$=16.96	<0.001, $f_{(1,314)}$=135.94	0.006, $f_{(1,314)}$=7.81	<0.001, $f_{(1,314)}$=19.25
Duration Highly Mobile (s)	<0.001, $f_{(1,314)}$=21.47	<0.001, $f_{(1,314)}$=401.72	0.001, $f_{(1,314)}$=11.37	<0.001, $f_{(1,314)}$=13.41

p Values cont.

Parameter	**Genotype x Treatment**	**Sex x Treatment**	**Genotype x Sex x Treatment**
Distance Travelled (cm)	0.549, ns	<0.001, $f_{(1,314)}$=45.04	<0.001, $f_{(1,314)}$=13.66
Velocity (cm/s)	0.521, ns	<0.001, $f_{(1,314)}$=45.03	<0.001, $f_{(1,314)}$=13.42
Duration Immobile (s)	0.037, $f_{(1,314)}$=4.39	<0.001, $f_{(1,314)}$=27.28	0.004, $f_{(1,314)}$=8.30
Duration Mobile (s)	<0.001, $f_{(1,314)}$=25.11	0.007, $f_{(1,314)}$=7.26	0.267, ns
Duration Highly Mobile (s)	0.034, $f_{(1,314)}$=4.53	<0.001, $f_{(1,314)}$=37.06	0.002, $f_{(1,314)}$=9.47

Table A2. P-values from Treatment part of Open Field experiment. Data were analysed using a three-way ANOVA, with genotype, sex and treatment as within subjects factors, time bin (2 and 3; 15 minutes each; test phase only) as a between subjects factor and each individual mouse nested within genotype, sex and treatment.

A2: Chapter 7, Standard Curves from Luminex Assay



< Figure A1: The standard curves of each cytokine from the Luminex assay containing maternal plasma, including their lower and upper limit of quantification.

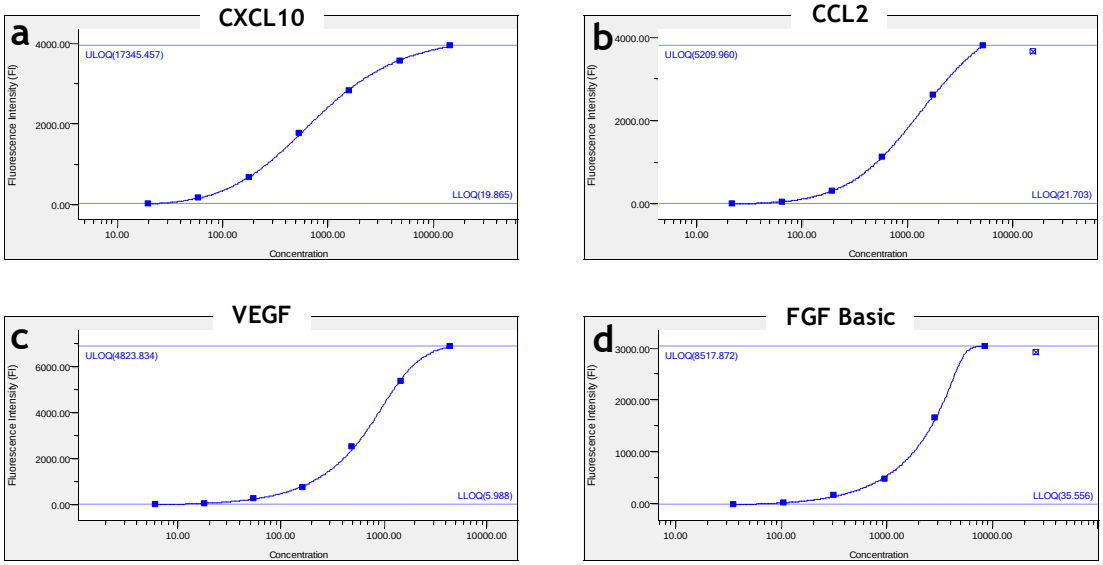


Figure A2: The standard curves of each cytokine from the Luminex assay containing embryonic brain tissue supernatant, including their lower and upper limit of quantification.