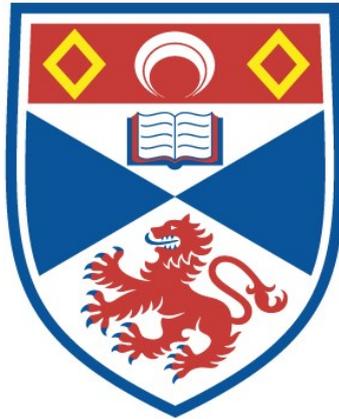


***IN VIVO AND IN VITRO* STUDIES OF  
POSITIVE ALLOSTERIC MODULATION OF  
THE NDMA RECEPTOR**

**Casmira T. Brazaitis**

**A Thesis Submitted for the Degree of Phd  
at the  
University of St Andrews**



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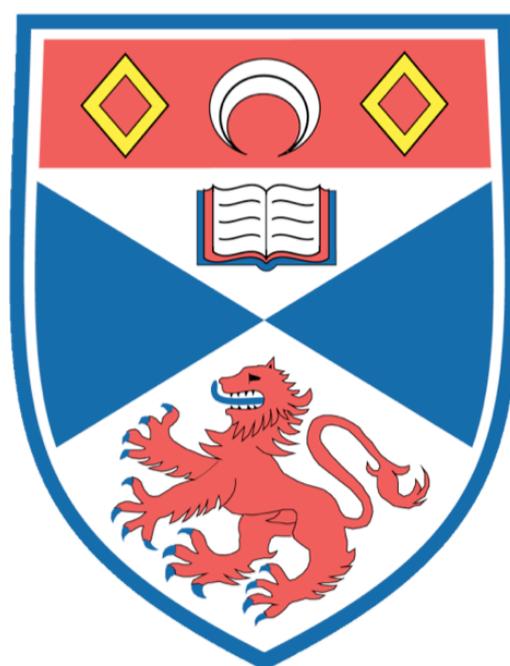
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# *In Vivo* and *in Vitro* Studies of Positive Allosteric Modulation of the NMDA Receptor

Casmira T. Brazaitis



This thesis is submitted in partial fulfilment for the degree  
of PhD at the University of St Andrews

Date of Submission

September 2016

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# Abbreviations

AC	adenylyl cyclase
AD	Alzheimer's disease
ADHD	attention deficit hyperactivity disorder
AM	acetoxymethyl
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ApoE	apolipoprotein E
APV	(2 <i>R</i> )-amino-5-phosphonovaleric acid
ATD	amino-terminal domain
BDNF	brain-derived neurotrophic factor
cAMP	cyclic AMP
CANTAB	Cambridge Neuropsychological Test Automated Battery
CGN	cerebellar granule neuron
CI	confidence interval
CNO	clozapine-N-oxide
CSF	cerebrospinal fluid
CTD	carboxyl-terminal domain
DAB	3,3'-Diaminobenzidine

DAPI	4',6-diamidino-2-phenylindole
df	degrees freedom
DI	discrimination index
DMS	dorsomedial striatum
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
D $\alpha$ AA	D- $\alpha$ -aminoadipate
EAA	excitatory amino acid
EAAT	excitatory amino acid transporter
ED	extradimensional
EPSC	excitatory post synaptic current
EPSP	excitatory post synaptic potential
GABA	<i>gamma</i> -Aminobutyric acid
GPCR	G-protein coupled receptor
HD	Huntington's disease
HET	heterozygote
HPBCD	hydroxypropyl beta-cyclodextrin
htt	huntingtin protein
ID	intradimensional
i.p.	intraperitoneal
LBD	ligand binding domain

LTD	long term depression
LTP	long term potentiation
LXR	liver X receptor
MCI	mild cognitive impairment
mAChR	muscarinic acetylcholine receptor
mGluR	metabotropic glutamate receptor
mhtt	mutant huntingtin protein
mPFC	medial prefrontal cortex
MSNs	medium spiny neurons
NMDA	N-methyl-D-aspartate
NOR	novel object recognition
NPR	novel place recognition
OFC	orbitofrontal cortex
PAM	positive allosteric modulator
PB	Phosphate buffer
PBS	phosphate buffered saline
PCP	phencyclidine
PDL	poly-d-lysine
pen/strep	penicillin/streptomycin
PFC	prefrontal cortex

RASSLs	Receptors Activates Solely by Synthetic Ligands
S-R	stimulus-response
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STS	sulfatase
SULT	sulfotransferase
TMD	transmembrane domain
TTC	trials to criterion
WCST	Wisconsin Card Sorting Task
WT	wildtype

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# Ethical Approval

All licenced procedures conformed to the Animals (Scientific Procedures) Act (1986). Licenced procedures on mice were carried out under PPL 70-8142 and PIL 60/13979, and licenced procedures on rats were conducted under PPL 60-4459 and PIL 60/13979, approved by the Animal Welfare Ethics Committee of the University of St Andrews.

# Abstract

Dysfunction of the N-methyl-D-aspartate (NMDA) receptor is thought to contribute to the cognitive deficits of many neurodegenerative diseases and psychiatric disorders. Cognitive symptoms of Alzheimer's disease can be treated with NMDA receptor antagonists or drugs targeting the cholinergic system; however, there are no effective treatments for cognitive deficits of schizophrenia or Huntington's disease. With the discovery of a potent and selective allosteric modulator of the NMDA receptor, there is the possibility of new treatments based on NMDA receptor functional-enhancement through neuroactive steroids, closely related in structure to the endogenous neurosteroid, cerebrosterol. The aim of this thesis was to examine steroidal modulation of the NMDA receptor both *in vitro* and *in vivo*. In chapter 2, NMDA receptor enhancement of both the synthetic and endogenous neuroactive steroids was assessed in neurons maintained in cell culture using calcium imaging techniques. Sulphation of the steroids greatly increased the efficacy of NMDA receptor enhancement compared to the unsulphated steroids. Chapters 3 and 4 investigate the potential for neuroactive steroids to treat cognitive impairments of Huntington's disease. Using a mouse model, tests were selected that were analogous to those in which patients are impaired; however, no impairments were found in the mouse model. Chapter 5, therefore, used a different model of cognitive impairment – namely, rats with a set-shifting impairment, as is seen in many psychiatric and neurological disorders, including Huntington's disease – to assess the effect of the synthetic steroid administration. Unfortunately, the rats did not show the expected impairment. The lack of reliable animal models compromised testing the efficacy of these promising NMDA receptor positive allosteric modulators. Nevertheless, the promising *in vitro* results suggest that there could still be therapeutic potential. In addition, the compound is a useful research tool for exploring NMDA receptor function in health and disease.

# Chapter 1

## General Introduction

“No one really starts anything new, Mrs. Nemur. Everyone builds on other men's failures. There is nothing really original in science. What each man contributes to the sum of knowledge is what counts.” – Daniel Keyes, *Flowers for Algernon* for Algernon



Illustration by C. Brazaitis

The discovery of a safe and effective cognitive-enhancing drug has been the focus of decades of neuroscience and pharmacology research to treat the cognitive deficits associated with a multitude of neurodegenerative diseases and psychiatric disorders. One treatment approach targets the glutamate system, where receptors and transporters of glutamate have been the focus of drug targets. This introduction will provide an overview of the glutamatergic system in health and disease, and describe why the N-methyl-D-aspartate (NMDA) receptor is a particularly good target, but will also explain the pitfalls that may occur with this approach. Then, an overview will be given of the tasks used to measure cognition, using the deficits seen in Huntington's disease (HD) as a focus disease model. Previous attempts to develop drugs targeting the NMDA receptor will be discussed, and finally, the background will be given to the molecules that are the focus of this thesis. As these molecules are also found endogenously, in addition to being used as possible treatments, their effects could help elucidate NMDA receptor modulation in health and disease states.

## 1.1 NMDA Receptors: History, Form and Function

### 1.1.1 Glutamate System

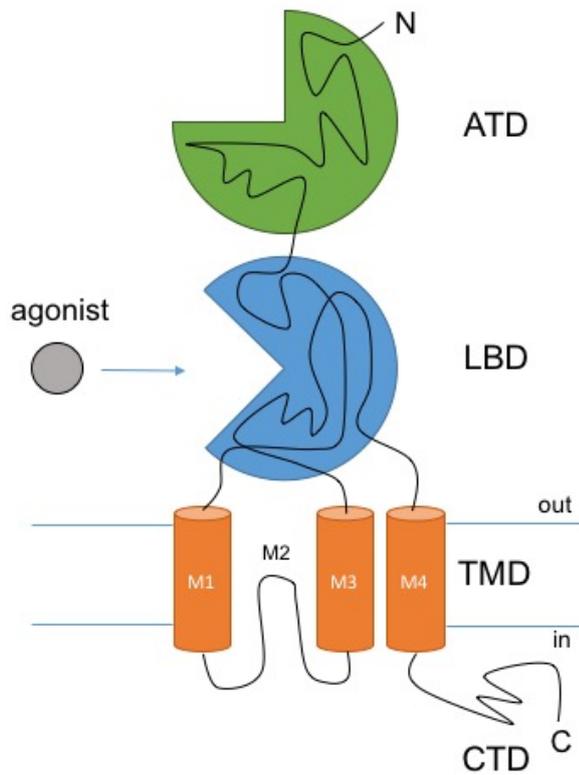
Glutamate is an excitatory amino acid (EAA) and is the major excitatory neurotransmitter in the mammalian central nervous system (Watkins and Jane, 2006). Glutamate is the most abundant amino acid in the brain (Zhou and Danbolt, 2014). The first clues that glutamate acts as a neurotransmitter were discovered in the 1940s in Japan by Hayashi (1954) (as cited by Takagaki (1996) and Watkins and Jane (2006)), who found that directly injecting glutamate into the motor cortex of dogs resulted in convulsions. Later, Watkins published a series of papers showing further support for the excitatory action of glutamate on neuronal activity using recordings from *in vivo* cat spinal cord neurons and isolated toad spinal cord preparations (Curtis

et al., 1961, Curtis et al., 1960, Curtis and Watkins, 1960). N-methyl-D-aspartate (NMDA) was the first synthetic ligand found, and it was proposed that it caused membrane depolarisation by binding to the receptor, causing a conformational change which opens a central ion pore, allowing for the influx of sodium ions (Curtis and Watkins, 1960, Curtis and Watkins, 1965). Later, other synthetic agonists were discovered such as kainate (Shinozaki and Ishida, 1981) and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Honore et al., 1982, Krogsgaard-Larsen et al., 1982). However, it wasn't until the availability of selective glutamate receptor antagonists that the existence of multiple receptors could be confirmed, where magnesium ( $Mg^{2+}$ ) was the first antagonist to separate NMDA receptors from the others, as addition of  $Mg^{2+}$  to the isolated frog spinal cord caused a dramatic decrease in NMDA-induced, but not kainate- or AMPA-induced responses (for review see: Watkins and Jane, 2006). Later the NMDA receptor specific antagonist D-2-amino-5-phosphonopentanoate (APV) was discovered, as well as the AMPA receptor selective and potent quinoxalinediones (Honore et al., 1988). Biscoe et al. (1977) crucially showed that the selective NMDA antagonist D- $\alpha$ -amino adipate ( $D\alpha AA$ ) could block non-cholinergic, but not cholinergic, excitation in cat spinal cord Renshaw cells *in vivo* showing that glutamate is a neurotransmitter of synaptic NMDA receptors. At the time aspartate, another EAA, was hypothesised as the endogenous neurotransmitter. However, Olverman et al. (1984) showed that glutamate had a 10-fold higher affinity for the NMDA receptor than aspartate, promoting it to the position of the major excitatory neurotransmitter in the brain.

Glutamate binds to two different classes of receptors in the CNS: metabotropic and ionotropic glutamate receptors. Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors, which activate second messenger systems in the post synaptic neuron and couple to specific ion channels (Conn and Pin, 1997, Niswender and Conn, 2010). Depending on the mGluR subtype, distinct second messenger pathways are activated: group I

activate the phospholipase C and phosphoinositide pathways, or the adenylyl cyclase (AC) pathway; group II and III inhibit AC (Conn and Pin, 1997, Niswender and Conn, 2010). These different pathways lead to a variety of cellular processes being activated or inhibited.

As suggested above, ionotropic receptors can also be further divided into three categories: AMPA, kainate, and NMDA receptors. Each receptor is an integral membrane protein made up of four large subunits encoded by different gene families (Alexander et al., 2008, Dingledine et al., 1999, Traynelis et al., 2010). Distinct from the metabotropic receptors, binding of a ligand to the receptor directly gates an associated cation-conducting ion channel, which allows permeation of specific ions in and out of the cell. Ionotropic receptors consist of an ion channel (transmembrane domain, TMD), a ligand binding (or agonist binding) domain (LBD), and an amino terminal (or modulatory) domain (ATD) (Figure 1.1). The ligand or agonist (e.g. glutamate) binds to the LBD, causing a conformational change that opens the ion channel allowing passage of ions. All three receptor subtypes allow influx of sodium ions ( $\text{Na}^+$ ) and efflux of potassium ions ( $\text{K}^+$ ) upon activation by an agonist, resulting in depolarisation of the neuron, which can be detected as an excitatory post synaptic potential (EPSP). Note certain receptor isoforms are additionally permeable to calcium ions ( $\text{Ca}^{2+}$ ) (see next section 1.1.2). AMPA receptors, in particular, mediate the fast depolarisation component of the EPSP (Lester et al., 1990). In addition to gating ion channels, ionotropic receptors can also activate intracellular pathways, as shown by the activation of the Mitogen-activated protein kinase (MAPK) pathway by the AMPA receptor (Hayashi et al., 1999). All three receptor subtypes share a similar protein sequence, suggesting that they share a similar structure (Traynelis et al., 2010).



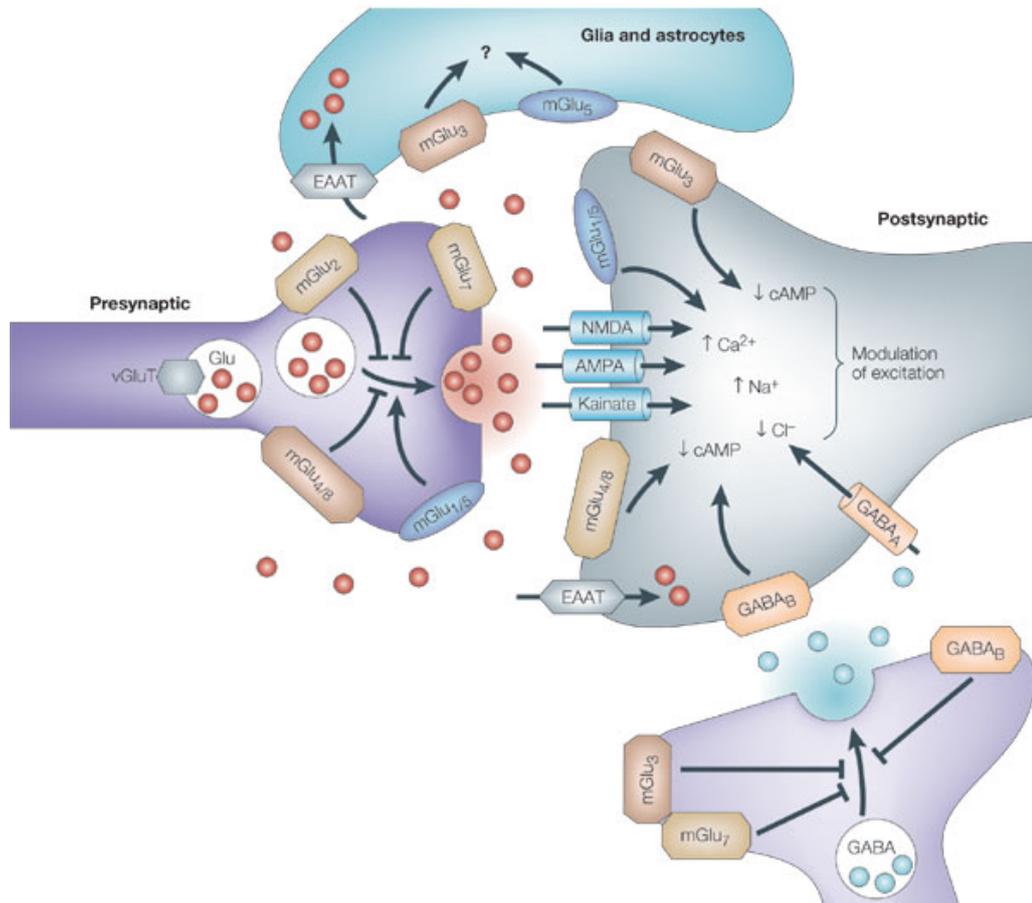
**Figure 1.1: A schematic of the structure of one of the four subunits of an ionotropic glutamate receptor.** Depicted are the extracellular amino terminal domain (ATD, green) and ligand-binding domain (LBD, blue), the transmembrane domain (TMD) consisting of membrane-spanning helices (M1, M3, and M4, orange) and the membrane re-entrant loop (M2). The intracellular C-terminal domain (CTD) is also shown. Adapted from Traynelis et al. (2010).

Glutamate is released from nerve terminals via exocytosis of synaptic vesicles (Danbolt, 2001) into the extracellular space, and binds to specific glutamate receptors expressed on neuronal and glial cell membranes. Glutamate can also be released via non-vesicular mechanisms, such as the reversal of the glutamate reuptake system, as occurs during cerebral ischaemia (Rossi et al., 2000) or via anion channels (Wang et al., 2013). It is also possible, though debated, that astrocytes are able to release glutamate via exocytosis (Bezzi et al., 2004, Zhou and Danbolt, 2014). The concentrations of extracellular glutamate are tightly regulated to enhance the transmission of the signal (Danbolt, 2001).

Following release of glutamate into the synaptic cleft, controlled regulation of glutamate levels is achieved by the efficient reuptake of glutamate from the synaptic cleft by specific glutamate transporters (see Figure 1.2). This controlled regulation also prevents excitotoxicity caused by an excess of extracellular glutamate (Danbolt, 2001). If glutamate uptake is inhibited, the constant release of glutamate into the extracellular fluid causes glutamate build up in a matter of seconds (Jabaudon et al., 1999). On the other hand, too little glutamate is also deleterious as it is required for healthy synaptic transmission; therefore, a delicate balance is required. The transporters that are responsible for efficient glutamate reuptake are called excitatory amino acid transporters and there are 5 subtypes (EAAT 1-5), each expressed in specific brain regions and cells (Kim et al., 2011). The most common transporter is EAAT2, which is largely expressed in astrocytes and is responsible for the majority of glutamate reuptake from glutamatergic synapses (Kim et al., 2011). Low EAAT2 expression in the hippocampus, temporal lobe (Shan et al., 2013), and prefrontal cortex (Spangaro et al., 2012) has been associated with schizophrenia. Despite the EAATs, low ambient levels of glutamate are still present extracellularly, and Le Meur et al. (2007) showed that these ambient extrasynaptic glutamate levels were able to activate specific extrasynaptic glutamate receptors in the rat hippocampus.

Glutamate receptors are expressed both in the synapse and outwith the synapse; whereby, the extrasynaptic receptors may be activated by synaptic spill-over or glutamate released outside of the synapse (Petralia, 2012).

Before the glutamate is cleared from the cleft, it is able to bind to receptors on the post synaptic neuron. These are the metabotropic and ionotropic receptors introduced above. The metabotropic receptors transmit signals slower, as second messenger systems result in modulation of synaptic transmission and excitability (Niswender and Conn, 2010). In contrast, when glutamate binds to the ionotropic receptors, depolarisation of the postsynaptic neuron causes rapid signal transmission. The AMPA and kainate receptors are activated first, followed by the NMDA receptors. NMDA receptor function will be discussed in detail in the next section.



**Figure 1.2: A summary of the glutamatergic synapse.** The transmission of the signal moves from the presynaptic to the postsynaptic neuron. Glia and astrocytes act as support cells to remove glutamate from the cleft via excitatory amino acid transporters (EAAT). GABAergic neurons serve a modulatory purpose. After glutamate is released from the presynaptic neuron, molecules activate AMPA and kainate receptors, the resulting depolarisation allows the activation of the NMDA receptor which is additionally permeable to calcium ions. The ionotropic receptors mediate the fast transmission of signal. Metabotropic glutamate receptors (mGluR) mediates the slow transmission of signal causing downstream changes due to excitation. Reproduced with permission (Swanson et al., 2005).

### 1.1.2 NMDA Receptor

The NMDA receptor is a tetrameric protein with two binding sites for the endogenous agonist glutamate (or aspartate) and the co-agonist glycine on the LBD (Paoletti et al., 2013, Laube et al., 1998). The LBD of the subunit is often described as a clamshell (also illustrated in Figure 1.1) where the agonist binds and causes the clamshells to close (conformational change) which is tightly linked to channel opening (Traynelis et al., 2010). There are seven types of subunits which make up the receptor: NR1, NR2 (A, B, C, D), and NR3 (A, B).

These receptors assemble in a heteromeric fashion, where a receptor always contains two NR1 subunits and two regulatory subunits of either NR2, or NR2 and NR3 subunits combined (Yashiro and Philpot, 2008). NMDA receptor function varies depending on the subunit composition. Glycine, the co-agonist, binds to NR1 subunits, while glutamate, the agonist, binds to NR2; the kinetics and pharmacology also differ depending on subunit composition (Cull-Candy et al., 2001). Receptors consisting of NR1/NR2A and B subunits have a high single channel conductance and high  $Mg^{2+}$  sensitivity, while NR1/NR2C and B subunit assemblies have a reduced conductance and  $Mg^{2+}$  sensitivity (Sieglar Retchless et al., 2012). Incorporation of the NR3 subunit causes a marked decrease in single channel conductance,  $Ca^{2+}$  permeability, and  $Mg^{2+}$  sensitivity (Sasaki et al., 2002, Paoletti and Neyton, 2007). Due to differences in pharmacology, agonists and antagonists specific to certain subunits can be used to identify receptor assembly. For example, ifenprodil is a selective NR2B antagonist (Williams, 1993), and zinc is a selective NR2A antagonist at nanomolar concentrations (Paoletti et al., 1997). This can be used to examine NMDA receptor properties during development, differences in synaptic and extrasynaptic receptors (see also section 1.2.4), and to assess NMDA subtypes during specific functions such as synaptic plasticity (Cull-Candy et al., 2001).

A key difference between the activation of the NMDA receptor and other ionotropic receptors is its ability to act as a co-incidence detector, and in that it is permeable to  $Ca^{2+}$ . The NMDA receptor is able to act as a co-incidence detector because ion conduction is both voltage sensitive (Nowak et al., 1984, Mayer et al., 1984) and requires both glutamate and glycine binding for the channel to open (Laube et al., 1998). When the ion channel does open, it is permeable to  $Ca^{2+}$  (in addition to  $Na^+$  and  $K^+$ ), which flows into the cell activating many downstream pathways (Mayer et al., 1987, Kawamoto et al., 2012, Wang and Zhang, 2012). The voltage sensitivity is caused by a magnesium ion, which directly blocks the ion channel when the cell is in a resting state (Nowak et al., 1984, Mayer et al., 1984). Upon sufficient

membrane depolarisation by AMPA receptor activation, the  $Mg^{2+}$  is dislodged from the ion channel, and as long as sufficient agonist (i.e. glutamate) and co-agonist (i.e. glycine) are present, the ion channel is active. Therefore, the NMDA receptor requires both presynaptic glutamate release and post synaptic depolarisation to become active. This is why AMPA receptors and kainite receptors are activated first by glutamate in the synaptic cleft, which then provide the conditions necessary to additionally activate NMDA receptors.

Not only is the NMDA receptor distinct from the AMPA receptor with regard to its  $Mg^{2+}$  sensitivity, it also differs in its kinetics. The NMDA receptor has a much slower onset of excitatory post synaptic current (EPSC, which is responsible for the EPSP and is measured in voltage-clamp conditions, which is a recording of the current shift required to hold the voltage) and also a longer lasting EPSC (hundreds of milliseconds) due to the high affinity and prolonged binding of glutamate to the receptor (Lester et al., 1990, Paoletti et al., 2013, Hestrin et al., 1990).

### 1.1.3 Long Term Potentiation: A Neural Correlate of Learning and Memory?

NMDA receptors are involved in a multitude of functions, including pain sensitisation (Petrenko et al., 2003), locomotion (Dale and Roberts, 1985), neuronal development (Van Horn et al., 2013), and cell death and survival (Kaufman et al., 2012). Of particular interest is the mediation of synaptic plasticity by NMDA receptors, particularly during long term potentiation (LTP), which is a putative neural correlate of learning (Bliss and Collingridge, 1993). LTP can be induced using many different mechanisms, but here only NMDA-dependent LTP in the CA1 of the hippocampus will be discussed. LTP can also be observed in other brain regions such as the cerebral cortex (Feldman, 2009), amygdala (Dityatev and Bolshakov, 2005), and cerebellum (Grasselli and Hansel, 2014). The proposed mechanism of LTP in the CA1 of the hippocampus is the pre-synaptic glutamate induced activation of postsynaptic

ionotropic receptors. This activation causes the influx of sodium ions, depolarizing the cell. This depolarisation relieves the magnesium ion block of the NMDA receptor; the resulting influx of calcium ions through the NMDA receptor activates downstream pathways strengthening the transmission between the pre- and postsynaptic neurons (Bliss and Collingridge, 1993). LTP may cause synaptic strengthening via many different mechanisms both presynaptic and postsynaptic. A likely presynaptic mechanism is increased glutamate release probability; while postsynaptic mechanisms involve AMPA receptor changes, including increased channel conductance, and insertion of additional AMPA receptors in the postsynaptic synapse (for review see Bliss and Collingridge (2013)).

Early *in vivo* experiments that demonstrated an involvement of NMDA receptors in learning were performed in rats, which had to remember where a platform was located in a water maze, testing hippocampal dependent, episodic like, spatial memory (Morris, 1989). The NMDA receptor is an especially good candidate for facilitating episodic like memory due to its coincidence detecting properties that allow association of distributed information. This means that associations can be formed between two stimuli, where one can predict the other, but the predictor does not need inherent value or relevance (Morris, 2013). Morris (1989) showed that a rat with chronic intraventricular infusion with the selective NMDA receptor antagonist, APV, took much longer to find the platform than controls with vehicle infusions. However, if APV was given after the location of the platform had already been learned, performance was equivalent to controls, indicating that NMDA receptors are involved in acquisition of spatial memory, but not in retrieval. These experiments were based on *in vitro* findings that APV prevented the induction of LTP in CA1 of rat hippocampal slices, suggesting that NMDA receptors are involved in LTP induction (Collingridge et al., 1983). The slower kinetics of the NMDA receptor response, compared to other ionotropic receptors, allows for summation of responses and is crucial to LTP

induction, and it depends on specific frequencies of stimulation (Bliss and Collingridge, 1993).

From the explanation above, it is clear that NMDA receptors are crucial for synaptic plasticity and healthy cell signalling; however, NMDA receptor activation can also be the cause of cell death. At first this was thought to be due to the degree of calcium influx through NMDA receptor activation, according to an inverted U-shaped relationship, where a moderate calcium influx is beneficial, and excessive calcium becomes toxic (Hardingham and Bading, 2003). This model has more recently been replaced with the NMDA receptor location hypothesis; more specifically, synaptic NMDA receptor activation is pro-survival and even neuroprotective (Hardingham et al., 2002), while extrasynaptic NMDA receptor activation is pro-cell death (Hardingham and Bading, 2010). This difference in cell signalling simply due to calcium influx at different locations could be due to three factors: first, the receptor signalling complex could be different in the two locations; second, the receptor subunit composition could be different, particularly the NR2B and NR2A subunits are thought to promote cell death and survival, respectively (Liu et al., 2007b); and finally, synaptic and extrasynaptic receptors are usually differentially activated by transient trans-synaptic glutamate and chronic ambient glutamate, respectively, which could result in a possible mechanism for a difference in cell survival signalling (Hardingham and Bading, 2010). The central role that NMDA receptors play in such a wide range of cellular processes, from synaptic plasticity, to cell death and survival make it a well-studied receptor both in health and disease.

## 1.2 NMDA Receptor Dysfunction and Disorders

NMDA receptors are involved in many disorders due to glutamate being a widespread neurotransmitter. This section will focus on the cognitive deficits associated with NMDA receptors in a range of disorders. Modulation of the NMDA receptor is an avenue for the possible treatment of the cognitive

deficits associated with neurological and psychiatric disorders. Specifically, potentiating the NMDA receptor could facilitate cognition, potentially by the modification of LTP mediated plasticity.

### 1.2.1 Schizophrenia

Schizophrenia is a psychiatric disorder that can have a wide range of symptoms, including psychosis, negative symptoms (e.g. anhedonia, alogia) and cognitive impairments (Tsai and Coyle, 2002). There are many theories of disease aetiology, including the dopamine hypothesis; and indeed dopamine receptor antagonists have been important in treating psychoses related to schizophrenia, but treatment of negative symptoms and cognitive deficits have proven to be a more difficult task (Tsai and Coyle, 2002). The glutamate system, and in particular the NMDA receptor, have been implied in the aetiology of the disease as NMDA receptor antagonists such as ketamine have produced symptoms that are similar to those of schizophrenic patients (Krystal et al., 1999). Phencyclidine (PCP) is a dissociative anaesthetic and NMDA receptor antagonist, which causes schizophrenic-like symptoms by blocking the ion channel and preventing ion influx (Javitt and Zukin, 1991, Beraki et al., 2008). Rats treated with ketamine (also a NMDA receptor ion channel blocker, like PCP) showed an increase in dopamine release in the prefrontal cortex, likely due to increased glutamate release and therefore, AMPA receptor activation; additionally, these rats were impaired in memory tests requiring the prefrontal cortex (Moghaddam et al., 1997). This indicated that glutamate disruption may contribute to abnormal dopamine levels. Dopamine and glutamate must also be functionally-coupled, as antipsychotic drugs that act on the D1 receptor also enhance the function of NMDA receptors through phosphorylation (Goff and Coyle, 2001, Wittmann et al., 2005). To highlight the difference in the roles played by glutamate and dopamine in schizophrenia, volunteers were given both PCP and amphetamine (which increases dopamine levels), resulting in amphetamine producing positive symptoms (i.e. psychosis) of schizophrenia; whereas, PCP was capable of

inducing positive, negative and cognitive symptoms (Javitt and Zukin, 1991). These results indicate that while dopamine dysfunction can contribute to positive symptoms, PCP is capable of producing a much broader spectrum of schizophrenic symptoms. Mice with the NR1 subunit knocked out of excitatory neurons in the medial prefrontal cortex (mPFC) showed cognitive deficits (in prepulse inhibition of the auditory startle reflex and object recognition memory tests) also seen in schizophrenia (Rompala et al., 2013), indicating that NMDA receptor hypofunction may play a role in cognitive impairment. Chronic administration of antipsychotic drugs has also been shown to increase mRNA expression of NMDA receptor subunits NR1 and NR2, which may be combating reduced NMDA receptor function (Goff and Coyle, 2001).

### 1.2.2 Aging

The cognitive deficits associated with aging are primarily memory loss and loss of executive function (Jagust, 2013). Age related changes include a reduction in glutamate binding to the NMDA receptor (Magnusson and Cotman, 1993) both in the cerebral cortex and hippocampus, as well as reduced NR1 subunit expression; these changes were found in a variety of species (Magnusson et al., 2010). These deficits have been correlated with reduced performance in spatial memory tasks using the water maze (Magnusson, 1998). Similarly, aged rats that were impaired in set-shifting (a task of executive function) exhibited reduced glutamate binding to NMDA receptors (Nicolle and Baxter, 2003). Barnes et al. (1996) showed a reduced EPSP in the CA1 region of the hippocampus in aged rats, that was likely to be NMDA receptor independent and may have been due to loss of synapses due to old age. Although the NMDA receptors may still be functioning normally, further enhancing their function could potentially make up for synaptic loss in aging.

### 1.2.3 Epilepsy

There are many types of epilepsy, however, in general, it is described as a seizure caused by aberrant excitatory activity. This is usually due to alterations

in both inhibitory circuits and excitatory circuits. There is evidence to suggest that increased NMDA receptor expression plays a part in the pathophysiology of seizures in temporal lobe epilepsy (Mathern et al., 1999). Additionally, changes in NMDA receptor subunit expression may be associated with focal cortical dysplasia where increased expression of NR2B subunits was found in temporal lobe tissue with epilepsy (Liu et al., 2007a). NMDA receptor antagonists show promising results in treating epilepsy, and it is possible that current antiepileptic drugs also target the NMDA receptor, contributing to its therapeutic effect (Ghasemi and Schachter, 2011). As NMDA receptors and glutamatergic neurotransmission plays such a crucial role in epilepsy, drugs that change these levels of excitation, particularly if this activity is being increased, pose a potential threat not only to enhance the risk of excitotoxicity (see section 1.1.3), but also seizures such as those seen in epilepsy.

#### 1.2.4 Huntington's Disease

NMDA receptor loss of function can be detrimental to cognition as described above. However, excessive activation of the NMDA receptor can have deleterious consequences. Many neurodegenerative disorders are associated with cognitive deficits, such as Parkinson's, Alzheimer's, and Huntington's disease (HD); and NMDA receptor dysfunction is also implicated in disease aetiology (Xu et al., 2012). HD is a dominant autosomal genetic disorder caused by a polyglutamine repeat in the ubiquitous huntingtin protein (htt), coded by an expanded CAG repeat in exon 1 of the gene that encodes the protein (Abada and Ellenbroek, 2016). HD is unusual as the genetic link to disease development is very strong; the onset of the disease is inversely proportional to the number of CAG repeats. An individual with 37 repeats and upwards is likely to develop the disease during their lifetime (Abada and Ellenbroek, 2016). HD causes cognitive deficits, mood changes and motor impairments (Fernandes and Raymond, 2009). It is primarily known for the motor symptoms of chorea (involuntary movement) followed by akinesia during disease progression. The motor symptoms are often considered for

diagnosis but are not required; however, up to 15 years prior to motor symptom onset, cognitive deficits may be apparent (Paulsen, 2011).

The role of wildtype (WT) htt remains to be elucidated; however, mice with the gene deleted are not viable past embryonic day 8 and the viable heterozygotes have motor and cognitive dysfunctions (Nasir et al., 1995). These findings indicate that the gene plays a crucial role in development and health. Mutant huntingtin (mhtt) impairs vesicular transport of brain-derived neurotrophic factor (BDNF) which is needed for neurotrophic support (Gauthier et al., 2004). mhtt also impacts upon calcium regulation and mitochondrial function, and is implicated in transcriptional dysregulation. These factors all contribute to the cell death strongly seen in the striatum of Huntington's patients (Abada and Ellenbroek, 2016).

As the genetic origin of HD is straightforward, a number of genetic mouse models have been developed to study the disease. These can be grouped into three categories: fragment models, which have expanded CAG repeats only in exon 1 of the huntingtin gene; knock-in models, where healthy CAG repeats are replaced with the expanded pathological repeats in the endogenous mouse gene; and full-length models, where mice express the full human mutated human huntingtin gene (Abada and Ellenbroek, 2016). The R6/2 model is an example of a fragment model, and shows an aggressive disease onset: motor deficits at 4.5 weeks, neurodegeneration at 12 weeks, and onset of cognitive impairments between 3.5 and 8 weeks (Lione et al., 1999, Carter et al., 1999). Knock-in models include the HDH<sup>Q111</sup> and HDH<sup>(CAG)150</sup> mice, which show mhtt nuclear inclusions in striatal neurons, late onset motor dysfunctions, and cognitive impairments (Wheeler et al., 2002, Wheeler et al., 2000) (also see section 1.3.6). Full length models include the 128 YAC mice which also display cognitive impairments (Brooks et al., 2012c, Brooks et al., 2012b) and motor impairments starting at 4 months (Brooks et al., 2012a).

HD is characterised by the loss of striatal neurons, where medium spiny neurons (MSNs) of the indirect pathway are particularly vulnerable, due to excitotoxicity. Neuronal death caused by excitotoxicity has been related to NMDA receptor function. Zeron et al. (2001) have shown that expression of mutant huntingtin protein with NMDA (NR1A/NR2B) receptors in HEK293 cells increased apoptotic cell death after NMDA application. Zeron et al. (2002) then showed that MSNs were vulnerable to NMDA application in a YAC mouse model, expressing the full-length mutant huntingtin protein, which was reversible with the NR2B subunit specific antagonist, ifenprodil. Quinolinic acid is a NMDA receptor agonist, and striatal lesions using this drug have produced characteristics, both in behaviour and physiology, that are similar to HD (Beal et al., 1986). Therefore, potentiating the NMDA receptor to enhance cognitive deficits must take into account that NMDA receptor potentiation may mediate some of the disease progression. As mentioned above (section 1.1.3), extrasynaptic NMDA receptors are thought to be involved in cell death, and these extrasynaptic receptors may also play a role in the cell death associated with HD (Parsons and Raymond, 2014, Milnerwood et al., 2010) due to mitochondrial dysfunction and intracellular calcium release (Raymond, 2016). As it is particularly the MSNs of the striatum that are targeted by degeneration in HD, it is interesting to note that the YAC72, YAC128, and R6/2 models showed increased NMDA receptor currents in the striatum in response to stimulation of glutamatergic afferents, and that mature MSNs have increased expression of NR2B subunits, which are linked to cell death signalling (Raymond et al., 2011). Finally, Okamoto et al. (2009) found a direct relationship between NMDA receptor location and mhtt: synaptic NMDA receptor activation increased mhtt inclusions, resulting in neuroprotection as damaging proteins are sequestered; while extrasynaptic NMDA receptor activation caused a decrease in inclusions and increased cell death in cortical neurons.

As NMDA receptor signalling plays a critical role in synaptic plasticity, it is vital for the functioning of cognition. In HD, as well as other neurodegenerative diseases (and also schizophrenia and aging mentioned above), loss of cognitive functions is one of the early and most detrimental symptoms of the disease. HD patients have a wide range of cognitive deficits including in emotion recognition, attention, working memory, spatial memory, pattern recognition, episodic memory, and verbal fluency (Johnson et al., 2007, Kirkwood et al., 2000, Lawrence et al., 1998, Lawrence et al., 1996). Paulsen (2011) describes a general decrease in the speed of cognitive processing in prodromal HD, where an ordinary mental task becomes “more tiring and takes more time to achieve the same outcome”. Paulsen suggests this is due to relying on alternative processing routes and lack of automation.

HD is an ideal model system to study the effects of NMDA receptor modulation on cognition, as NMDA receptor dysfunction is a prominent component of disease progression and cognitive deficits are likely associated with this NMDA receptor dysfunction. This thesis uses Huntington’s as a focus for the study of the NMDA receptor’s involvement in cognition by investigating a range of behaviours in rodent models, which were developed to measure different areas of cognition, from spatial memory to executive functions. These are described in detail below, along with the role NMDA receptors in particular, play in these processes.

## 1.3 Behavioural Tasks: Memory

### 1.3.1 Novel Object Recognition and Novel Place Recognition

Novel object recognition (NOR) is widely used for assessing memory in rodents. It is very popular as it requires no specialist equipment, does not require any training, and capitalises on the innate behaviours of the rodent to spontaneously explore novelty. More specifically, NOR is often used to test episodic memory, where a battery of task variations assess the what-where-

which aspects of this memory (Mumby et al., 2002). In the basic NOR test, the subject is presented with two identical objects, after a short interval, one of the objects is replaced with a new object. If the subject preferentially explores the new object, we can infer that an implicit memory for the familiar object has been formed. In a similar fashion, spatial memory can be tested with the novel place recognition (NPR) task. Here the subject is again presented with two identical objects, however instead of changing the object identity, one of the objects changes location. If the object in the new location is preferentially explored, we can conclude that a memory for the familiar location has been formed.

### 1.3.2 The Role of the Hippocampus and NMDA Receptors in NPR

The hippocampus has been placed in centre stage for spatial memory, with NMDA receptors playing a critical role in synaptic plasticity through LTP (see section 1.1.3). Therefore, it is not surprising that it has also been shown to play a role in NPR tasks. For example, rats with lesions to the hippocampus were still able to perform the NOR task; however, rats no longer preferred novelty in the NPR task and a contextual task (Mumby et al., 2002, Barker and Warburton, 2011). In contrast, perirhinal cortex lesions were shown to decrease preference for the novel object in the NOR task (Aggleton et al., 2010), but place recognition remained intact (Glenn and Mumby, 1998, Barker and Warburton, 2011). Collectively, these studies indicate a dissociated role for the perirhinal cortex and hippocampus for object and place recognition, respectively. Further, only tests which require allocentric (rather than egocentric) spatial memory seem dependent on the hippocampus (Langston and Wood, 2010). This supports the role of the hippocampus as creating a “cognitive map” of the environment (O'Keefe and Nadel, 1978), where place and grid cells provide the mechanism to create these spatial “place fields” (Burgess and O'Keefe, 2011). Shapiro (2001) describes a key role of NMDA receptors in creating place fields, which are spatial locations where place cells fire when the animal is that location. This is supported by a number of studies

which show that NMDA receptors are involved in NPR tasks. For example, NMDA receptor antagonists that were given intraperitoneally before the sample phase, but not before the test phase, of the NPR task, reduced preference for the novel place (Larkin et al., 2008). APV infusions directly to the hippocampus before the sample phases also impaired rats in the NPR task, but not the NOR task (Barker and Warburton, 2015). Additionally, APV infusion via various paths (dentate gyrus, CA3, and CA1) to the hippocampus also impaired preference of the novel place (Hunsaker et al., 2007). This was also found in juvenile rats (Jablonski et al., 2013).

### **1.3.3 Morris Water Maze**

The Morris water maze (hereafter referred to as “water maze”) was developed by Morris (1984) in St Andrews for rats. In this classic test, rats are required to swim in a pool of water to locate a submerged and invisible platform, which is the only means of escape from the water. The rat is placed into the pool from different release sites chosen randomly during each trial, and required to use extra-maze cues to locate the platform. The transfer test, also called probe trial, tests for spatial memory by removing the platform completely after training, and recording the swim paths of the subjects. If memory for the platform location has been established, the subjects will spend more time swimming in the proximity of the old platform location.

### **1.3.4 The Role of the Hippocampus and NMDA Receptors in the Water Maze**

The hippocampus is thought to be critical for spatial learning in the water maze, a concept supported by lesion studies both in rats (Pearce et al., 1998, Morris et al., 1982, Morris, 1984) and mice (Cho et al., 1999). In particular, the place cells of the hippocampus are implicated in spatial learning, as demonstrated by impaired platform location acquisition in conditions where place cell learning is disrupted (Bures et al., 1997) and by place cell firing during exploration of enclosed environments (Poucet et al., 2000). Place cells

also show place specific firing in mice (Silva et al., 1998). The hippocampus is thought to be needed for acquisition, retrieval and storage of spatial memory required for water maze learning (D'Hooge and De Deyn, 2001). Other brain regions, such as the striatum, are also implicated in spatial learning, as well as the connections between these regions and the hippocampus (Miyoshi et al., 2012).

As described above (section 1.1.3), NMDA receptors are implicated in the formation of spatial memory, possibly through the process of LTP. NMDA receptor involvement in water maze spatial learning was first suggested by Morris (1989), where intraventricular infusions of APV (an NMDA antagonist) caused spatial memory impairments in the water maze. Other studies showed that impaired LTP in the CA1 region, created by a knockout of the NMDA receptor, caused spatial impairments (Wilson and Tonegawa, 1997, Sakimura et al., 1995). Transgenic disruption of LTP downstream signalling also caused impaired spatial learning in the water maze (Silva et al., 1992, Bourtchuladze et al., 1994) as did protein synthesis inhibitors (Meiri and Rosenblum, 1998).

Bannerman's group (2014) has challenged the established view that hippocampal NMDA receptors are needed for spatial learning in the water maze. They used transgenic mice lacking NMDA receptors in the CA1 region and dentate gyrus of the hippocampus in a water maze task, where the mice were required to discriminate between two beacons associated with the platform based on location (where only one beacon signalled the platform location). Transgenic mice were unimpaired compared to WT in learning the platform location when released from sites equidistant between the beacons. However, transgenic mice were impaired at inhibiting the urge to visit the incorrect beacon when released near it (Taylor et al., 2014). If two visually distinct beacons were used, where one predicted the platform location, and the other did not, the mice lacking NMDA receptors were unimpaired (Bannerman et al., 2012). These findings indicate that while NMDA receptors of the hippocampus may not be needed for spatial learning per se, they are

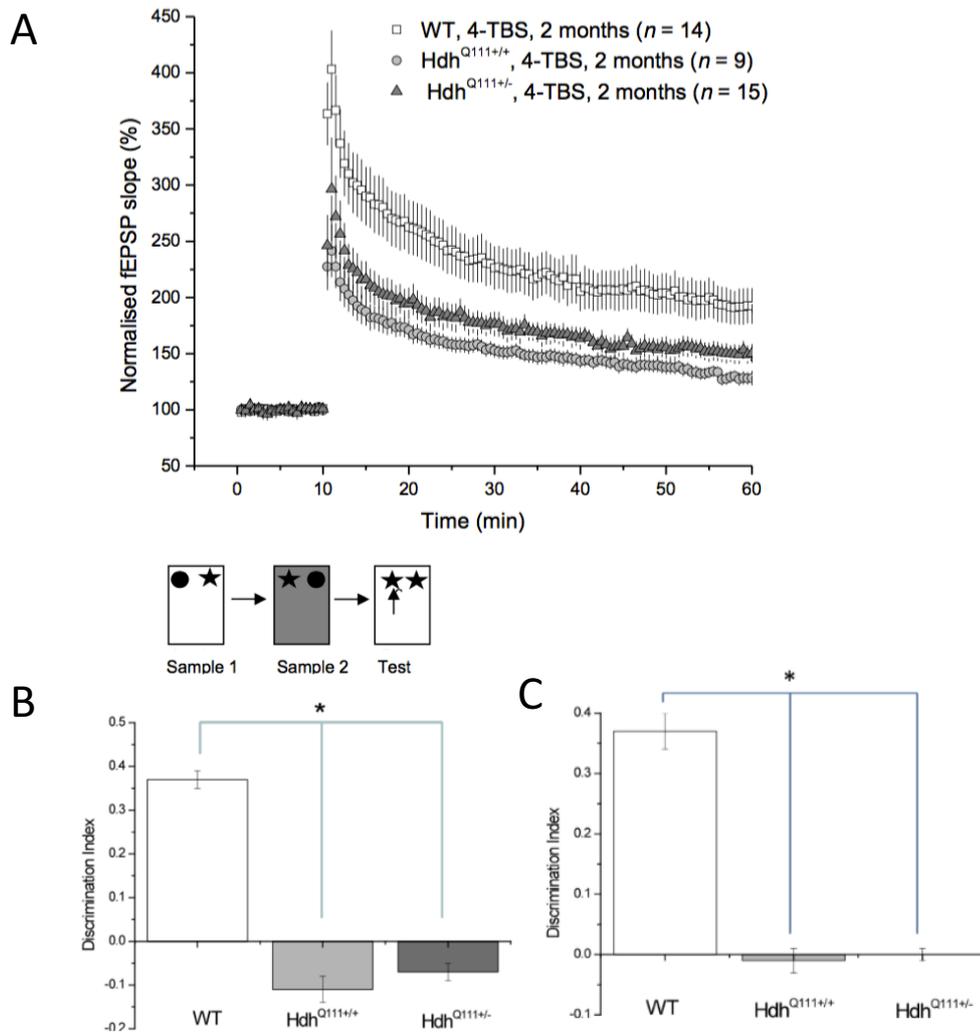
critical for inhibiting inappropriate responses when confronted with conflicting information.

### 1.3.5 Spatial Memory Impairments in HD Patients

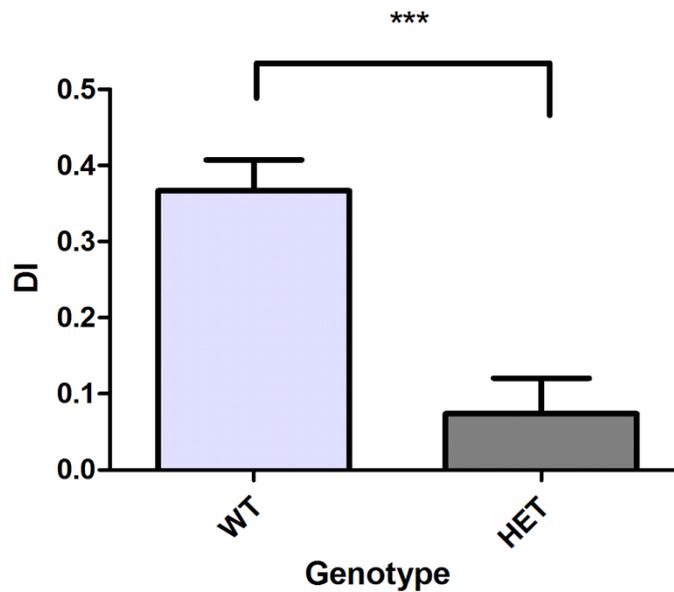
HD patients also show spatial memory impairments. Lawrence et al. (2000) found that patients in early stages of HD were impaired in spatial memory compared to a control group. Subjects were required to remember the locations of five squares on a touchscreen and recall these locations when paired with a square in a new location. A later study showed that HD patients were impaired compared to control subjects, Parkinson's disease, and AD patients, in a task requiring the subjects to place 9 stimulus cards in the same locations as they were previously shown (Brandt et al., 2005). Both authors suggest the spatial impairments are due to the two different visual information processing streams: the ventral and dorsal streams within the cerebral cortex, which are associated with object cognition and visuospatial cognition, respectively. As HD is primarily considered a disease which targets the striatum and the cortex, and the connectivity between them, it is clear why spatial memory impairments are explained in these terms. However, many HD mouse models also show hippocampal deficits (Gauntlett-Gilbert et al., 1999, Gil et al., 2005, Kolodziejczyk et al., 2014, Murphy et al., 2000), which may contribute to spatial memory dysfunctions. Specifically, the R6/2 line showed a clear spatial impairment in the water maze compared to WT controls, both during platform acquisition, and in the probe trial (Murphy et al., 1992, Lione et al., 1999). The HDH<sup>Q111+/-</sup> mouse is discussed in the next section. Interestingly, Begeti et al. (2016), noticing the trend of spatial impairments in the water maze in mouse models, set out to replicate these findings in HD patients. The authors used a virtual reality version of the water maze with early HD patients and pre-manifest patients. Early HD patients were impaired compared to controls, and performance correlated with the estimated years to diagnosis for pre-manifest patients.

### 1.3.6 Memory Impairments in the HDH<sup>Q111+/-</sup> Mouse Model of Huntington's Disease.

The hippocampus is connected to both the frontal cortex (Barker and Warburton, 2011) and to the striatum (Delcasso et al., 2014), areas which are known to be affected in HD (see section 1.2.4). NMDA receptor dysfunction has also been shown to play a role in HD (see section 1.2.4). To find a model to test possible therapeutics for this aspect of cognitive dysfunction in HD, the Lambert/Langston laboratory has investigated cognitive impairments in the HDH<sup>Q111+/-</sup> mouse model in an attempt to identify a phenotype similar to that observed in HD patients. Previous findings include: a LTP deficit in the CA1 region of the hippocampus (Figure 1.3A); a deficit in the episodic-like memory ("object-place-context" task, Figure 1.3B); and long-term (24 hour) NOR task (Figure 1.3C) (Mitchell, 2012). Finally, there is evidence that the HDH<sup>Q111+/-</sup> mouse has a reduced preference for the novel place in the NPR task, compared to WT controls (Chandler (2014), Figure 1.3).



**Figure 1.3: The phenotype of the HDH<sup>Q111+/-</sup> mouse model of HD at 2 months.** (A) The heterozygote (HDH<sup>Q111+/-</sup>) and homozygote (HDH<sup>Q111+/-</sup>) is impaired at a 4-theta-burst stimulation (TBS) compared to wildtype (WT) controls ( $p < 0.05$ ). (B) Both the heterozygous and homozygous mouse is impaired at the episodic-like (“object-place-context”) memory task ( $p < 0.05$ ). (C) Both the heterozygous and homozygous mouse is impaired at the 24-hour NOR task ( $p < 0.05$ ). A positive discrimination index indicates a preference for the novel object. Data from Mitchell (2012).



**Figure 1.4:** The HDH<sup>Q111+/-</sup> mouse (HET) shows no preference for the novel location in the NPR task compared to wildtype (WT) controls ( $p < 0.001$ ) at 5 months of age. A positive discrimination index (DI) indicates a preference for the novel place. Data collected by Chandler (2014).

As the HDH<sup>Q111+/-</sup> mouse seems to show cognitive impairments well before motor impairment onset (which only becomes mildly apparent starting at 9 months (Yhnell et al., 2016a)), it is an ideal candidate for behavioural testing. Not only is there no concern of motor impairments interfering with behavioural measurements, but it also more closely mimics the patient disease progression (Roos, 2010). The HDH<sup>Q111+/-</sup> mouse also more closely models the human disease as cognitive impairments are present in the heterozygous (not just the homozygous) animal; likewise, HD is an autosomal dominant genetic disease, therefore, only one copy of the gene is required for disease onset.

## 1.4 Behavioural Tasks: Executive functions

### 1.4.1 Attentional Set-Shifting task

Forming attentional set is a cognitive function shared by humans and other animals. Having an attentional set can be regarded as a cognitive short-cut; it

works as a filter so that limited processing resources can be preferentially assigned to stimuli that are currently most relevant. However, such a filter, whilst highly efficient in many circumstances, also requires processes that will over-ride it in certain circumstances. For example, a particularly salient stimulus will capture attention, in spite of an attentional set. Attentional set also must not be static and fixed; the attentional set must shift if circumstances change. The ability to shift an attentional set is impaired in certain human illness; for example, in schizophrenia (Pantelis et al., 1999, Ceaser et al., 2008) and following frontal lobe damage (Owen et al., 1993, Pantelis et al., 1999). Therefore, tests of attentional set-shifting have been used for many decades as an archetypal test of behavioural flexibility.

The classic and most well-known test of attentional set-shifting is the Wisconsin Card Sorting Task (WCST). However, the intradimensional/extradimensional (ID/ED) task (particularly varieties that have a 'total change design' (Slamecka, 1968), with novel stimuli at each acquisition stage) is a more 'pure' test of the ability to form and shift attentional set. Furthermore, it has been adapted for numerous other species enabling inter-species comparisons to be made. The Cambridge Neuropsychological Testing Automated Battery (CANTAB) contains an ID/ED task often used for patient assessment (Potter et al., 2012).

In the rat version of the ID/ED task, introduced by Birrell and Brown (2000), rats are required to dig in bowls to find a food reward. The rat is presented with two bowls that differ in both smell and the digging medium within the bowl. The rat learns that only one of the stimulus dimensions (the odour or the medium) is relevant to finding the food and consequently, over relatively few discriminations, attention is directed preferentially to the stimuli in the relevant dimension; this demonstrates that an attentional set for this dimension has been formed. Set-shifting is a test for how easily this attentional set can be moved to another dimension. The number of trials needed to dig in the bowls to reach criterion to move to the next stage (trials

to criterion; TTC) reflects the behavioural flexibility of the subject to shift attentional set to another dimension.

Birrell and Brown (2000) not only introduced the ID/ED task for rats, showing that rats form attentional set, they also showed that a lesion of the medial prefrontal cortex (mPFC) impaired their ability to shift attentional set. This coincides with marmoset literature showing lateral lesions to the prefrontal cortex (PFC) caused set-shifting deficits (Dias et al., 1997), and above mentioned human frontal lobe damage (Owen et al., 1993, Pantelis et al., 1999).

#### 1.4.2 Role of the NMDA Receptor in Attentional Set-Shifting

The NMDA receptor is vital for a wide range of cognitive abilities and attentional set-shifting is not an exception. Both pharmacological manipulations of the NMDA receptor and genetic mutations have been studied in rodents to assess the role of the NMDA receptor in set-shifting.

Kos et al. (2011) showed that ketamine administration increased TTC at the ED stage of a set-shifting task modified for mice. These authors also found that the NR2B antagonist, Ro 25-6981, decreased the TTC at the ED stage. This indicates that the NR2B subunit may have a specific role in behavioural flexibility, however, as there was no reversal cost it is likely that a set was not formed in the first place and conclusions of behavioural flexibility cannot be drawn. Marquardt et al. (2014) showed that a GluN2A knockout mouse took significantly more TTC at the ED stage of the ID/ED task compared to WT and heterozygous littermates, indicating a set-shifting impairment. While Thompson et al. (2015) set out to further explore the role of the NR2B subunit, showing that mice lacking these subunits were unable to form attentional set in the first place. Although this particular finding cannot give information about set-shifting, it does indicate the NR2B subunit plays an important role in set formation and learning. Together these studies suggest that the plasticity required to allow the cortical regions to shift strategies could involve the

NMDA receptor, in a similar fashion to its established role in memory formation.

### 1.4.3 Set-Shifting in Huntington's Disease

Patients with HD have a variety of executive function deficits. HD patients were shown to be impaired in the WCST due to increased perseverative responding (Josiassen et al., 1983). Patients in the early phases of HD also showed a deficit at the ED stage of the CANTAB set-shifting task (Lawrence et al., 1996). The cortex has many feedback loops to the basal ganglia in order to allow for appropriate action selection. Although HD is classically known as a disorder that primarily causes degeneration in the striatum, it can be postulated that disruption to the corticostriatal loops from striatal damage can cause set-shifting impairments. Monkey anatomy suggests that the dorsolateral prefrontal cortex (analogous to the mPFC in rats) projects to the dorsal striatum (Alexander et al., 1986). Additionally, there is good evidence that dysfunction of cortical pyramidal neurons, which project to the striatum, are impaired in HD as seen in patients as well as mouse models (Estrada-Sanchez and Rebec, 2013). NMDA receptors may be involved in cortical dysfunction as Dallerac et al. (2011) found LTP impairments in the prefrontal cortex of the R6/1 mouse.

Behavioural flexibility has also been measured in mouse models for HD, such as in the Barnes maze, where the R6/1 mouse was unable to change search strategies (Nithianantharajah et al., 2008). Brooks et al. (2006) attempted an ID/ED task in the  $Hdh^{CAG(150)}$  mouse, claiming to have found an ED deficit; however, as there was no ID/ED difference for the experimental or WT control group, this cannot be concluded as no set was formed in the first place.

### 1.4.4 Reversal Learning

Reversal learning, as well as attentional set formation and set-shifting, come under the umbrella term of "executive function". This term includes any behaviour that requires complex cognitive functioning to select actions such

as planning and rule learning and changing in response to stimuli (Talpos and Shoaib, 2015). Reversal learning is the reversal of an established stimulus-outcome (or stimulus-reward) contingency. Usually the subject first learns a discrimination task, and discrimination is then reversed so that the previously rewarded stimulus is no longer rewarded, and the previously unrewarded stimulus becomes rewarded. Reversal learning consists of 4 stages: (1) detection of the learned action no longer resulting in reward (2) inhibition of the now unrewarded action (3) changed behaviour to discover new stimulus-reward contingency (4) learning of the new contingency (Talpos and Shoaib, 2015).

Lesion studies have shown that the PFC is required for reversal learning in rodents and non-human primates; specifically, lesions of the orbitofrontal cortex (OFC) selectively cause reversal learning impairments (Dias et al., 1996, Dias et al., 1997, McAlonan and Brown, 2003). Human studies also show increased OFC activation during reversal learning (Hampshire and Owen, 2006, Cools et al., 2002) and lesions of the striatum impair reversal learning (Cools et al., 2006). The frontal cortex does not work in isolation, and corticostriatal circuits have also been implicated in reversal learning. The OFC also has strong glutamatergic projections to the basal ganglia, particularly the nucleus accumbens and dorsomedial striatum (DMS) (Haber et al., 1995). The basal ganglia serve as a feedback system for action selection; it receives glutamatergic inputs not only from the cortex, but also the thalamus, amygdala and hippocampus, which it integrates with dopaminergic inputs from the ventral midbrain (Voorn et al., 2004). Striatal contribution is further supported by lesions to the DMS impairing reversal learning in rats (Castane et al., 2010, Ragozzino, 2007, Graybeal et al., 2011). DMS lesions also caused reversal impairments in mice in a cue-based water maze task (Lee et al., 2014). Clarke et al. (2008) found that medial striatum lesions in marmosets caused similar reversal learning impairments as OFC lesions. DMS lesions in rats also

impaired attentional set-formation (Lindgren et al., 2013), which also occurs with OFC lesions (Chase et al., 2012).

#### 1.4.5 NMDA Receptors and Reversal Learning

As the NMDA receptor is required for some forms of synaptic plasticity, it seems likely to be involved in behavioural flexibility tasks such as reversal learning that require adaptive behaviours. An acute block of NMDA receptors in the dorsal striatum impaired reversal learning in rats (Palencia and Ragozzino, 2004). However, the involvement of NMDA receptors in reversal learning is not clear cut as some laboratories found deficits with NMDA receptor antagonist administration (Abdul-Monim et al., 2007), while others found no difference (Janhunen et al., 2015). Reduced NMDA receptor expression in the dorsal striatum due to gene (*Grin1*) inactivation caused a deficit in rule shifting but not reversal learning in mice (Darvas and Palmiter, 2015). The NR2B subunit may be particularly important for reversal learning, as systemic administration of the selective antagonist, Ro25-6981, impaired reversal learning, but not initial discrimination learning in rats (Dalton et al., 2011). Others found that localised antagonist administration or genetic deletion of the NR2B subunit in the OFC caused reversal deficits due to perseveration in mice (Brigman et al., 2013, Thompson et al., 2015).

#### 1.4.6 Reversal Learning in Huntington's Disease

Executive function deficits are some of the early symptoms of HD (Paulsen, 2011), and as the striatum and cortex are the main targets for neurodegeneration and dysfunction, it is not surprising that HD patients in the early stages (Lawrence et al., 1999) and preclinical (Lawrence et al., 1998) stages of the disease were shown to have a probabilistic reversal learning deficit compared to age matched controls. In contrast, in late HD progression, simple reversal learning is impaired due to perseverative responding (Lawrence et al., 1996). Habits and automated processes increase processing speed; therefore, if more cortical control is required, due to a less efficient

striatum, actions may become slower. Similarly, if the striatum is no longer able to respond efficiently to the glutamatergic signals to change behaviour and induce plasticity, perseverative behaviour can occur. This would also slow behaviour, as inappropriate responses would be preferentially executed.

There have been a few reports of mouse models of HD showing reversal learning impairments. For example, the R6/2 mouse model has been reported to show an age dependent reversal learning deficit at 6.5 weeks of age in a water maze environment discriminating between a bright and a dimmed light, but without an acquisition impairment (Lione et al., 1999). Impaired switching between delayed match to position and delayed non-match to position tasks in the HDH<sup>Q111+/-</sup> mouse was interpreted as a 'reversal learning' impairment, although this task is clearly more complex than simply reversal learning (Yhnell et al., 2015). Furthermore, the HD and WT mice were not performing at the same level before 'rules' were switched. Therefore, there is clearly a need for additional study of the putative reversal impairments in HD mouse models before concluding that they have similar deficits to those seen HD patients.

## 1.5 NMDA Receptor Pharmacology for Cognitive Enhancement

As NMDA receptor dysfunction is implicated in the aetiology of many disorders associated with cognitive impairments, it seems able to be an ideal target for treatments of cognitive dysfunction. However, care must be taken with any drug development to minimise side effects; especially NMDA receptor modulation must be approached with caution, as it is so central to very basic and important neuronal properties and networks. As mentioned in section 1.1.3 and 1.2.4, both too little and too much excitatory signalling can have detrimental effects to neuronal functioning and care must be taken to prevent epileptic activity. Previous drugs have focused on NMDA receptor antagonism such as memantine, which is the first drug used to treat AD that is

not focused on the cholinergic system. Memantine preferentially blocks extrasynaptic NMDA receptors, implicated in excitotoxicity, largely leaving synaptic receptors to carry out their normal physiological functions (Xia et al., 2010). Alternatively, treatments can focus on NMDA receptor enhancement, such as with NMDA receptor co-agonists, or positive allosteric modulators (PAMs), which will be the focus for this thesis.

### 1.5.1 NMDAR Co-Agonists

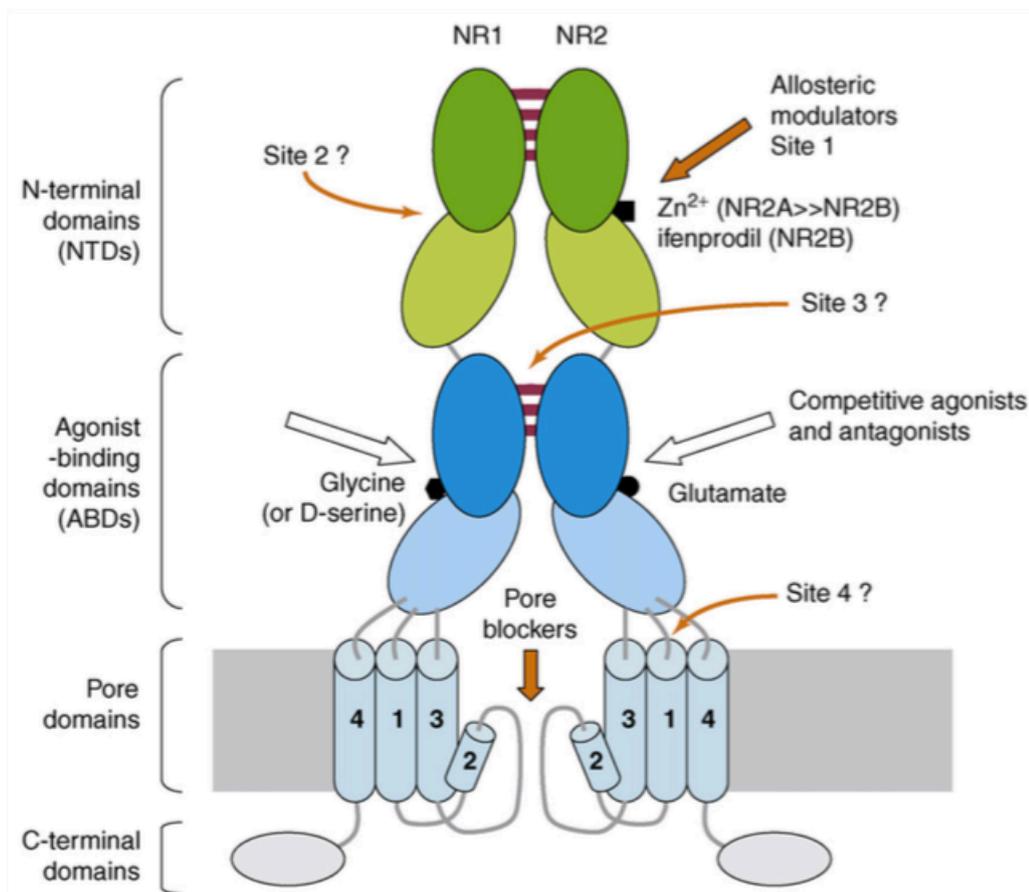
The D-serine site and glycine binding site are the same site, and it is situated on the LBD of the NMDA receptor (see Figure 1.5), and allows binding of both glycine (Ascher and Nowak, 2009) and D-serine (Nagai et al., 2012). This site must be occupied in addition to the glutamate binding site to allow NMDA receptor activation (Curras and Pallotta, 1996). Papouin et al. (2012) have shown that synaptic NMDA receptors are gated by D-serine; whereas, extrasynaptic receptors use glycine as the co-agonist. As this site is essential to NMDA activation, increasing the concentration of available endogenous ligands may increase channel opening and, therefore, potentiation of NMDA receptor function. Glycine has been shown to increase EPSPs in the neocortex (Thomson et al., 1989) as well as reduce negative symptoms in schizophrenic patients (Coyle and Tsai, 2004). Unfortunately, glycine has poor blood brain barrier permeability (Hashimoto et al., 2013).

D-serine was able to reverse polyriboinosinic-polyribocytidilic acid induced cognitive deficits in mice, and this reversal was inhibited with an NMDA antagonist, suggesting that D-serine is modulating the NMDA receptor to enhance cognition (Nagai et al., 2012). D-cycloserine is also a ligand of the D-serine site and has been shown to enhance cognition (Thompson et al., 1992). D-cycloserine was also able to decrease negative symptoms of schizophrenia in patients as measured by an improvement of reaction time needed on tests requiring the PFC (Goff et al., 1995). However, it is only a partial agonist, and

therefore, less effective at increasing NMDA receptor activity than full agonists like D-serine (Heresco-Levy and Javitt, 2004).

Alternatively, the concentration of available co-agonist can also be increased by preventing the reuptake by glycine transporters. This approach has led to the development of glycine transporter inhibitors, that prevent the uptake of glycine from the synaptic cleft. One such drug, Bitopertin, showed improvements in the negative symptoms of schizophrenia (Umbricht et al., 2014) but unfortunately these results did not replicate in phase III clinical trials (Lencz and Malhotra, 2015).

Currently, a promising modulator (partial agonist of an allosteric site) of the glycine site, is under development for treatment-resistant major depressive disorder (Moskal et al., 2016). There is also data indicating that Rapastinel, developed by Allergan, has a cognitive enhancing effect (Moskal et al., 2016). The approach of targeting the co-agonist site of the NMDA receptor may present a good alternative to direct activation of the NMDA receptor to enhance cognition.



**Figure 1.5: The structure and pharmacology of the NMDA receptor.** The glycine binding site is located on the NR1 subunit, while the glutamate binding site is located on the NR2 subunit. Modulatory sites are present on the N-terminal domains, where zinc or ifenprodil bind, for example. The transmembrane domain is the binding site of channel blockers, where magnesium binds at resting membrane potentials. It is also the binding sites of synthetic drugs such as MK801, PCP, and ketamine. Reproduced with permission (Paoletti and Neyton, 2007).

### 1.5.2 Indirect Modulation of the NMDA Receptor

As described in section 1.1.2, NMDA receptor activation is dependent on preceding AMPA receptor mediated depolarisation of the postsynaptic neuron. Therefore, positive modulation of the AMPA receptor has the potential to result in downstream enhanced NMDA receptor activity. PAMs of the AMPA receptor (known as ampakines) have been shown to enhance memory encoding in preclinical studies, however, conclusive translation into humans has not yet been reached but development of ampakines is ongoing

(Lynch et al., 2011). As with NMDA receptors, AMPA receptor modulation must also be approached with care as too much excitatory activity could lead to epileptiform activity (see section 1.2.3).

## 1.6 Neuroactive Steroids as Positive Allosteric Modulators of the NMDA Receptor

Another method to enhance NMDA receptor function is by positive allosteric modulation. The advantage of allosteric modulation is that it only has an enhancing effect on receptor function in the presence of a receptor agonist. PAMs usually work by prolonging the time the agonist binds to the receptor or by increasing the probability that the agonist will bind to the receptor. Therefore, the PAM should only enhance a signal that is already present. This may have the advantage of reducing excitotoxicity that is related to NMDA agonists, or other methods such as AMPA enhancement described in the section above, and prevent epileptic activity (see section 1.1.3).

Section 1.2 has highlighted NMDA receptor malfunction in a variety of psychiatric and neurological conditions. In other areas of receptor research, PAMs have provided useful avenues for treatments (e.g. benzodiazepines on the gamma-aminobutyric acid (GABA)<sub>A</sub> receptor). However, to date no selective NMDA receptor PAMs have progressed to clinical use. Here I will review the current literature regarding NMDA PAMs and then discuss unpublished findings that identify highly selective PAMs. Importantly, these studies have identified a potent PAM, which is made within the brain and may act as an endogenous modulator of the NMDA receptor to fine tune neural excitation.

### 1.6.1 Pregnenolone and Pregnenolone Sulphate

Pregnenolone is a precursor to all other steroids in the brain; however, it also has many other functions as its molecular targets include the sigma 1 receptor, microtubules, and the type-1 cannabinoid receptor (Vallee, 2015).

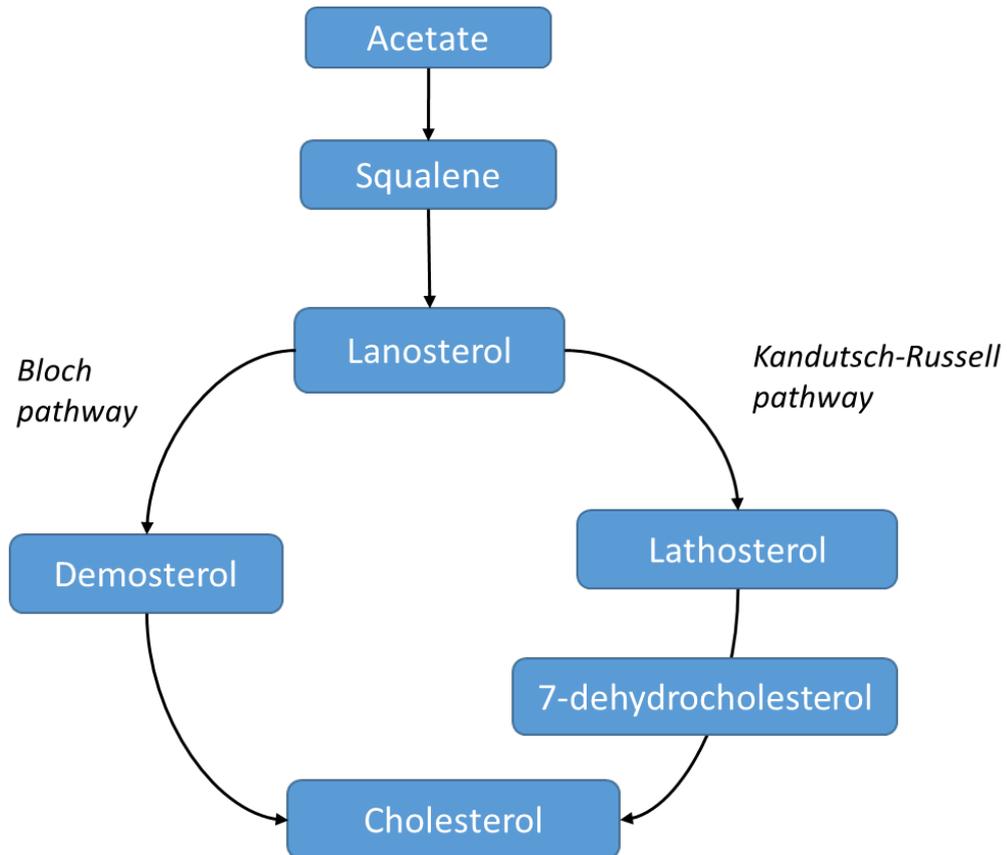
Pregnenolone sulphate is an endogenous neurosteroid, it is a metabolite of cholesterol, which binds to the NMDA receptor extracellularly and increases receptor function as a PAM (Wu et al., 1991, Weaver et al., 1998, Malayev et al., 2002). The term neurosteroid was coined by Baulieu (1997) to describe cholesterol metabolites that are synthesised in the CNS. Pregnenolone and pregnenolone sulphate have been shown to increase memory function in rodents using a variety of behavioural tests (Marx et al., 2011). Pregnenolone was also able to improve the functional capacity (ability to perform everyday tasks) of schizophrenic patients (Marx et al., 2014). Other preliminary clinical trials with small sample sizes have shown that administration of pregnenolone sulphate has no side effects, and can improve attentional and learning deficits in patients with schizophrenia, although larger trials will be necessary to confirm these results (Marx et al., 2011). Pregnenolone in its unsulphated form is not active on the NMDA receptor; however, pregnenolone can be sulphated in the brain via the hydroxysteroid sulfotransferase (SULT) enzyme, where the SULT2B1 enzyme is specific for 3"-hydroxysteroids including pregnenolone (Meloche and Falany, 2001). Marx et al. (2014) suggested that a possible mechanism for the symptomatic improvement of schizophrenic patients is due to the increase in pregnenolone sulphate from higher available levels of pregnenolone to undergo sulphation.

### 1.6.2 Synthetic Neuroactive Steroids

The demonstration that the steroid pregnenolone sulphate enhances NMDA receptor function encouraged the search for more potent and selective steroidal PAMs of the NMDAR. Organon screened a large library of steroid compounds in a medium throughput assay for NMDA receptor activity. This led to the identification of a potent and selective NMDA receptor PAM named Org49209 ((3 $\beta$ )-26,27-dinoregost-5-ene-3,24-diol). Intriguingly, the structure of this steroid was closely related to that of a naturally occurring brain steroid, cerebrosterol (Figure 1.7), raising the exciting prospect that brain NMDA receptors may be influenced by this neurosteroid.

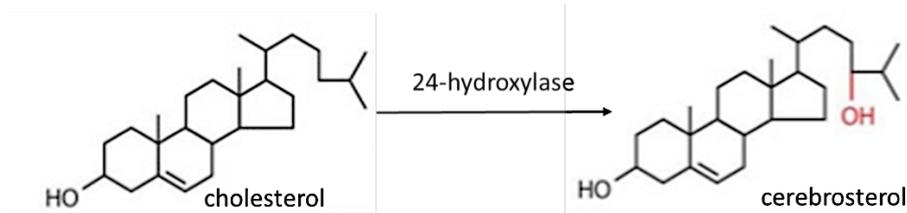
### 1.6.3 Cerebrosterol: History, Synthesis, and Functions

Cerebrosterol was given this name due to its abundance in the horse and human brain, where it was first discovered by Ercoli et al. (1953) (as cited by Bjorkhem (2007)). Cerebrosterol (also known as 24-S hydroxycholesterol) is an endogenous neurosteroid, and is the major metabolite of cholesterol in the brain, among other oxysterols (oxidised derivatives of cholesterol) such as 25- and 27- hydroxycholesterol (Bjorkhem et al., 1998). Cerebrosterol is involved in the balance of cholesterol in neurons, as cholesterol concentrations are usually quite low compared to glial cells, and cerebrosterol has the ability to activate neuronal cholesterol transporters (Björkhem, 2007). Cholesterol is synthesised *de novo* in the brain, both neurons (via the Kandutsch-Russel pathway) and astrocytes (via the Bloch pathway) are able to synthesise cholesterol (Figure 1.6); however, in the adult synthesis is mainly left to the endoplasmic reticulum of astrocytes (Zhang and Liu, 2015).



**Figure 1.6: Pathways of cholesterol biosynthesis.** Both the Bloch pathway of astrocytes and Kandutsch-Russel pathway of neurons is shown, which form different cholesterol precursors.

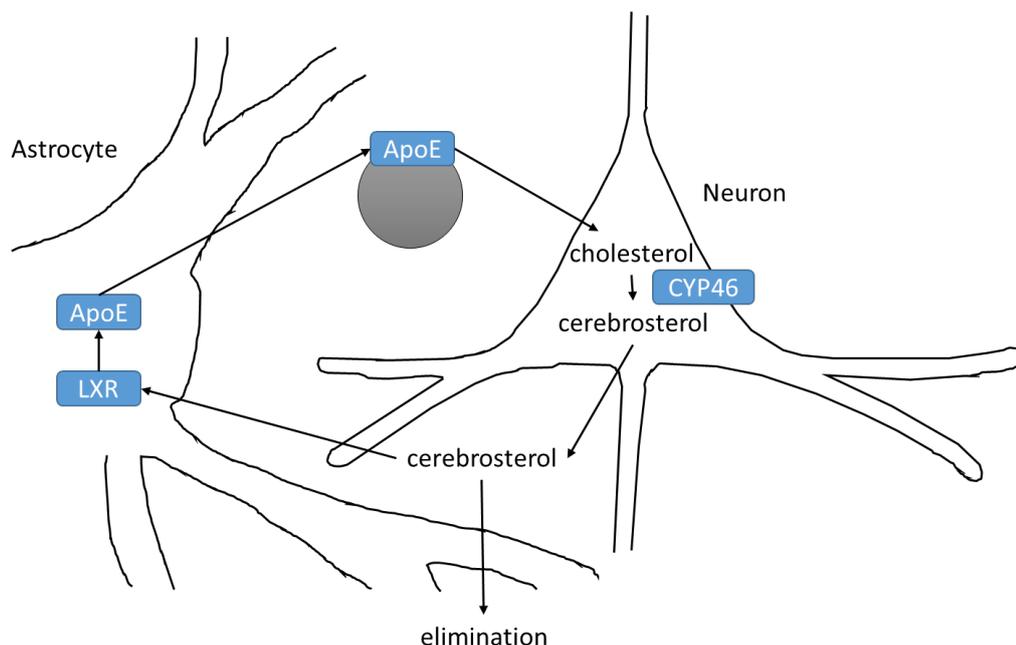
Movement of free cholesterol across the blood brain barrier is very slow, which means that cholesterol synthesis and metabolism is separate from the rest of the body (Zhang and Liu, 2015). Cholesterol is metabolised to cerebrosterol by the P450 enzyme cholesterol 24-hydroxylase (Bjorkhem (2007), Figure 1.7). This is a P450 enzyme coded by the CYP46A1 gene. 24-hydroxylase expression is very low in glial cells, but expressed in the ER of the cell bodies and dendrites of selective neuronal types: pyramidal neurons of the hippocampus and cortex, Purkinje cells of the cerebellum, and hippocampal and cerebellar interneurons (Ramirez et al., 2008). Cerebrosterol can cross cell membranes and thus reach the blood circulation, where it is catabolised by the liver (Zhang and Liu, 2015).



**Figure 1.7: The metabolism of cholesterol to cerebrosterol.** The enzyme cholesterol 24-hydroxylase converts cholesterol to cerebrosterol.

Cholesterol levels are held constant in the brain by producing new cholesterol as the old cholesterol is metabolised (Zhang and Liu, 2015). Even when plasma cholesterol levels fluctuate or are very high, brain cholesterol levels remain constant as cholesterol does not cross the blood brain barrier (Bjorkhem and Meaney, 2004). Cerebrosterol is thought to be one of the messengers that is able to signal between astrocytes and neurons to maintain cholesterol homeostasis. *In vitro*, cerebrosterol is able to activate the liver X receptor (LXR) (Lehmann et al., 1997) and is a more effective ligand than all other common oxysterols in the brain (Janowski et al., 1999). The LXRs are nuclear receptors which are crucial in the regulation and feedback mechanisms of cholesterol metabolism (Zhao and Dahlman-Wright, 2010). Pfrieder (2003) suggest that cerebrosterol is involved in the regulation of cholesterol moving from glia (the production site) to neurons via apolipoprotein E (ApoE), which is regulated by a cholesterol transporter under the control of the LXR (Figure 1.8). As cerebrosterol is an efficient agonist of the LXR, increased levels of the cholesterol metabolite would increase the flux of cholesterol from glia to neurons. In support of this model, cerebrosterol was shown to induce ApoE transcription, synthesis, and secretion from astrocytes via the LXR (Abildayeva et al., 2006). However, the role of cerebrosterol in cholesterol homeostasis is not clear-cut, as Shafaati et al. (2011) have shown that the overexpression of

cerebrosterol in transgenic mice did not result in the expected activation of LXR signalling genes.



**Figure 1.8: An illustration of the proposed role of cerebrosterol in cholesterol homeostasis in the brain.** Cerebrosterol is produced in neurons from cholesterol, and bind to the liver X receptor (LXR) in astrocytes. This increases cholesterol biosynthesis in astrocytes and release via bound apolipoprotein E (ApoE) to the neuron. Adapted from Bjorkhem (2007).

#### 1.6.4 Cerebrosterol Sulphate

It is of relevance to note that cerebrosterol sulphate is also found endogenously in the brain (Cook et al., 2009). Cerebrosterol is known to be sulphated (or glucuronidated) in the liver on its journey to bile acids prior to excretion (Bjorkhem et al., 2001). Human cytosolic sulfotransferase (SULT) is able to sulphate cerebrosterol (Javitt et al., 2001, Fuda et al., 2007).

Three isoforms of SULT are active on cerebrosterol: SULT2A1, SULT1E1, and SULT2B1b (Cook et al., 2009). SULT2B1b and SULT1E1 only sulphate cerebrosterol on the 3-position of the steroid ring (to form 24-hydroxycholesterol-3-sulphate), while SULT2A1 is able to sulphate both at the 3- and 24- position, creating a disulphate (Cook et al., 2009). Of these isoforms, only SULT2B1b is present in the brain (Geese and Raftogianis, 2001). Sulphation can be a reversible reaction, where hydrolysis by the steroid

sulfatase (STS) enzyme is able to de-sulphate most steroids, primarily oestrogen sulphates and hydroxysteroid sulphates (Iwamori, 2005). STS is also present in the brain (Kriz et al., 2005). With respect to cerebrosterol, the 3-sulphate is readily hydrolysed by STS, while the 24-sulphate is resistant, and the disulphate can only be hydrolysed at the 3-position (Cook et al., 2009).

Interestingly, Cook et al. (2009) found that while oxysterols act as LXR agonists, the sulphated oxysterols are significantly better antagonists of the LXR. This suggests that sulphation and de-sulphation of cerebrosterol may play a role in regulating cholesterol homeostasis through LXR modulation.

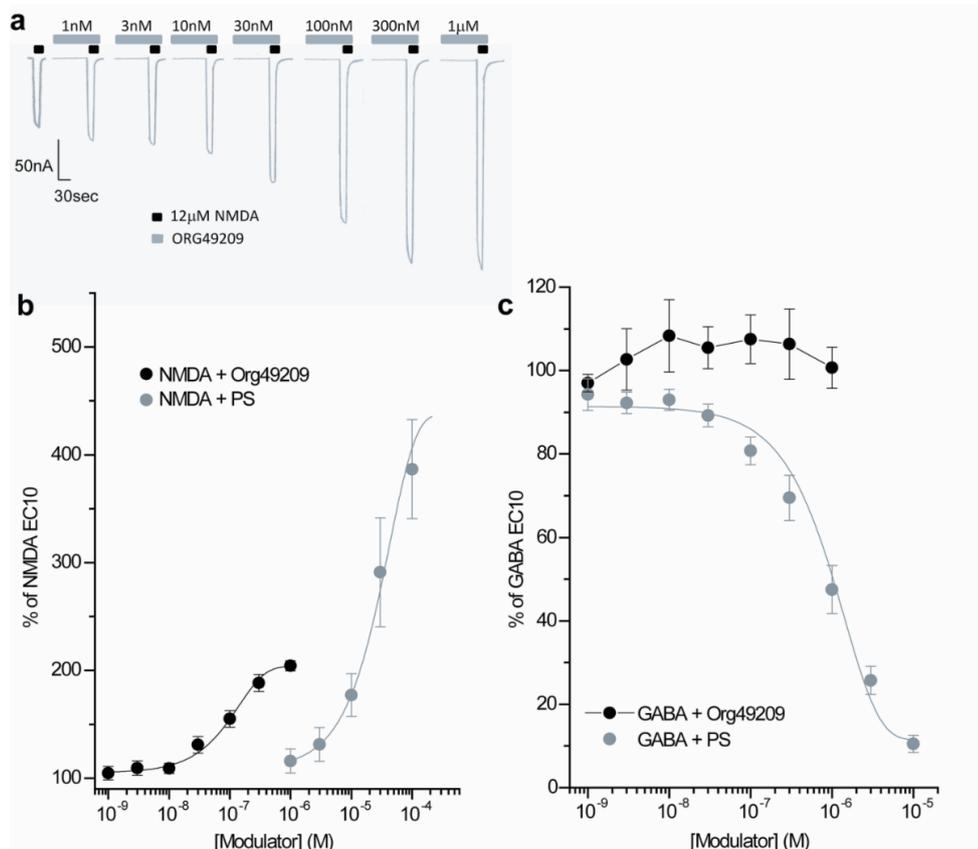
### 1.6.5 Cerebrosterol and the NMDA Receptor

Unpublished data from the Lambert laboratory showed that cerebrosterol is also capable of modulating the NMDA receptor (Table 1.1). In these experiments recombinant NMDA receptors were expressed in *Xenopus leavis* oocytes. The current induced by activation of the receptors by a submaximal concentration of NMDA ( $EC_{10}$ ) was enhanced by pregnenolone sulphate in a concentration dependent manner. Org49209 also enhanced such currents, but was active at far lower concentrations and exhibited a reduced maximal effect of pregnenolone sulphate (Figure 1.9 A and B). Oocytes expressing GABA<sub>A</sub> receptors exhibited a concentration dependent decrease in GABA-mediated current with pregnenolone sulphate, but Org49209 was inactive (Figure 1.9 C). These findings indicate that Org49209 is a selective NMDA receptor PAM. The sulphated Org49209 (3-sulfate of (3 $\beta$ )-26,27-dinorergost-5-ene-3,24-diol, Org49230) was also tested and was shown to be just as potent as cerebrosterol and Org49209, but had a much greater efficacy (Table 1.1). Therefore, it is of interest that cerebrosterol sulphate is also found in the brain (see section 1.6.4). As the synthetic Org49209 is similar in structure to cerebrosterol and shows similar enhancement of the NMDA receptor cerebrosterol (Table 1.1), Org49209 studies can be putatively extrapolated to suggest the effects cerebrosterol would have on the NMDA receptor. The

difference in the range of the maximum response values of sulphated and unsulphated steroid steroids introduces an important point on striking a balance between physiological stimulation and stimulation that could be excitotoxic. Although Org49230 may have a large effect, this may be less use in the clinic compared to a potent, but moderately efficacious steroid such as Org49209.

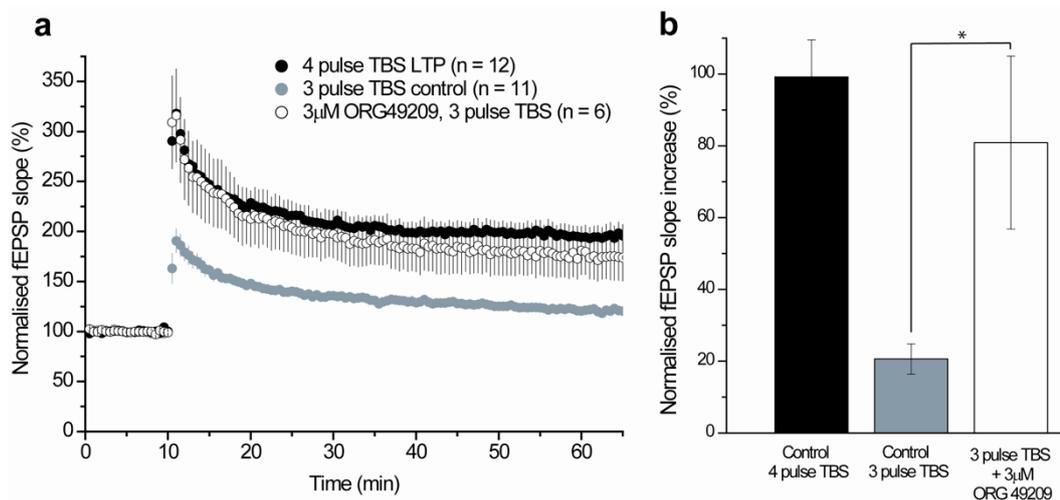
**Table 1.1: The maximum responses and EC<sub>50</sub> values for NMDA receptor-active steroids in oocytes.** Note the high efficacy of sulphated steroids (pregnenolone sulphate, cerebrosterol sulphate, and Org49230) compared to unsulphated steroids (cerebrosterol and Org49209). Data from Etherington (Lambert laboratory), unpublished.

Compound	Maximum (% of control)	EC50
Pregnenolone sulphate	460 ± 65	36 ± 6.7 µM
Cerebrosterol	162 ± 10	5.2 ± 1.6 nM
Cerebrosterol sulphate	493 ± 71	71.8 ± 7.2 nM
ORG 49209	227 ± 27	95 ± 13 nM
ORG 49230	1189 ± 34	121.9 ± 11 nM



**Figure 1.9: *Org49209 is a potent and selective PAM of the NMDA receptor in oocytes.*** (A) The raw trace of the NMDA-induced current with applications of increasing concentrations of Org49209. (B) Comparison of pregnenolone sulphate (PS) and Org49209 on the NMDA-induced current. (C) Comparison of PS and Org49209 on the GABA-induced current. Figure from Etherington (Lambert laboratory), unpublished.

Paul et al. (2013) showed that Org49209 is not selective for any particular NMDA receptor subunit, and that it could reverse ketamine induced suppression of LTP in slices. They also showed that both Org49209 and cerebrosterol increase NMDA-evoked currents in cultured hippocampal neurons. Additionally, the potency of cerebrosterol at NMDA receptors is much higher than at LXR receptors, further suggesting an endogenous role for cerebrosterol in modulating NMDA receptor (Paul et al., 2013). The Lambert laboratory also showed that Org49209 is able to increase reduced LTP due to a submaximal (3-pulse theta burst) stimulation to the level of normal (4-pulse theta burst) stimulation in hippocampal slices (Etherington (2012), unpublished observations, Figure 1.10).



**Figure 1.10: *Org49209* is able to increase low hippocampal LTP. (A)** The average field excitatory post synaptic potential (fEPSP) slopes with a low (3-pulse theta burst stimulation, TBS), a normal 4-pulse TBS, and the low TBS with bath applied *Org49209* (3  $\mu$ M). **(B)** *Org49209* significantly increased the 3-pulse TBS fEPSP after 60 minutes ( $p < 0.05$ ). Figure from Etherington (Lambert laboratory), unpublished.

Cerebrosterol has also been shown to induce both cell death in neuronal cultures (Yamanaka et al., 2011), as well as promote survival (Okabe et al., 2013). Cell death may be due to esterification of cerebrosterol, which may itself be cytotoxic, or the formation of lipid droplets may trigger cell death; cell survival is likely due to LXR activity rather than NMDA receptor activation. Low concentrations of *Org49209* were not toxic to neurons on their own, but increased cell death caused by mild hypoxic stress, which is largely prevented with an NMDA receptor antagonist (Sun et al., 2016). Additionally, reduced cholesterol levels due to Simvastatin application, and reduced steroid levels due to beta-cyclodextrin application, reduced the NMDA-induced toxicity in cortical neurons (Zacco et al., 2003). Although these experiments do not directly imply cerebrosterol activity on the NMDA receptor, Simvastatin and beta-cyclodextrin could both also reduce cerebrosterol levels. Therefore, the ability for cerebrosterol to act as a PAM may play a role in the toxicity of NMDA in these cortical cultures. However, it remains uncertain whether NMDA receptor activation by cerebrosterol can contribute to neuronal cell death.

## 1.7 Cerebrosterol and Cognition

A growing body of evidence suggests that cerebrosterol is responsible for more than the elimination of cholesterol from the brain. It is possible that through the interaction with NMDA receptors as mentioned above, cerebrosterol has an effect on synaptic plasticity and cognitive functioning. Some of this evidence is outlined below.

### 1.7.1 Biomarkers for Neurodegenerative Diseases

As 24-hydroxylase, the enzyme responsible for the production of cerebrosterol, is predominantly expressed in the brain, and the ability for cerebrosterol to cross the blood brain barrier is the main route of cholesterol leaving the brain, circulating cerebrosterol plasma levels are largely of cerebral origin (Lutjohann et al., 1996, Bjorkhem et al., 1998). Therefore, it is possible that plasma levels of cerebrosterol are able to give insight to the state of cholesterol homeostasis in the brain. Cerebrosterol has been implicated as a biomarker for AD, as increased levels are observed in AD patients (Zuliani et al., 2011, Popp et al., 2012), and cerebrosterol can increase AD associated protein aggregation in neurons (Gamba et al., 2011). Cerebrosterol, as well as 27-hydroxycholesterol have been identified as possible candidates of biomarkers for AD as well as mild cognitive impairment (MCI). Cholesterol metabolism seems dysfunctional in AD, as shown by Wang et al. (2016) who found increased levels of both 24S-hydroxycholesterol and 27-hydroxycholesterol in the cerebrospinal fluid (CSF) of MCI and AD patients compared to age-matched controls (also shown by Leoni et al. (2006)). MCI is often a precursor to AD and early detection and treatment of MCI can be valuable in preventing the onset of AD (Sperling et al., 2011). Dysfunctional cholesterol metabolism is further supported by abnormal 24-hydroxylase activity in the glial cells of patients with AD (Bogdanovic et al., 2001, Brown et al., 2004). Finally, Heverin et al. (2004) found a decrease in cerebrosterol levels in the brains of patients with AD. Although there is no consensus

regarding the levels of cerebrosterol in plasma of AD patients, there is agreement that levels are altered, and this could be a biomarker for this neurodegenerative disease.

### 1.7.2 “Knockout” Mice and CYP46A1 Mutations

CYP46A1 is the gene which encodes 24S-hydroxylase. CYP46A1 single nucleotide polymorphisms in humans are associated with an increased risk for AD (Garcia et al., 2009, del Pozo et al., 2006, Jia et al., 2016). These polymorphisms may also be associated with cognitive decline in the elderly (Fu et al., 2009). Mouse models have been studied to a greater extent than humans, which help elucidate the role of 24S-hydroxylase and cerebrosterol in the brain. Djelti et al. (2015) used an adeno-associated virus vector to decrease CYP46A1 gene expression in the hippocampus of mice. This knockout caused: increased cholesterol accumulation in hippocampal CA3 neurons; impaired spatial learning in the water maze; and prior to cell death, increased deleterious amyloid peptides associated with AD. Lund et al. (2003) showed that CYP46A1 knockout mice kept stable cholesterol levels, suggesting a decrease in cholesterol synthesis. The knockout mice were severely impaired at spatial learning in the water maze task (Kotti et al., 2006), and LTP in the CA1 of the hippocampus was also impaired in these animals (Kotti et al., 2006, Kotti et al., 2008). Collectively, these mutation studies indicate that cerebrosterol, the product of 24S-hydroxylase, may play a role in the cognitive symptoms of neurodegenerative diseases and disease aetiology.

### 1.7.3 Sulphation Mutations

As cerebrosterol sulphate has a greatly increased efficacy compared to cerebrosterol in the enhancement of NMDA receptor activity, it is possible that alterations in sulphation could affect cognition. Mutations of the steroid STS gene have been found in neurodevelopmental disorders such as schizophrenia (Milunsky et al., 1999) and autism (Shinawi et al., 2009). Stergiakouli et al. (2011) found that two single nucleotide polymorphisms

(SNPs) of the STS are associated with attention deficit hyperactivity disorder (ADHD). All of these disorders are known for their detrimental cognitive dysfunctions; however, it is unclear whether the mutation is directly related to these deficits.

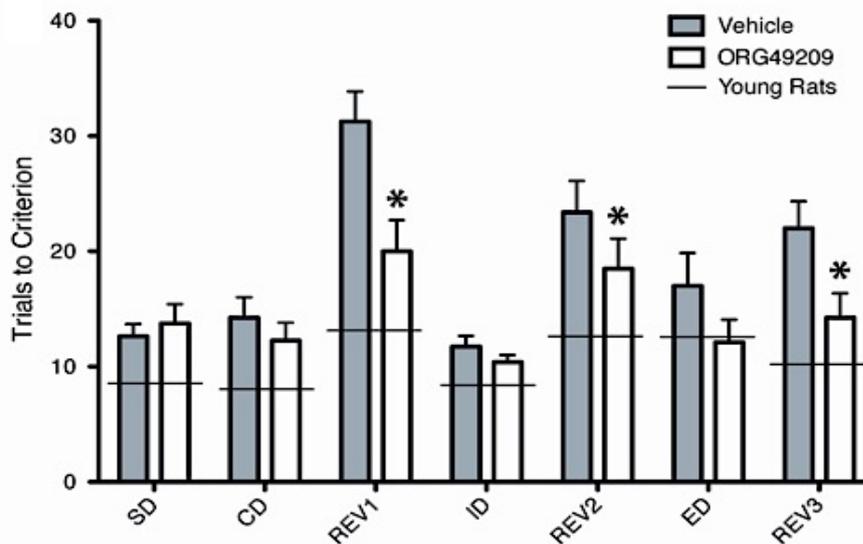
#### 1.7.4 Huntington's Disease and Cerebrosterol

As seen in AD (see section 1.7.1), cerebrosterol has also been suggested as a possible biomarker in HD; increased plasma cerebrosterol has been found in early stages of HD (Leoni et al., 2008). In contrast, a decrease in plasma cerebrosterol was seen in HD patients relative to disease progression (Leoni and Caccia, 2014). HD mouse models have also shown disrupted cholesterol synthesis and metabolism (Valenza et al., 2007b, Valenza et al., 2007a, Valenza et al., 2010). In particular, the R6/2 mouse striatum and striatal cell lines expressing mhtt showed decreased levels of cerebrosterol (Boussicault et al., 2016).

Seeing the connection between HD and cerebrosterol dysregulation, Boussicault et al. (2016) studied this relationship in more detail by inhibiting CYP46A1 expression in the striatum in WT mice via an adeno-associated virus. This reproduced the HD phenotype seen in the R6/2 mouse, consisting of striatal neurodegeneration and motor deficits. Virus-mediated delivery of CYP46A1 to R6/2 mice improved motor deficits, reduced neuronal atrophy and decreased the size of mhtt aggregates. The authors, therefore, concluded that 24-hydroxylase activity has a neuroprotective role in HD. This may be due to increased levels of cerebrosterol; however, the authors assign the neuroprotective effects of increased 24-hydroxylase activity to an increase in the cholesterol precursors Lanosterol and Desmosterol, which were neuroprotective in mhtt expressing striatal neurons maintained in culture. Unfortunately, cognitive deficits were not tested in either of the mouse models.

### 1.7.5 Can Cerebrosterol Administration Improve Cognition?

Org49209 was able to reverse spatial working memory deficits of mice tested in the Y-maze after acute administration of MK-801, a NMDA receptor antagonist (Paul et al., 2013). The same authors used a modified structure of Org49209, which has an additional methyl group at the 3-position of the steroid ring, to reverse deficits in the NOR task and social interactions in rats treated with a subchronic administration of PCP. Work from the Brown laboratory administered Org49209 to middle-aged rats with a reversal learning impairment, and showed a reduction in trials to criterion (Chase (2013), Figure 1.11). Collectively, these data suggest that cerebrosterol may be an endogenous regulator of cognition, and Org49209 administration may have potential as a treatment for NMDA receptor induced cognitive impairments.



**Figure 1.11: Org49209 administration reduces reversal learning deficits of middle-aged rats.** Results from the ID/ED task (SD, simple discrimination; CD, compound discrimination, REV1, reversal of CD; ID, intradimensional acquisition; REV2, reversal of ID; ED, extradimensional acquisition; REV3, reversal of ED). Middle-aged rats were impaired at the reversal stages of the task (grey bars). Repeated acute administration (i.p.) reduced the trials to criterion (mean  $\pm$  SEM),  $p < 0.05$ . Data from Chase (2013).

## 1.8 Thesis Aims

Considering the NMDA receptor dysfunctions that are associated with the cognitive symptoms of HD, an enhancer of NMDA receptor function may be able to ameliorate some of these debilitating symptoms. A synthetic NMDA receptor-active steroid, Org49209, is a promising candidate. Additionally, the role of the endogenous steroid, cerebrosterol, which is also an enhancer of NMDA receptor function may play a role in cognition in both health and disease. The experiments presented in this thesis build on previous work to further investigate the potential of Org49209 in treating the cognitive symptoms of HD. Additionally, the effect of these neuroactive steroids has the potential to give insight into NMDA receptor function in both health and disease, and the role that the endogenous cerebrosterol may play in the brain. Therefore, the aim of this thesis is both preclinical as the effect of Org 49209 is investigated as a possible cognitive enhancer for HD, but also basic science to determine the effects of NMDA receptor modulation. Specifically, the experiments presented set out to answer the following questions:

### 1.8.1 Do cerebrosterol and Org49209 potentiate NMDA receptor-evoked responses in neurons?

Previous work both from the Lambert laboratory (see section 1.6.5) and others (Paul et al., 2013) have shown that Org49209 is a PAM of the NMDA receptor. The Lambert laboratory has also demonstrated that Org49209 is able to increase hippocampal CA1 LTP, a NMDA receptor dependent process. Although (Paul et al., 2013) also showed that both Org49209 and cerebrosterol increased NMDA receptor mediated currents in cultured hippocampal neurons, these findings had not been published when my experiments had started. Therefore, I set out to answer whether Org49209 and cerebrosterol were PAMs of the NMDA receptor in cultured cerebellar granule neurons.

### 1.8.2 What is the effect of sulphation of Org49209 and cerebrosterol on NMDA receptor-evoked responses in neurons?

As preliminary data from the Lambert laboratory indicated that sulphation of the 3-position of the steroid ring of Org49209 increased drug efficacy, and cerebrosterol is able to be sulphated in the brain by enzymes present in neurons, I wanted to show whether sulphation of both Org49209 and cerebrosterol produced an increase of efficacy in neuronal cultures. This would allow for the exciting prospect of an additional mechanism for the fine-control of NMDA receptor activity by cerebrosterol and by the synthetic steroid Org49209.

### 1.8.3 Can Org49209 administration recover the behavioural phenotype of the HDH<sup>Q111+/-</sup> mouse?

Once the action of Org49209, cerebrosterol and their sulphated analogues had been investigated *in vitro*, the use of this NMDA receptor modulator was investigated *in vivo*. NMDA receptor dysfunction had been suggested to play a role in the cognitive deficits associated with HD. Therefore, I used a mouse model of HD to investigate the possibility of using Org49209 as a treatment for cognitive function. Previous work from the Lambert/Langston laboratory has suggested that the HDH<sup>Q111+/-</sup> mouse has a spatial learning impairment (see section 1.3.6). In addition, HD patients have reversal learning impairments (see section 1.4.6) and there is some evidence for dysfunctional reversal learning in HD mouse models (see section 1.4.6). Therefore, spatial learning was tested in the HDH<sup>Q111+/-</sup> mouse using the NPR task and in the water maze. The water maze was also used for investigating the reversal learning abilities of the HDH<sup>Q111+/-</sup> mouse, for use as a potential behavioural test to examine the effects of Org49209 on treatment of executive function disorders.

#### 1.8.4 Can Org49209 administration recover the behavioural set-shifting deficit, caused by inhibition of glutamatergic neurons in the mPFC, in rats?

As HD patients are also impaired at set-shifting, a perturbation likely due to corticostriatal loop dysfunctions (see section 1.4.3), and there is some evidence for set-shifting impairments in HD mouse models, the effect of Org49209 was tested in rats with a set-shifting deficit due to chemogenic inhibition of the glutamatergic neurons in the mPFC.



## Chapter 2

# Synthetic and Endogenous Steroids Potently Enhance the Function of Neuronal NMDA Receptors

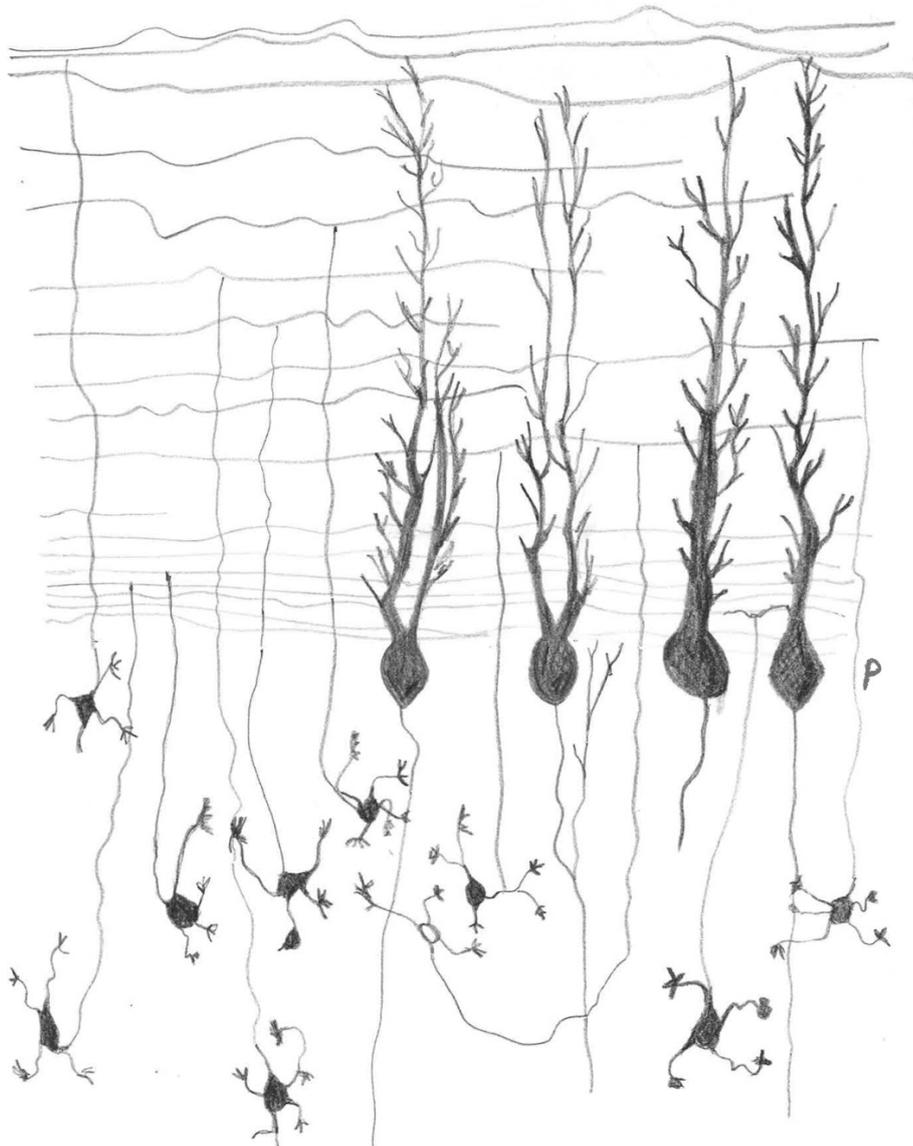


Illustration by C. Brazaitis

## 2.1 Introduction

Previous work demonstrated that both cerebrosterol and a related synthetic analogue steroid, Org49209, enhance NMDA receptor mediated cation currents. This was demonstrated both in the Lambert laboratory utilising *Xenopus laevis* oocytes expressing recombinant NMDA receptors and by Paul et al. (2013) in rat hippocampal neurons maintained in cell culture using voltage-clamp techniques.

Upon activation, the NMDA receptor undergoes a conformational change to cause the opening of an associated cation-selective ion channel. As described in section 1.1.2, although the channel is blocked in a use- and voltage-dependent manner by magnesium ions, it is highly permeable to calcium ions (Vyklícký et al., 2014). Consequently, single-cell calcium imaging provides a convenient method to study NMDA receptor function.

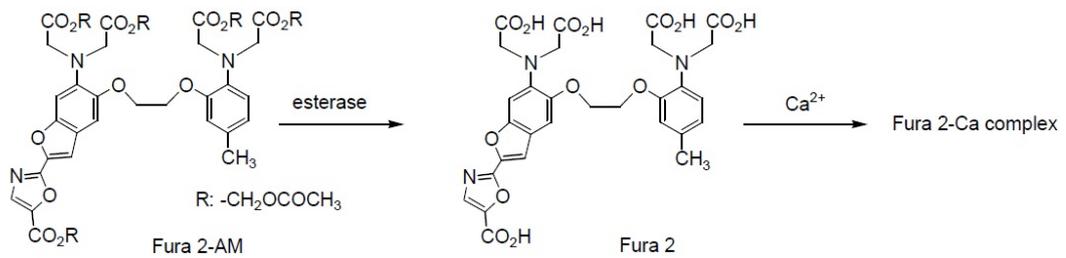
Cerebellar granule neurons (CGNs) were used in this assay, as the preparation provides a high neuron to glial cell ratio. CGNs are small glutamatergic neurons located in the internal granule layer of the cerebellum. There they receive excitatory inputs from mossy fibres and inhibitory inputs from Golgi neurons and project into the molecular layer of the cerebellum where they synapse with Purkinje cells (Butts et al., 2014). The NMDA receptor subunits expressed in CGNs include the mandatory NR1 subunit, and the NR2B subunit, which is gradually replaced with the NR2A subunit in the first 2 postnatal weeks of maturation (Llansola et al., 2005, Farrant et al., 1994). Between postnatal days 21 and 40 the incorporation of NR2C subunits is also expected (Cathala et al., 2000).

For recombinant NMDARs, Paul et al. (2013) have demonstrated that cerebrosterol is active on all four NR2 subunits subtypes when co-expressed with the NR1 subunit. Therefore, I expected to observe an increased NMDA receptor activation with cerebrosterol and its analogues. Additionally, 24-hydroxylase is highly expressed in Purkinje cells of the cerebellum and in Golgi

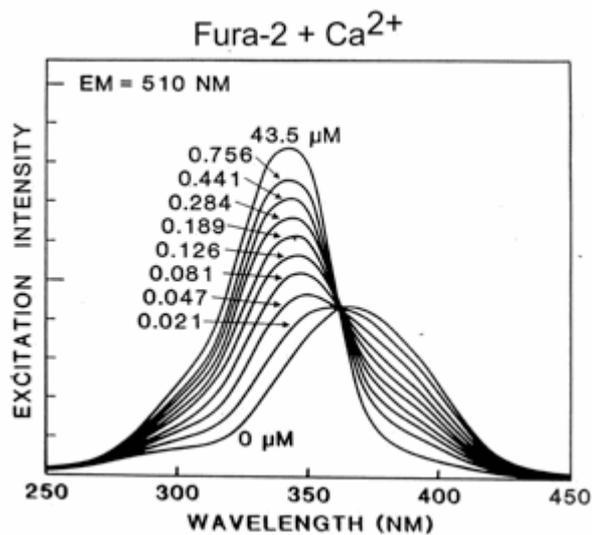
neurons of the granule cell layer (Ramirez et al., 2008), suggesting that cerebrosterol could putatively be produced in the cerebellum and therefore potentially be able to act as an endogenous modulator of NMDA receptors to influence cerebellar function.

## 2.2 Methods

Fura 2-acetoxymethyl ester (AM) is a ratiometric fluorescent dye that is used to measure changes in intracellular calcium levels. Due to the hydrophobic properties the AM group bestows on Fura 2-AM, it is membrane permeable; but once Fura 2-AM is inside the cell, the ester group is cleaved and the molecule becomes water soluble and membrane impermeable (Figure 2.1). This modification traps the indicator inside the cell, where it is able to bind with intracellular calcium. Coupled with increased excitation intensity, the excitation peak of Fura 2-AM shifts from 380 nm to 340 nm with bound calcium; the light is emitted at 510 nm (Figure 2.2). The ratio of bound calcium to free calcium provides a measure of the relative change in the intracellular calcium concentration (Nicholls, 1994). The ratiometric measurement of bound calcium is advantageous compared to approaches using single wavelength dyes as is it not affected by uneven dye uptake, dye leakage, variable cell sizes, and, importantly for long term imaging experiments, photo bleaching (Nicholls, 1994).



**Figure 2.1: The structural formula of Fura 2-AM.** Cleaving of the AM group occurs by an esterase once Fura 2-AM has entered the cell. Intracellular calcium can then bind to Fura 2 to form a Fura 2-Ca complex.



**Figure 2.2: The excitation spectrum of Fura 2 in response to a range of calcium concentrations.** Increased intracellular calcium results in increased excitation intensity of Fura 2, as well as a shift in the excitation peak from 380 nm to 340 nm. Image taken from <http://www.teflabs.com/ion-indicators>

### 2.2.1 Cell Culture Preparation

Cerebellar granule cell preparation was modified from M. Cousin's protocol (as described by Cheung and Cousin (2011)). CGNs were prepared from male and female C57BL6 mouse pups of developmental ages ranging between postnatal days 5-7. Sterile coverslips (13 mm  $\varnothing$ , Scientific Laboratory Supplies MIC3276) were coated in filter sterilised Poly-D-lysine (PDL, Sigma P6407) and 100 mM boric acid (BDH 100588W, at pH 8.5) overnight at room temperature on a roller. The following day coverslips were washed with sterile water 3 times

and left on the roller for one hour. The coverslips were then separated onto a sterile tissue in the tissue culture hood (Microflow Biological Safety Cabinet M51424/2) to dry fully before they were placed in a 24-well plate (TPP 92024). It was vital to ensure that all excess PDL was washed from the coverslip and the coverslip is dry as free PDL is toxic to neuronal cultures (Ahmed et al., 1983).

Each cerebellum was processed individually until the final cell count before cell plating. Pups were sacrificed by cervical dislocation followed by decapitation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. The cerebellum was dissected and placed into a dish containing 1 ml HBSS (Life Tech 14170088 Ca<sup>2+</sup>/Mg<sup>2+</sup> free; with 10 mM HEPES, Life Tech 15630-056). The tissue was minced up in the dish using fine forceps. The suspension was added to a 15 ml tube containing 1 ml of trypsin solution (0.05% trypsin in HBSS, as above). Trypsin is a proteolytic enzyme that breaks down the proteins responsible for cell adhesion and allows for the dissociation of cells. The tubes were left in a water bath at 37 °C and were agitated every 5 minutes by inverting the tube once. 3 ml of serum containing neutralisation solution (DMEM containing pyruvate, glutamine and high glucose (Life Tech, 41966029), supplemented with 10% FCS (Fisher Scientific 12329772) and 1% penicillin/streptomycin (pen/strep) (Life Tech 15140-122), and DNase (Invitrogen 18047-019, at a final concentration of 30 U/ml) was added to the cells to inactivate the trypsin. The cells were spun down in a centrifuge (Eppendorf 5804) at 1000 RPM for one minute. The supernatant was discarded and cells were re-suspended in 1 ml of neutralisation solution using a 1 ml pipette. Using the same pipette, the solution was triturated 30-35 times to fully dissociate the tissue. After the cells were left undisrupted for 2 minutes, the supernatant was taken and spun down at 1500 RPM for 2 minutes. The supernatant was discarded and cells were re-suspended in neutralisation solution (lacking DNase). At this stage all tissue was collected into one tube. A 10 µl sample of the cell suspension was diluted (1:10) in

Trypan blue. Trypan blue stains dead cells. Alive (unstained, bright) cells were counted using a haemocytometer. The cells were diluted in neurobasal growth media (Life Tech 21103-049, supplemented with 2% B27 (Life Tech 17504-044), 1X Glutamax (Life Tech 35050-038), and 1X pen/strep solution, 20 mM KCl and 1:5000 AraC (Sigma C1768-500mg)) plated on coverslips at a density of  $5 \times 10^5$  and left to adhere in an incubator (5% CO<sub>2</sub>, 37°C). After one hour the media was exchanged for 1 ml fresh medium and every three days a partial medium change (0.5 ml) was performed.

Penicillin was used in the media to prevent infections. Acute application of Penicillin has been shown to have an effect on electrophysiological properties of neurons, by antagonizing GABA<sub>A</sub> receptors, thereby causing cells to be more excitable (Tsuda et al., 1994). As it does not have a direct acute effect on NMDA receptors, and was not present during recording, it was still possible to use penicillin for cell culture maintenance although it could be argued that responses may be slightly altered compared to non-pen/strep treated cultures due to changes in synaptic plasticity.

## 2.2.2 Calcium Imaging

### 2.2.2.1 Fura 2-AM Loading

Cerebellar granule cells were used from DIV 6-20 for single neuron calcium imaging. One coverslip was placed from the culture medium into the incubation solution (in mM): 120 NaCl, 3.5KCl, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 5 NaHCO<sub>3</sub>, 1.2 Na<sub>2</sub>SO<sub>4</sub>, 10 glucose, 1.2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, and 20 N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES), pH adjusted to 7.4 with NaOH. The coverslip was then incubated in incubation medium containing Fura 2-AM (3 μM) for 45 minutes at room temperature, followed by a wash for 15 minutes in Fura 2-AM- free medium. The wash removes the extracellular dye, to minimise background fluorescence from free Fura 2-AM.

### 2.2.2.2 Imaging

The coverslip was placed under an inverted epifluorescence microscope with a 40X oil immersion objective (Zeiss Axiovert 200, Germany) and was stimulated with excitation light from a xenon arc lamp (Cairn, UK), after the wavelengths of 340 nm and 380 nm were selected by a monochromator (Cairn, UK). The light was delivered via a liquid fibre optic cable to the microscope. The resulting image from the 510 nm emitted light was captured by a camera (photometrics CoolSNAP HQ, Roper Scientific) and the data was viewed and recorded by MetaFlour software (version 7.8.2.0). All imaging was performed in a dark room to minimise photo bleaching. Using the software, regions of interest were selected to measure the change in fluorescent intensity of the cell bodies only. One region of interest was placed outside of the cell regions to serve as a reference by subtracting the background fluorescent signal from the signal obtained from the cell bodies. This design allowed for a more accurate comparison of the relative intracellular calcium concentration changes between coverslips. A perfusion system allowed for the constant exchange of imaging solution (the incubation solution contained a low [0.1 mM]  $\text{MgCl}_2$  concentration, coupled with 10  $\mu\text{M}$  glycine (G6761 Sigma), which are conditions designed to facilitate NMDA receptor function). Images were captured every 5 seconds with a 200 ms exposure to light, a protocol designed to gain good temporal and spatial resolution, without excessive photo bleaching over the course of long-term (120 mins) imaging. The shutter controlling the light intensity emitted from the xenon arc lamp was kept approximately 90% open, again to limit beaching, without compromising on image quality. A photodiode with narrow band pass filters for 380 nm and 340 nm was used to check and calibrate the excitation wavelengths.

## 2.2.3 Calcium Imaging Experimental Protocols

### 2.2.3.1 Characterisation

The NMDA receptor pharmacology of the cerebellar granule cells was characterised by obtaining a concentration response curve to NMDA (M3262, Sigma) with successive application of NMDA concentrations (1  $\mu$ M- 300  $\mu$ M) in the imaging solution (see above, containing reduced  $Mg^{2+}$  and 10  $\mu$ M glycine).

A curve was fitted to the data using the following equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{-(\text{LogEC50}-X) \cdot \text{HillSlope}}}$$

Where:

Bottom= The Y value at the bottom of the plateau. This is the minimum response.

Top= The Y value at the top of the plateau. This is the maximum response.

LogEC50= The X value half way between Bottom and Top. The EC50 is the effective concentration to reach half of the maximal response.

Hill Slope= The variable that describes the steepness of the curve.

To assess that the response to NMDA was caused by NMDA receptor activation, 30  $\mu$ M APV (an NMDA receptor competitive antagonist) was co-applied with NMDA to cells after submaximal (30  $\mu$ M) application of NMDA. APV occupies the agonist binding site, therefore preventing NMDA from binding to the receptor.

### 2.2.3.2 Neurosteroid Application

Steroids were repeatedly applied to the same cells at increasing concentrations, in a repeated measures design. To assess the effect of repeated application of NMDA resulting in submaximal receptor activation,

NMDA (30  $\mu$ M) was applied to the same coverslip six times. In between applications the cells were allowed to remain in wash for at least 5 minutes.

Various steroids were applied to CGNs maintained in cell culture to assess their effect on the NMDA induced calcium response of CGNs. The steroids tested were the known endogenous steroids pregnenolone (P9129 Sigma), pregnenolone sulphate (P162 Sigma); cerebrosterol and cerebrosterol sulphate (as well as a ketone variation, all provided by N. Hamilton); and the synthetic Org49209 (kindly provided by Prof. David O'Hagan) and Org49230 (provided by Niall Hamilton). Org49209 and Org49230 were originally discovered by the pharmaceutical company Organon, using a high throughput screen for NMDA receptor PAMs. The imaging solution was perfused until a stable baseline was obtained at which time 10-30  $\mu$ M NMDA (diluted from 10mM stock) was perfused on to the cells to obtain a submaximal NMDA response, followed by a wash after the NMDA response had peaked. For most experiments, 30  $\mu$ M NMDA was used; however, on rare occasions this concentration produced an unusually large response, in which case the concentration was decreased to 10  $\mu$ M NMDA. The cells were allowed to remain in the "wash" control solution for 5 minutes once the baseline was re-established after agonist washout.

Next, the steroid was applied in increasing concentrations- first in isolation (to assess any direct effects of steroid), followed by co-application with NMDA (to assess effects of the steroid on the NMDA-induced response). After each steroid/ NMDA co-application, a wash was applied and the calcium levels were allowed to reach baseline for 5 minutes before the next steroid concentration was applied. Steroid stock solutions were made up in 100% DMSO, and left in a heated sonicator bath until fully dissolved before further dilution and use. At the end of the experiment 40 mM of KCl (activating voltage-gated calcium channels) was perfused and again washed after the response peaked (see Figure 2.5 for a sample trace). This was to ensure that neurons responded with an expected large increase of intracellular calcium to the strong depolarisation

of KCl via an NMDA receptor independent mechanism. As astrocytes also express voltage-gated calcium channels, this method unfortunately could not be used to confirm recordings were only taken from neurons (Latour et al., 2003).

#### 2.2.4 Analysis

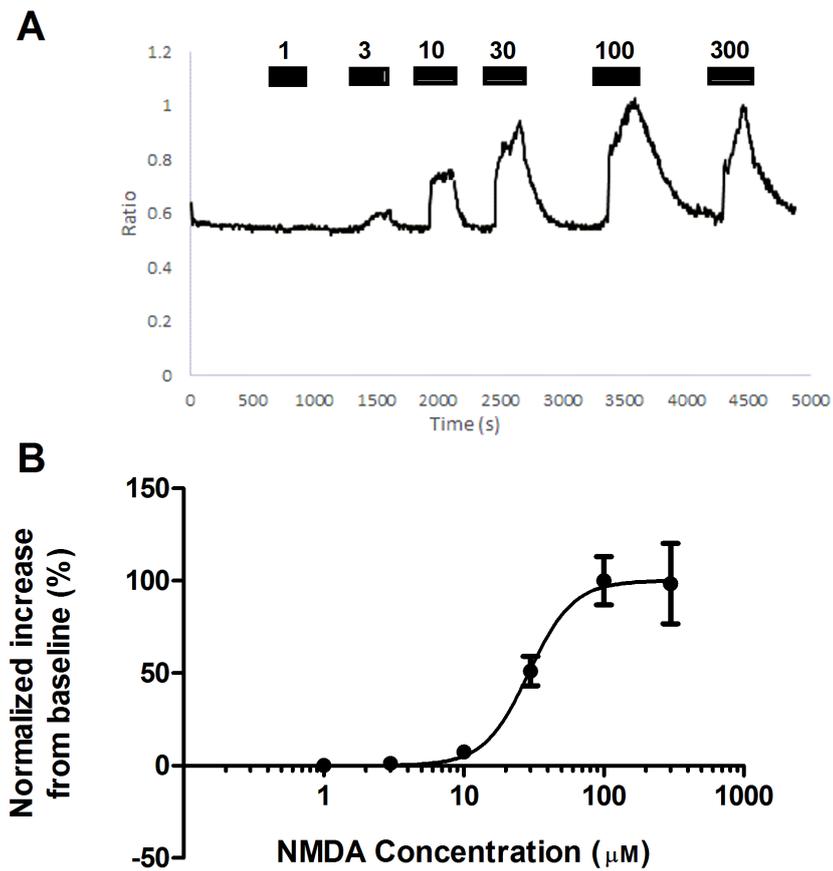
Data was saved as a text file by the Metaflour data recording software. Microsoft Excel was used to analyse the raw data and produce scatter graphs, the bar graphs and concentration responses curve were produced in Graph Pad. The maximum response value (ratio value) for each experimental condition was used for each region of interest corresponding to one cell. Each response was normalised to the baseline value and the percent increase was calculated as the difference between the NMDA-evoked response and the NMDA-evoked response in the presence of the steroid. Per treatment condition, a minimum of 15 cells (up to 50 cells) were averaged to produce one value. The 95% confidence interval (CI), plotted in the bar graphs, was calculated from the standard deviation of all cells in each treatment condition. The 95% CI is used to show the significance of the steroid-induced change of the NMDA-induced calcium response.

### 2.3 Results

#### 2.3.1 Characterisation of CGN Calcium Imaging Responses

##### 2.3.1.1 The NMDA Concentration Response Relationship

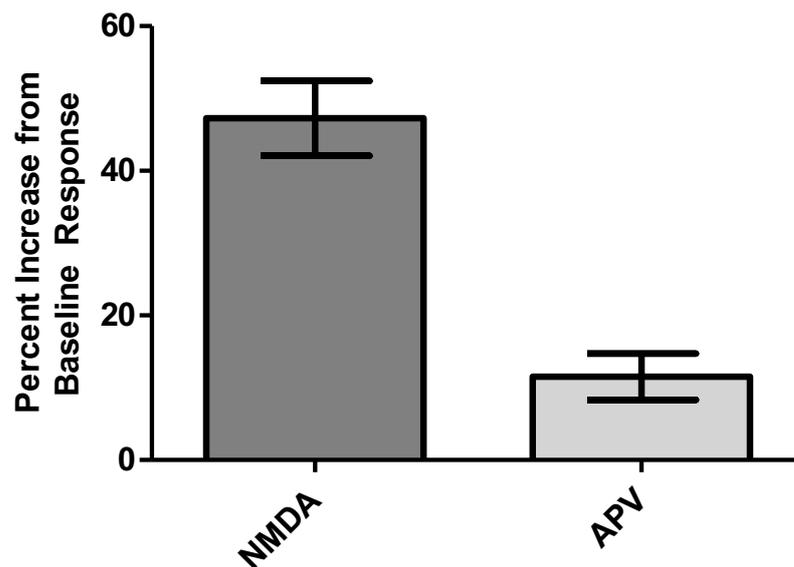
Examination of the NMDA concentration response relationship revealed that 10  $\mu\text{M}$  of the agonist to be a threshold concentration, with 100 – 300  $\mu\text{M}$  NMDA producing a maximal effect (Figure 2.3). From further inspection of the concentration-response relationship the  $\text{EC}_{50}$  for NMDA was found to be 29.1  $\mu\text{M}$  with a confidence interval (CI) ranging from 23.5 to 36.1  $\mu\text{M}$  (Figure 2.3,  $n=6$ ). The Hill slope was found to be  $2.7 \pm 1.1$ .



**Figure 2.3: NMDA produces a concentration-dependent increase of intracellular calcium in CGNs (DIV 5 – 19).** (A) A representative trace of the raw data showing the response ratio of intracellular calcium with increasing concentrations of NMDA (black bars, showing in order: 1, 3, 10, 30, 100, 300  $\mu\text{M}$ ). (B) The concentration response relationship plotting the influence of the NMDA concentration (on a log scale) vs the NMDA-induced change of the intracellular calcium concentration (a linear scale) expressed as a percentage of the response (100 %) to a maximal concentration of NMDA (300  $\mu\text{M}$ ) determined for each cell (n=6). Note all ratio values were normalised to the baseline response, maximum and minimum values were normalised to 100% and 0% respectively. Error bars show SEM.

### 2.3.1.2 NMDA Response is Reduced by the NMDA Receptor Antagonist APV

At low concentrations of NMDA (30  $\mu\text{M}$ ) that evoke a submaximal response, the application of APV (30  $\mu\text{M}$ ) reduced this response 4 fold, from  $47.3 \pm 2.6\%$  to  $11.5 \pm 1.6\%$  of the baseline calcium levels (Figure 2.4). Not only is NMDA a selective NMDA receptor agonist, but the strong effect of this selective competitive antagonist demonstrates that NMDA receptor activity was measured using this calcium imaging technique.

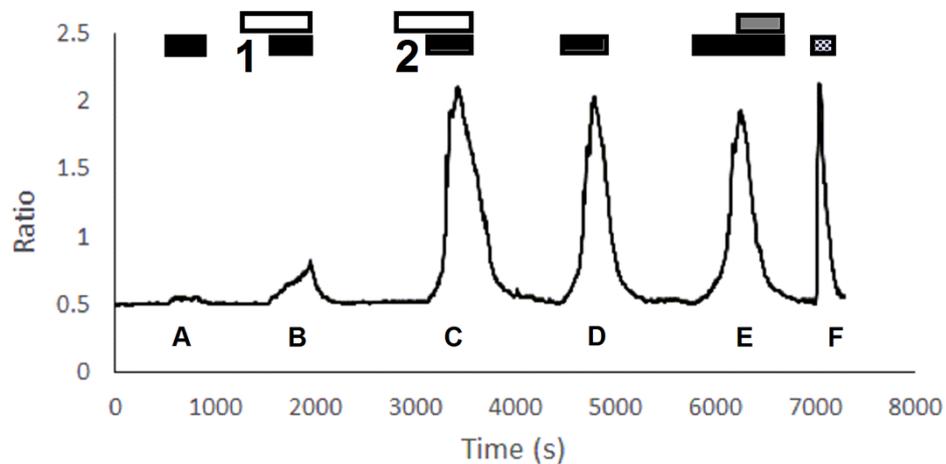


**Figure 2.4:** *The calcium response to submaximal NMDA (30  $\mu\text{M}$ ) is decreased by 4 fold with the NMDA receptor antagonist, APV (30  $\mu\text{M}$ ).* Application of 30  $\mu\text{M}$  NMDA alone produced an increase in the baseline response by  $47.3 \pm 2.6\%$ , which 30  $\mu\text{M}$  APV reduced to  $11.5 \pm 1.6\%$ ,  $n=4$ . Error bars show 95% CI.

### 2.3.2 Modulation of NMDA Receptors by Neuroactive Steroids

To elucidate the effect of the various neuroactive steroids on NMDA receptor function, the steroid was applied at increasing concentrations in conjunction with NMDA. DMSO at a final dilution of 1:1000 (which was used for steroid applications) was also applied as a control and had no effect on intracellular calcium levels of CGNs (data not illustrated). In each experiment a submaximal

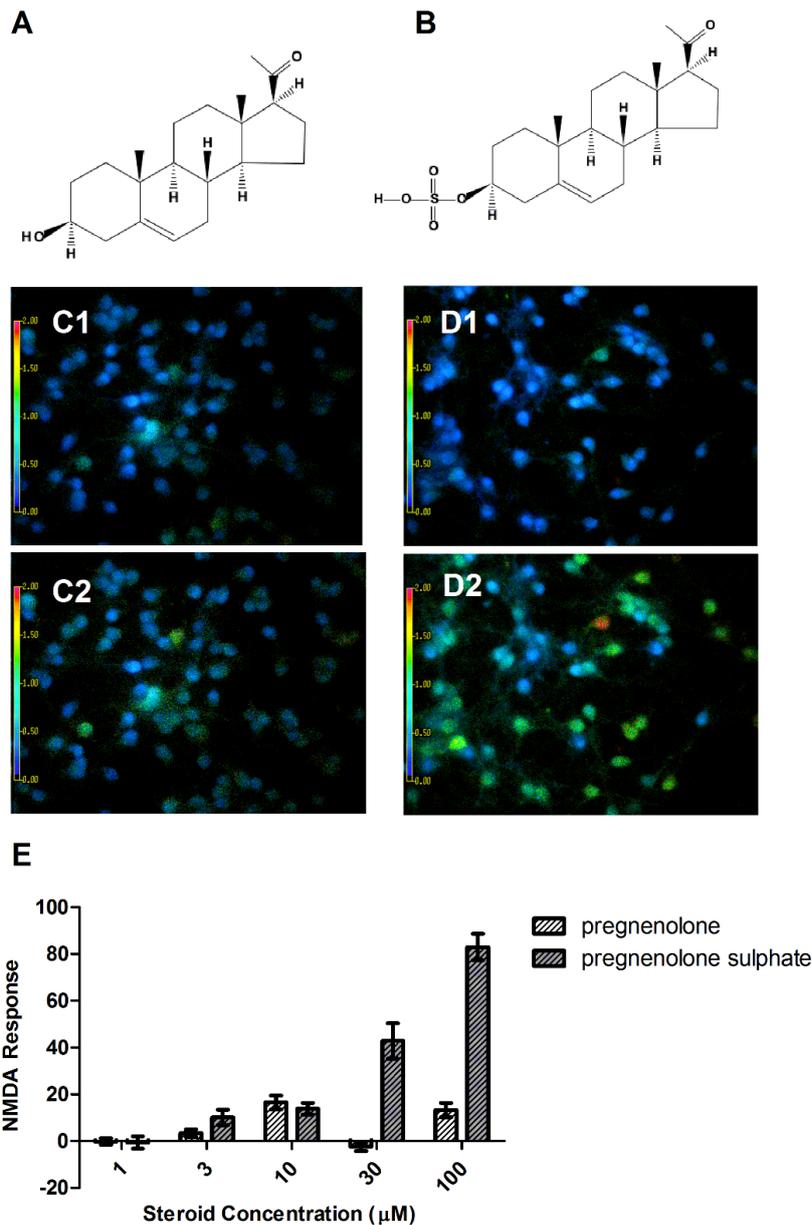
concentration of NMDA (10-30  $\mu$ M) was applied to the cells first, followed by a wash. Each steroid was applied in isolation before it was co-applied with the same submaximal concentration used initially. The preparation was then washed again and after the cells recovered to baseline, the next concentration was applied. Figure 2.5 shows an example of the trace from the raw data of a sample experiment recording from a single neuron. Importantly, this trace also illustrates the resistance for the steroid effect to be reversed upon washout. It was therefore essential to apply the steroid in increasing concentrations; i.e., in a cumulative escalating concentration paradigm. Finally, the trace also shows the antagonizing effect of APV (as shown in Figure 2.4).



**Figure 2.5 A sample trace following one cell through a series of drug applications.** The trace shows the response ratio of a single cell in different drug conditions: **(A)** 30  $\mu$ M of NMDA (black bars) produced a submaximal response **(B)** Pre-application of an un-named steroid (100 nM, labelled **(1)**, white bar) alone gave no response; however, the co-application of the steroid with NMDA increased the calcium response, followed by a return to baseline in the wash (steroid free) solution; **(C)** Pre-application of 300 nM steroid again had no effect (labelled **(2)**, white bar), co-application with NMDA, however, caused an even larger increase, followed by return to baseline in wash saline. **(D)** The response remained elevated *cf* control when NMDA was applied alone. **(E)** This NMDA response was attenuated by the NMDA receptor antagonist, APV (grey bar). **(F)** KCl (40 mM, chequered bar) caused calcium influx through voltage gated calcium channels, an NMDA receptor independent mechanism.

### 2.3.3 Pregnenolone Sulphate: A Known PAM of Neuronal NMDA Receptors.

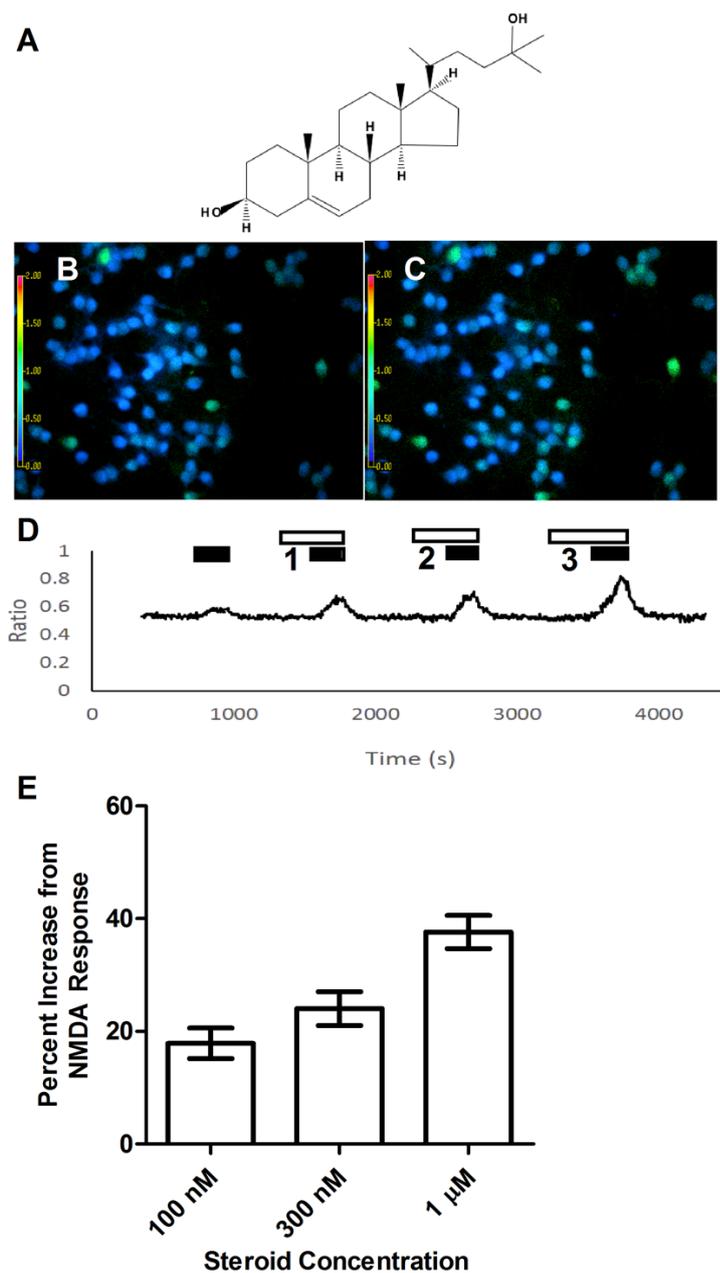
Pregnenolone sulphate is a known PAM of the NMDA receptor and acts to enhance cognitive performance in certain rodent behavioural paradigms (Wu et al. (1991), see also section 1.6.1). However, this steroid is not selective for NMDA receptors as it also inhibits GABA<sub>A</sub> receptors and strychnine-sensitive glycine receptors- actions that may contribute to the cognitive effects of this steroid (Schumacher et al., 2008). By contrast, the non-sulphated derivative, pregnenolone was previously shown to be inactive at the NMDA receptor (Vallee, 2015, Wu et al., 1991). Therefore, before investigating the actions of novel steroids in the CGN assay, I first compared the actions of pregnenolone and pregnenolone sulphate on NMDA-evoked increases of intracellular calcium. In agreement with published studies, here pregnenolone (1 – 30 µM) had little or no effect on the NMDA-evoked response. However, at the greater concentration of 100 µM this steroid caused a modest enhancement of the NMDA (30 µM)-induced response ( $13.3 \pm 1.5\%$  (n=4)). By contrast, pregnenolone sulphate (3 – 100 µM) produced a clear concentration-dependent increase of the NMDA (30 µM)- evoked response, with (30 µM pregnenolone sulphate causing a  $42.8 \pm 3.8\%$  increase and 100 µM producing an  $83.0 \pm 2.8\%$  increase (n=4), Figure 2.6).



**Figure 2.6: Pregnenolone sulphate, but not pregnenolone enhances NMDA receptor function of cerebellar granule cells.** (A) The molecular structure of pregnenolone and (B) pregnenolone sulphate. (C) Representative images of CGNs containing intracellular Fura 2-AM showing responses to (1) submaximal (30 μM) NMDA and (2) the same field of cells with NMDA + 100 μM pregnenolone. (D) Images showing (1) submaximal (30 μM) NMDA (2) the same field of cells with NMDA + 100 μM pregnenolone sulphate. The scale bars indicate the relative calcium concentration starting at low concentrations (blue), continuing to high concentrations (red). (E) A bar graph comparing the effects of pregnenolone and pregnenolone sulphate on the NMDA (30 μM)-evoked response. Note that pregnenolone (1 -30 μM) had little, or no effect on the NMDA-evoked response, although the greater concentration of 100 μM produced a modest enhancement of  $13.3 \pm 1.5\%$  ( $n=4$ ). By contrast, pregnenolone sulphate produced a clear concentration-dependent (3 – 100 μM) enhancement of the NMDA (30 μM)-evoked response, where 30 μM pregnenolone sulphate produced an  $42.8 \pm 3.8\%$  increase and 100 μM produced an  $83.0 \pm 2.8\%$  increase ( $n=4$ ). The columns represent the means with 95% CI.

#### 2.3.4 Org49209: A Potent PAM of Neuronal NMDA Receptors

Previous studies from the Lambert laboratory utilising recombinant receptors expressed in *Xenopus laevis* oocytes demonstrated that in contrast to pregnenolone sulphate, Org49209 at concentrations that enhanced NMDA receptor function had no effect on GABA<sub>A</sub> receptors (Chapter 1, Figure 1.9). Here Org49209 (100 nM – 1 μM) produced a concentration-dependent increase of the NMDA (30 μM)-evoked increase of intracellular calcium in CGNs. Specifically, 100 nM increased the response by  $18 \pm 1.4\%$  (n=6), 300 nM increased the response by  $24 \pm 1.5\%$  (n=5) and 1 μM increased the response by  $38 \pm 1.5\%$  (n=9) (Figure 2.7).

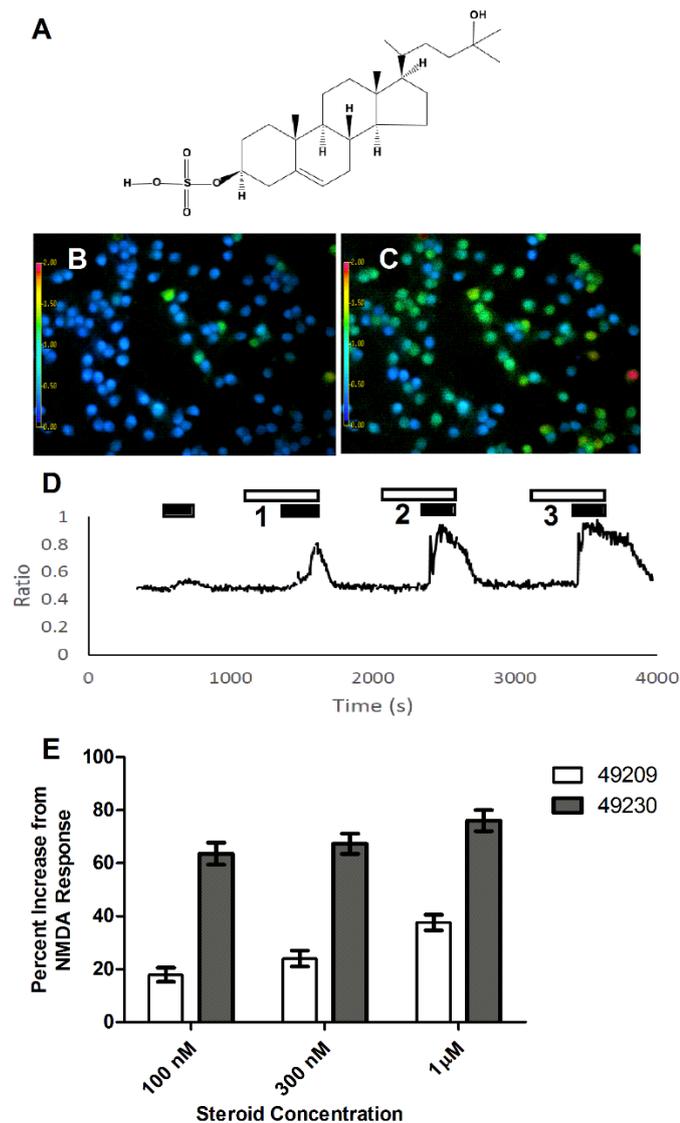


**Figure 2.7: *Org49209* is a potent, but relatively low efficacy PAM of the NMDAR. (A)** The molecular structure of *Org49209*. **(B)** Representative images of CGNs containing intracellular Fura 2-AM showing responses to submaximal (30  $\mu$ M) NMDA and **(C)** the same field of cells now in the presence of NMDA + 1  $\mu$ M *Org49209*. The scale bars indicate the relative calcium concentration starting at low concentrations (blue), continuing to high concentrations (red). **(D)** A sample trace of raw data showing the response ratio of one cell to a range of drug applications: submaximal (30  $\mu$ M) NMDA (black bars) followed by wash with agonist free saline **(1)** 100 nM of *Org49209* (white bars), followed by co-application with NMDA and wash with drug free saline. **(2)** 300 nM *Org49209* followed by co-application with NMDA and wash. **(3)** 1  $\mu$ M *Org49209* followed by co-application with NMDA and wash. **(E)** A bar graph showing the effects of *Org49209* on the NMDA (30  $\mu$ M)-evoked response. *Org49209* increased the NMDA response by 17.9 $\pm$ 1.4% at 100nM (n=6), increasing to 24.0 $\pm$ 1.5% at 300nM (n=5) and 37.6 $\pm$ 1.5% at 1  $\mu$ M (n=9). The columns indicate the mean with 95% CI.

### 2.3.5 Org49230: A Highly Potent PAM of Neuronal NMDA Receptors

Given that pregnenolone sulphate is an efficacious PAM of the NMDA receptor, whereas the non-sulphated parent steroid pregnenolone is relatively inert in this respect, it was of interest to investigate whether this chemical structural modification of Org49209 would similarly enhance the efficacy of this potent PAM of the NMDA receptor. Org49230 is the sulphated derivative of Org49209. In common with pregnenolone sulphate, Org49230 is sulphated at the 3-position of the A ring of the steroid (Figure 2.8A).

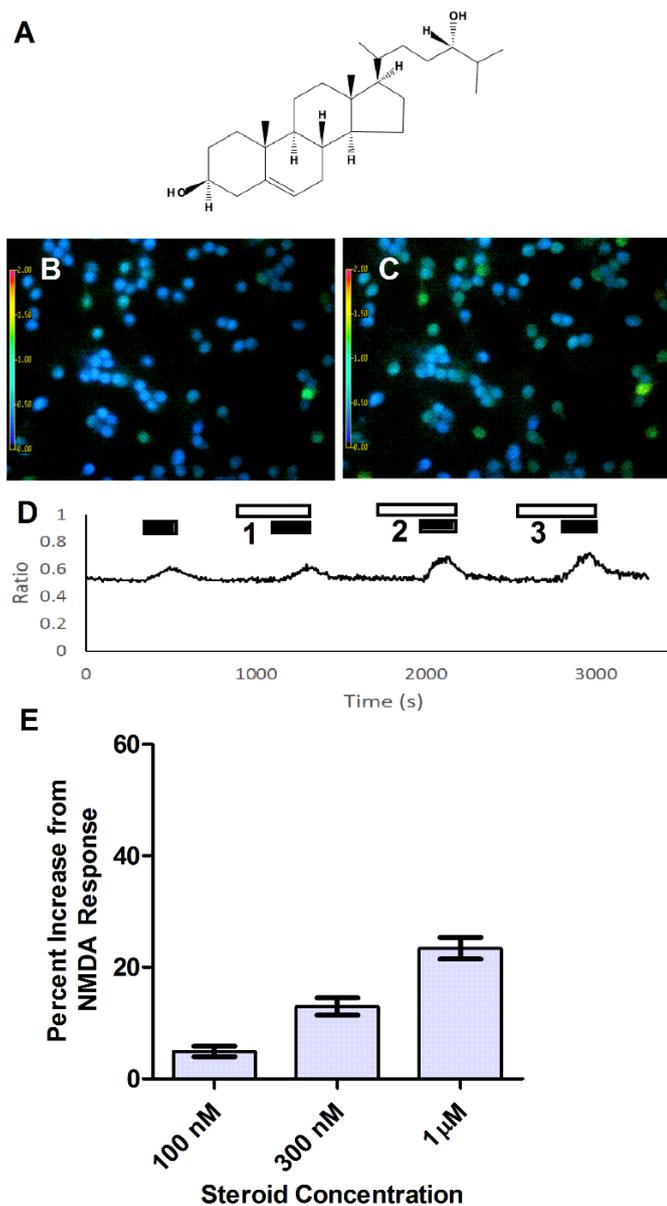
Org49230 (100 nM - 1  $\mu$ M) produced a concentration-dependent enhancement of the NMDA (30  $\mu$ M)-induced response of CGNs. Although active at similarly low concentrations to Org49209, this sulphated analogue was far more potent, increasing the NMDA-induced response by  $63.6 \pm 2.1\%$  at 100 nM, increasing to  $67.4 \pm 2.0\%$  at 300 nM, and  $76.1 \pm 2.1\%$  at 1  $\mu$ M (Figure 2.8E). The response to the sulphated steroid compared to the non-sulphated steroid increased 3.5 fold at 100 nM, 2.8 fold at 300 nM, and 2.0 fold at 1  $\mu$ M. As an asymptotic concentration for Org49209 was not reached, it is not possible to deduce that Org49230 is more efficacious than Org49209, however, it is more potent at the same concentrations.



**Figure 2.8: Sulphation of Org 49209 to produce Org49230 greatly increased the efficacy of the steroid to enhance NMDA receptor function. (A)** The molecular structure of Org49230. **(B)** Representative images of CGNs containing intracellular Fura 2-AM showing responses to submaximal (30  $\mu$ M) NMDA and **(C)** the same field of cells now in the presence of NMDA + 1  $\mu$ M Org49230. The scale bars show the relative calcium concentration starting at low concentrations (blue), continuing to high concentrations (red). Note how the presence of the neuroactive steroid has greatly increased the NMDA-induced response. **(D)** A sample trace of raw data showing the response ratio of one cell to a range of drug applications: submaximal (30  $\mu$ M) NMDA (black bars) followed by wash with agonist free saline **(1)** 100 nM of Org49230 (white bars), followed by co-application with NMDA and wash with drug free saline. **(2)** 300 nM Org49230 followed by co-application with NMDA and wash. **(3)** 1  $\mu$ M Org49230 followed by co-application with NMDA and wash. **(E)** A bar chart comparing the NMDA-enhancing action of Org 49209 and Org 49230. Here Org49230 increased the NMDA response by  $64 \pm 2.1\%$  at 100 nM ( $n=8$ ),  $67 \pm 2.0\%$  at 300 nM ( $n=6$ ), and  $76 \pm 2.1\%$  at 1  $\mu$ M ( $n=8$ ). The enhancement of the NMDA-evoked response by Org49230 in comparison to Org49209 was 3.5 fold at 100 nM, 2.8 fold at 300 nM, and 2.0 fold at 1  $\mu$ M. The columns indicate the mean with 95% CI.

### 2.3.6 Cerebrosterol: A Potent PAM of Neuronal NMDA Receptors.

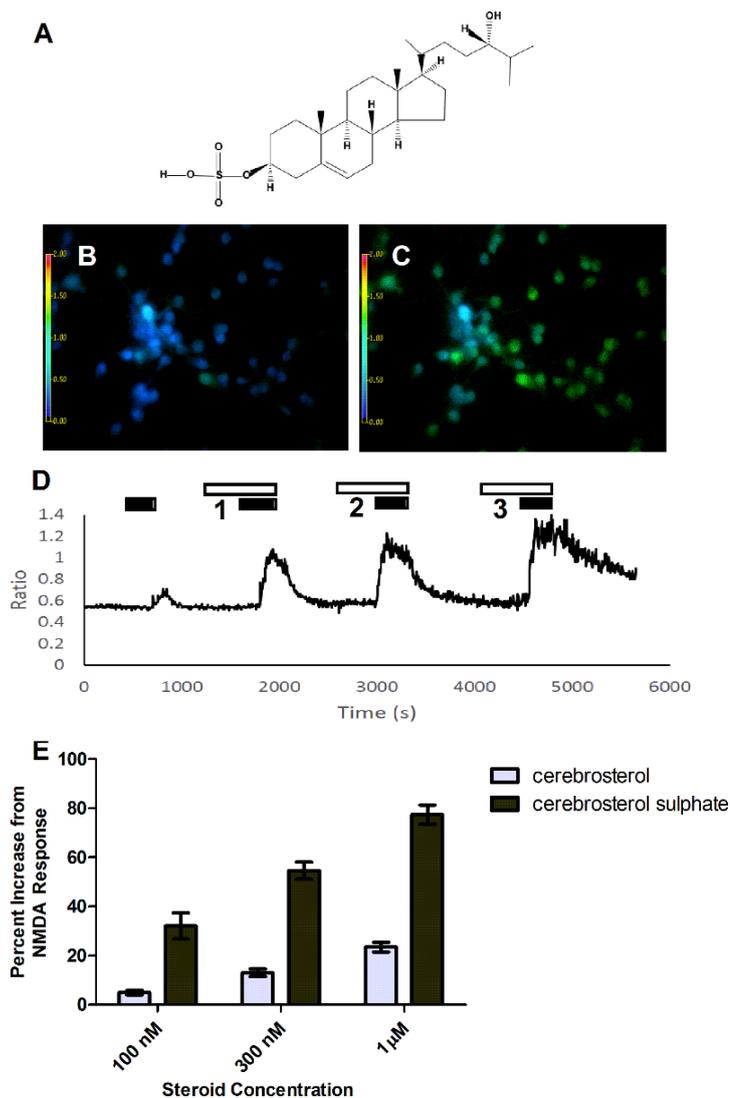
The endogenous neurosteroid cerebrosterol is structurally related to the synthetic neuroactive steroid Org49209 (compare Figures 2.9A and 2.7A). Therefore, cerebrosterol was tested in the calcium imaging assay to determine whether this steroid, the major metabolite of cholesterol in mammalian brain (Bjorkhem et al., 1998), could enhance neuronal NMDA receptor function in a similar fashion to Org49209. Importantly, cerebrosterol (100 nM – 1  $\mu$ M) increased the NMDA-evoked response of CGNs in a concentration-dependent manner. The threshold concentration of cerebrosterol was 100 nM (increasing the NMDA- evoked response by  $4.9 \pm 0.5\%$ , n=4), while 300 nM increased the response by  $13 \pm 0.8\%$  (n=4) and 1 $\mu$ M increased the response by  $23 \pm 1.0\%$  (n=11) (Figure 2.9). Given the impact of sulphation on the NMDA-enhancing actions of the endogenous neurosteroid pregnenolone, and the synthetic neuroactive steroid Org49209 described above it is clearly of interest to investigate whether the sulphation similarly impacts upon the ability of cerebrosterol to enhance NMDA receptor function.



**Figure 2.9: Cerebrosterol is a potent low efficacy PAM of the NMDAR.** (A) The molecular structure of cerebrosterol. (B) Representative images of CGNs containing intracellular Fura 2-AM showing responses to submaximal (30  $\mu$ M) NMDA and (C) the same field of cells now in the presence of NMDA + 1  $\mu$ M cerebrosterol. The scale bars show the relative calcium concentration starting at low concentrations (blue), continuing to high concentrations (red). (D) A sample trace of raw data showing the response ratio of one cell to a range of drug applications: submaximal (30  $\mu$ M) NMDA (black bars) followed by wash with agonist free saline (1) 100 nM of cerebrosterol (white bars), followed by co-application with NMDA and wash with drug free saline. (2) 300 nM cerebrosterol followed by co-application with NMDA and wash. (3) 1  $\mu$ M cerebrosterol followed by co-application with NMDA and wash. (E) A bar graph showing the effects of cerebrosterol on the NMDA (30  $\mu$ M)-evoked response. Cerebrosterol increased the NMDA response by  $4.9 \pm 0.5\%$  at 100 nM (n=4),  $13 \pm 0.8\%$  at 300 nM (n=4), and  $23 \pm 1.0\%$  at 1  $\mu$ M (n=11). The columns indicate the mean with 95% CI.

### 2.3.7 Cerebrosterol Sulphate: A Highly Potent PAM of Neuronal NMDA Receptors

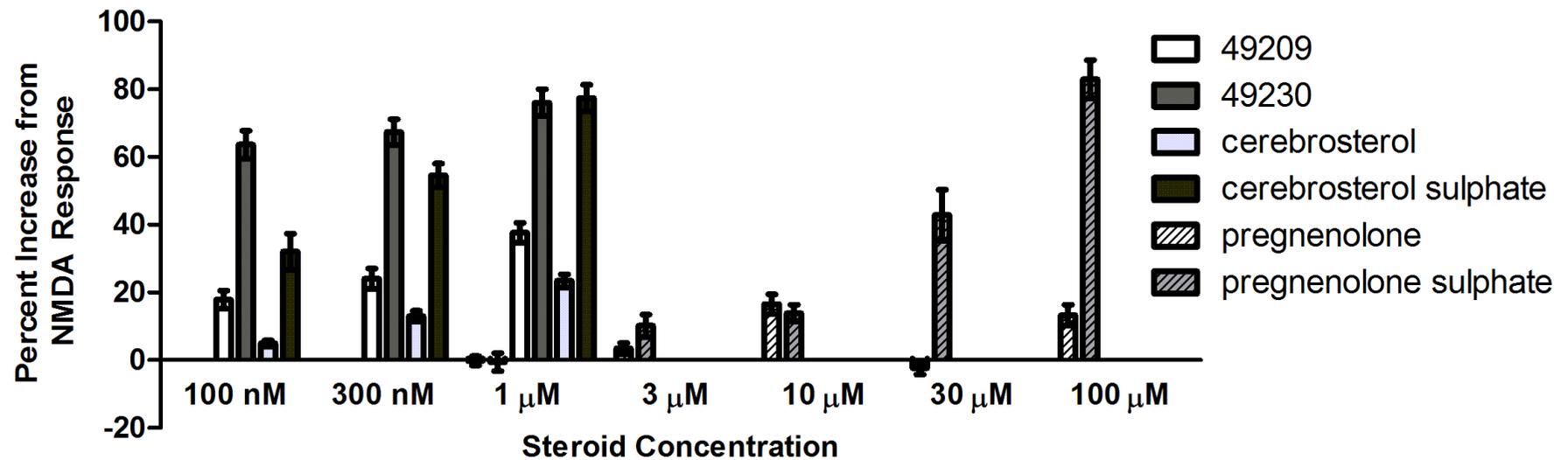
Cerebrosterol sulphated at the 3-position of the steroid A ring was synthesised by Dr. Niall Hamilton and kindly provided by him for experimentation. As described above, given the impact of sulphation on the efficacy of these steroids as NMDA receptor PAMs, it was of interest to investigate whether sulphation similarly influenced the interaction of cerebrosterol with the NMDA receptor. Furthermore, it is conceivable that cerebrosterol may be sulphated in the brain by the resident sulphotransferases and desulphated by sulphatases (Javitt et al., 2001, Iwamori, 2005). My experiments revealed sulphated cerebrosterol to be a more potent PAM of neuronal NMDA receptors and cerebrosterol. Relatively low concentrations of cerebrosterol sulphate increased the NMDA (30  $\mu$ M)-evoked response in a concentration-dependent manner (100 nM =  $32 \pm 2.7$  % increase, n = 6; 300 nM =  $55 \pm 1.8$  % increase, n = 6; 1  $\mu$ M =  $77 \pm 2.0$  % increase, n = 3; Figure 2.10). In comparison to the parent steroid, cerebrosterol sulphate was more potent at each concentration tested with the magnitude of enhancement of the NMDA-evoked response produced by cerebrosterol sulphate being 6.5 fold for 100 nM; 4.2 fold for 300 nM and 3.3 fold for 1  $\mu$ M than produced by equivalent concentrations of cerebrosterol. As with the synthetic steroids, efficacy could not be compared as an asymptotic concentration was not reached with cerebrosterol.



**Figure 2.10: Cerebrosterol sulphate is a highly potent and efficacious PAM of neuronal NMDA receptors.** (A) The molecular structure cerebrosterol sulphate. (B) Representative images of CGNs containing intracellular Fura 2-AM showing responses to submaximal (30  $\mu$ M) NMDA and (C) the same field of cells now in the presence of NMDA + 1  $\mu$ M cerebrosterol sulphate. The scale bars show the relative calcium concentration starting at low concentrations (blue), continuing to high concentrations (red). Note how the presence of the neuroactive steroid has greatly increased the NMDA-induced response. (D) A sample trace of raw data from a single cell showing the response ratio of one cell to a range of drug applications: submaximal (30  $\mu$ M) NMDA (black bars) followed by wash with agonist free saline (1) 100 nM of cerebrosterol sulphate (white bars), followed by co-application with NMDA and wash with drug free saline. (2) 300 nM cerebrosterol sulphate followed by co-application with NMDA and wash. (3) 1  $\mu$ M cerebrosterol sulphate followed by co-application with NMDA and wash. (E) A bar chart comparing the NMDA-enhancing action of cerebrosterol and cerebrosterol sulphate. Here cerebrosterol sulphate increased the NMDA response by  $32 \pm 2.7\%$  at 100 nM (n=6),  $55 \pm 1.8\%$  at 300 nM (n=6), and  $77 \pm 2.0\%$  at 1  $\mu$ M (n=3). The enhancement of the NMDA-evoked response by cerebrosterol sulphate in comparison to cerebrosterol was 6.5 fold at 100 nM, 4.2 fold at 300 nM, and 3.3 fold at 1  $\mu$ M. The columns indicate the mean with 95% CI.

### 2.3.8 Summary of Neuroactive Steroids

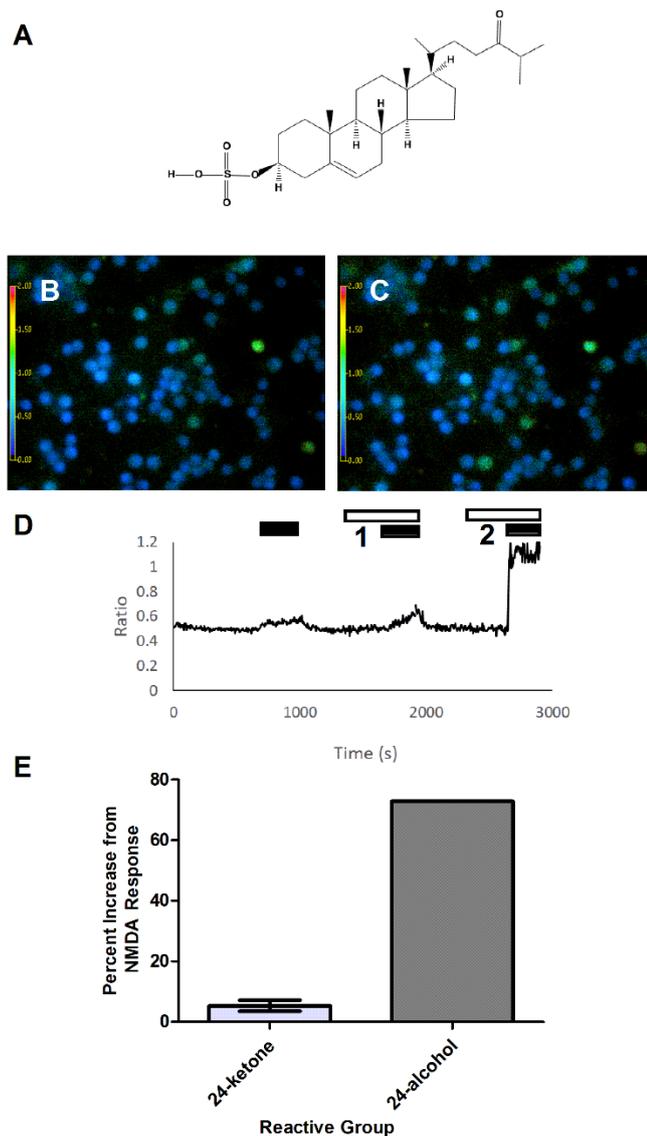
To more easily compare the effects of each of the steroids, Figure 2.11 provides a summary of the means of the above presented steroids, at varying concentrations. Particularly noticeable is the difference in potency between pregnenolone sulphate and the other neuroactive steroids, as well as the difference in efficacy of the sulphated steroids compared to the non-sulphated steroids.



**Figure 2.11: A comparison of the NMDA-enhancing actions of neuroactive steroids.** Both cerebrosterol and its synthetic analogue, Org49209, are active at relatively low concentrations (100 nM – 1 μM). The sulphated steroids Org49230 and cerebrosterol sulphate are active at the same low concentrations as their non-sulphated steroid counterparts, but in comparison a far more efficacious. Pregnenolone sulphate is only active at higher concentrations (30 – 100 μM), while pregnenolone is mostly inactive. The columns indicate the mean with 95% CI.

### 2.3.9 Ketone Group Properties

To assess the properties of the steroid that are needed for NMDAR modulation, a compound was synthesised (by Dr. Niall Hamilton) that was identical to cerebrosterol sulphate, however, the 24-alcohol was replaced with a 24-ketone. This steroid did not increase the NMDA induced response at 1  $\mu\text{M}$  (n=5, Figure 2.12). Cerebrosterol sulphate was tested post ketone application as a control, and showed the expected increase as seen above (n=2).



**Figure 2.12: Cerebrosterol sulphate with a 24-ketone replacing the 24-alcohol is inactive on the NMDAR.** (A) The molecular structure of cerebrosterol sulphate 24-ketone. (B) Representative images of CGNs containing intracellular Fura 2-AM showing responses to submaximal (30  $\mu$ M) NMDA and (C) the same field of cells now in the presence of NMDA + 1  $\mu$ M cerebrosterol sulphate (24-ketone). The scale bars show the relative calcium concentration starting at low concentrations (blue), continuing to high concentrations (red). (D) A sample trace of raw data showing the response ratio of one cell to a range of drug applications: submaximal (30  $\mu$ M) NMDA (black bars) followed by wash with agonist free saline (1) 1  $\mu$ M cerebrosterol sulphate (24-ketone, white bars), followed by co-application with NMDA and wash with drug free saline. (2) 1  $\mu$ M cerebrosterol sulphate (24-alcohol) followed by co-application with NMDA and wash. (3) 1  $\mu$ M cerebrosterol sulphate followed by co-application with NMD. (E) A bar chart comparing the NMDA-enhancing action of cerebrosterol sulphate (24-ketone) and cerebrosterol sulphate (24-alcohol). Here cerebrosterol sulphate (24-ketone) did not increase the NMDA mediated response (n=5). Cerebrosterol sulphate (24-alcohol) was applied as a control on the same cells and showed the previously established enhancement of the NMDA induced response (see Figure 2.10, n=2). The columns indicate the mean with 95% CI.

## 2.4 Discussion

This chapter describes a protocol for assessing the ability for certain steroids to modulate NMDA receptors in neuronal cell cultures. Here, calcium imaging was used as an indirect measure of NMDA receptor activity in CGNs. Steroids originally discovered and synthesised by Organon as potential PAMs of the NMDA receptor were tested, as well as structurally-related endogenously occurring steroids.

Before the activity of the steroids were tested, the CGNs were characterised for their response to NMDA. In the presence of glycine, NMDA produced a concentration dependent increase of intracellular calcium as inferred by changes in fluorescence with an  $EC_{50}$  comparable to responses observed by others in cultured neurons (Sinor et al., 2000). The Hill slope was higher than predicted from others using patch clamp recordings (Patneau and Mayer, 1990, Verdoorn and Dingledine, 1988, Hahn et al., 2015). This is likely due to the fact that calcium imaging is an indirect measure of NMDA receptor activity, and whole-cell patch clamp preparations are needed to accurately estimate NMDA receptor pharmacology. However, APV (a competitive NMDA receptor antagonist) significantly reduced the response to submaximal NMDA application (Figure 2.4). Not only is NMDA a selective NMDA receptor agonist, but the clear effect of this selective antagonist gives a strong indication that NMDA receptor activity is being measured using this technique.

Pregnenolone sulphate was tested first as a well-documented PAM of the NMDA receptor (Wu et al., 1991). In agreement with others, this steroid increased the NMDA-evoked response in CGNs (Figure 2.6). Pregnenolone sulphate showed a clear and large enhancement of the NMDA-evoked response at 30  $\mu$ M, consistent with the literature (Wu et al. (1991), see also Section 1.6.1). In accordance with literature pregnenolone had little or no effect on the NMDA-evoked response for most concentrations, surprisingly a slight response was seen at the highest concentration tested (100  $\mu$ M, Figure

2.6). This is inconsistent with the literature which shows pregnenolone to be inactive on the NMDA receptor (Vallee, 2015). Although pregnenolone sulphate is clearly very efficacious, relatively high steroid concentrations are required to produce this effect and there is debate as to whether such concentrations are achieved under physiological or pathophysiological conditions (Smith et al., 2014). Indeed, levels detected both in rat and human brains have not been above the high nanomolar range (Schumacher et al., 2008). Furthermore, at similar concentrations to those required for activity at the NMDA receptor, pregnenolone sulphate acts to inhibit GABA<sub>A</sub> receptor-mediated responses (Majewska et al. (1988), see also Chapter 1, Figure 1.9), an action that may contribute to the cognitive enhancing properties of this steroid. In summary, not only is pregnenolone sulphate not selective for the NMDA receptor, but it may not be potent enough to act as a PAM of the NMDA receptor in the brain under physiological or pathophysiological conditions. Therefore, it is of particular interest that Organon was able to identify synthetic compounds that are both potent and selective NMDA receptor PAMs.

The first novel steroid tested was the Organon compound, Org49209, which was effective at relatively low concentrations (100 nM), but only modestly increased the NMDA-evoked response of the CGNs even at the highest concentration tested (1  $\mu$ M, Figure 2.7); i.e., it is a potent PAM of the NMDA receptor but exhibits relatively limited efficacy at the concentrations measured. As pregnenolone sulphate, but not pregnenolone, is active as a PAM of the NMDA receptor, it was of interest to investigate the effect of sulphation of Org49209 at the same 3-position of the steroid A ring i.e. Org49230. In the CGN assay this structural modification produced a large increase in NMDA receptor potency of the steroid, demonstrated by large increase in response at the same concentrations as Org49209 (Figure 2.8). Indeed, inspection of the concentration response relationship for these two steroids suggested that sulphation may have also increased the NMDA

receptor potency of the steroid by lowering the concentration needed to produce a minimum response. Should time permit it would be of interest to investigate the NMDA receptor actions of lower concentrations of Org49230. These data are in agreement with an electrophysiological study from the Lambert laboratory comparing the effects of Org49209 and Org49230 on NMDA-evoked currents mediated by recombinant NMDA receptors expressed in *Xenopus laevis* oocytes (see Table 1.1).

Investigation of the molecular structure of Org49209 revealed a structural similarity to the endogenous cholesterol metabolite, cerebrosterol. Examination of this effect of cerebrosterol revealed that in common with Org49209, this naturally occurring steroid is a potent PAM of the NMDA receptor (Figure 2.9). However, although active at relatively low concentrations, cerebrosterol is not as efficacious at these concentrations as Org49209, or Org 49230. These observations raise the exciting prospect that the activity of a major excitatory ionotropic glutamate receptor in the mammalian CNS could potentially be under the fine control of a locally produced neurosteroid. Furthermore, given that excessive activation of NMDA receptors are known to be neurotoxic and potentially pro-convulsant, the limited efficacy of cerebrosterol might be desirable for an endogenous modulator i.e. producing a subtle change in receptor function. However, cerebrosterol is known to be sulphated in the mammalian CNS (Cook et al., 2009). Therefore, the effect of cerebrosterol sulphate was also investigated in CGNs. In common with cerebrosterol, the sulphated cerebrosterol was a potent PAM of the NMDA receptor, but produced a much higher response at these concentrations (Figure 2.10). This observation raises the exciting prospect that cerebrosterol not only modulates NMDA receptor activity, but that sulphation of this steroid provides an additional method for fine-tuning the enhancement of NMDA receptor activity.

Finally, the possibilities to modify the basic structure of Org49209 to gain an understanding of the structure-activity relationship with regard to the NMDA

receptor is a crucial tool both for understanding the mechanism of the endogenous neurosteroids (i.e. pregnenolone sulphate, cerebrosterol, cerebrosterol sulphate) but also for the development of effective and viable drugs. For this purpose, we began investigating the role of the hydroxyl group on the 24-position of the steroid ring. We discovered that this group is critical for the enhancing effect of cerebrosterol sulphate on the NMDA receptor (Figure 2.12). While modifications at the 3-position, such as sulphation, are able to greatly change the efficacy of the steroid on NMDA receptor activity, it seems that the 24-alcohol is crucial for any steroid activity.

In summary, this set of experiments illustrates the effect of neuroactive steroid in cultured CGNs using calcium imaging techniques. The synthetic steroid Org49209, and the endogenous steroid cerebrosterol are more potent than the well-documented PAM of the NMDAR, pregnenolone sulphate. The sulphated steroids, Org49230 and cerebrosterol sulphate, retain the same potency as their unconjugated steroids, however, have a much higher efficacy at equivalent concentrations. As this chapter shows Org49209 as a promising candidate for enhancing NMDA receptor function, the remainder of the thesis will investigate the potential of this steroid to ameliorate cognitive deficits associated with HD in animal models.



## Chapter 3

# HDH<sup>Q111+/-</sup> Mice Improve in Novel Place Recognition with Practice



Illustration by C. Brazaitis

## 3.1 Introduction

This chapter uses the HDH<sup>Q111+/-</sup> mouse model of HD, which has previously been shown to have both episodic and spatial memory impairments (see section 1.3.6). In this chapter the novel place recognition (NPR) task is used as a spontaneous spatial memory task. This task was selected as HDH<sup>Q111+/-</sup> mice have previously been shown to be impaired in this task (Chandler (2014) Chapter 1, Figure 1.4). Furthermore, the task is also known to be hippocampal dependent and the HDH<sup>Q111+/-</sup> mouse has LTP deficits in the hippocampus (see Chapter 1, Figure 1.4A). For this reason, it is important to replicate the HDH<sup>Q111+/-</sup> mice NPR deficit in order to establish if it is a reliable assay in which putative treatments may be tested. The novel object recognition (NOR) task was expected to be unimpaired, based on previous observations (see Chapter 1, Figure 1.4C), and therefore, this would be used as a control task.

It has been established that NMDA receptor function plays a key role in NPR performance (see section 1.3.2); therefore, a NMDA receptor modulator would be an ideal candidate for therapy. Org49209, specifically, is a good candidate as it is able to reverse the LTP deficit of a 3-pulse theta burst to the “normal” levels of a 4-pulse theta burst *in vitro* (see Chapter 1, Figure 1.10).

### 3.1.3 Study Aims

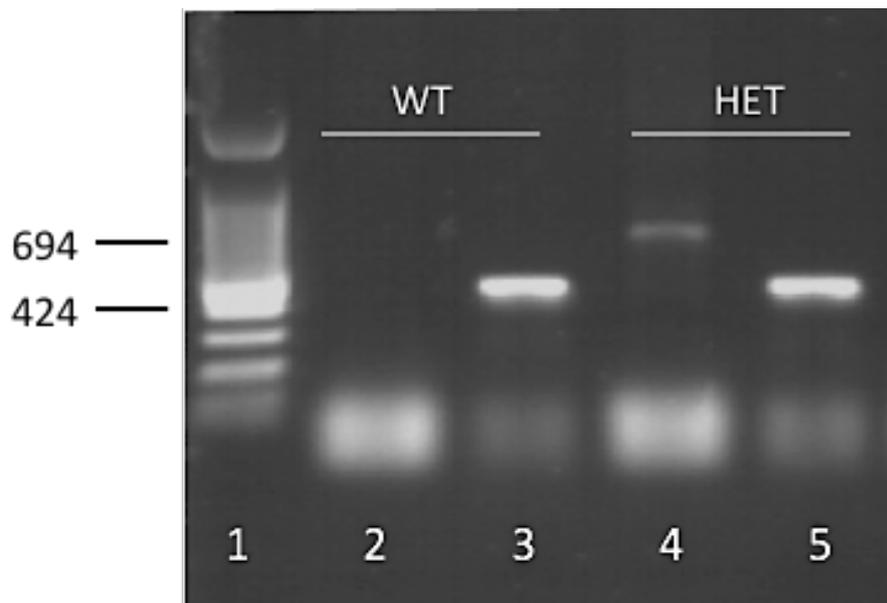
This chapter explores the possibility for the NMDA receptor PAM, Org49209, to improve spatial memory impairments. The aims of this study are two-fold: (1) to replicate the NPR deficit; (2) to reverse the deficit with Org49209.

## 3.2 General Methods

### 3.2.1 HDH<sup>Q111</sup> Mouse Model of Huntington’s Disease

Homozygotes (HDH<sup>Q111+/+</sup>) were purchased from Jackson Laboratories and crossed with wildtype (WT) C57/BL6 mice to produce heterozygote (HDH<sup>Q111+/-</sup> or HET) mice in an inbred colony. To identify the genotype of the resulting

mixed litters of HDH<sup>Q111+/-</sup> and WT mice, DNA was extracted from ear clips (Machery & Nagel and Qiagen Hotstar Taq Plus polymerase kit) and amplified (in the C1000 thermal cycler, Biorad, Annealing temp 56 °C). The resulting DNA samples were run on agarose gels. The WT Huntington gene forms a band at 424 base pairs, while the knock-in Huntington gene forms a band at 694 base pairs. Heterozygotes are identified with the formation of both bands while the WT will only form one band at 424 base pairs (Figure 3.1). The gel was visualised and image captured using the Uvipro Silver gel documentation system by Uvitec.



**Figure 3.1: Genotyping the HDH<sup>Q111+/-</sup> mouse.** Agarose gel image showing the ladder on the far left (lane 1), a WT band (lanes 2&3), and a HDH<sup>Q111+/-</sup> (HET) band (lanes 4&5). The heterozygote is distinguished by the additional band in lane 4 at 694 base pairs, which is absent in the WT lane 2 which only has a line at 424 base pairs.

### 3.2.2 Subjects

15 HDH<sup>Q111+/-</sup> (male and female) mice were used for all object recognition tasks. Mice were 3-4 months of age at the start of testing. Mice were kept in standard housing conditions on a 12 hour light/dark cycle from 7 AM to 7 PM. Food and water was available *ad libitum*.

### 3.2.3 Apparatus

Experiments were conducted in a rectangular arena (25x36x22 cm) with plastic wood-effect floor and white walls (Figure 3.2). Cues were hung in the top north-west and north-east corners of the box to act as spatial cues. Velcro strips were used to secure an object to one of the four corners of the arena. Between every trial, the box and objects were wiped with lemon scented antibacterial wipes (Tesco) to reduce cues from odours. Objects used for NOR were selected from an extensive object pair database from the Langston laboratory, which minimised object bias based on previous data. A holding box was used during testing when the mouse was not in the arena. The holding box was a large white opaque plastic box, roughly the same size as the arena, with a perforated lid, and plenty of sawdust to provide a comfortable environment for the mouse. Mouse exploration was viewed on a screen (LG) connected to an overhead video camera (Sony HandyCam) mounted on the wall above the arena. The video camera also recorded the input, which enabled re-scoring at a later date if needed.



**Figure 3.2: The object recognition arena.** Two objects are shown in the northern corners of the arena. Velcro strips can be seen in the south corners of the arena, these were used to adhere the objects to the floor. Hanging cues can be seen in top corners of the north wall, which act as spatial markers.

### 3.2.4 Experimental Procedures

During the object recognition experiments, mice were timed at how long they spent exploring the objects in the arena at each phase of the task. Exploration was recorded manually with two key press-activated timers- one for each object. Exploration was defined as sniffing at, chewing, scratching, climbing on, or looking at the object from a close (approx. 1 cm) distance. If the mouse was on top of the object, but otherwise not interacting with it, this was not counted as exploration. All testing was carried out during the light phase starting at about 9 AM.

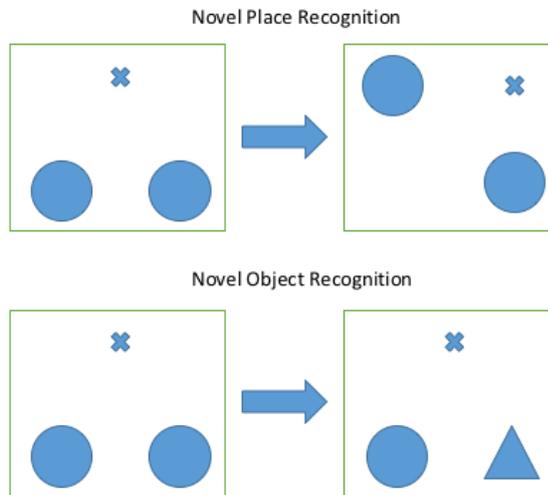
#### **3.2.4.1 Handling and Habituation**

Prior to testing mice were handled for five days in the experimental room to get them used to the transport from the holding room to the experimental room, and to habituate them to the experimenter. They were then habituated to the testing apparatus and experimental procedure over two days. On day one, mice were placed in the arena together with cage mates for 10 minutes in the morning. In the afternoon, individual mice were placed briefly in the holding box and then the testing arena for three minutes. On day two, mice were again placed in the holding box, and then into the arena with two random objects (that are not used for testing) in the NE and NW corners of the box. Exploration was recorded and used to ensure mice were exploring objects sufficiently.

#### **3.2.4.2 Novel Object and Novel Place Recognition Protocol**

In this study, both novel object and novel place recognition experiments were used. Both tasks consist of a 3-minute sample phase and a 3-minute test phase, with a 1-minute break in between. The mouse was placed in the holding box briefly before the sample phase to prepare it for testing, and during the 1-minute break in between sample and test phases. For NOR (Figure 3.3, bottom), two identical objects were placed in the north side (far end of experimenter), the mouse was placed into the arena on the opposite side (south), facing the wall, and exploration times were scored. After 3 minutes, the mouse was removed from the arena and placed back into the holding box while the box was cleaned and objects set up for the test phase. During the test phase, one of the objects was replaced with a novel object. The familiar object was a third copy of the two objects used during the sample phase to minimise the use of odour cues. The mouse was placed back in the arena for another 3 minutes and exploration was scored. NPR (Figure 3.3, top) used the identical procedure, except that the same two objects were used in the sample and test phase, but one was moved from the corner of the north wall to the corner of the south wall in the test phase. Again, the test phase

objects were identical copies to those used in the sample phase. The mouse entry point was adjusted to lie equidistant between the two objects. The novel object and novel place was counterbalanced to minimise effects of object or location preference.



**Figure 3.3: A diagram of the experimental procedure for novel place recognition (NPR, top) and novel object recognition (NOR, bottom).** For NPR two identical objects (circles) were presented, and after a 1-minute break, the same two objects were presented again but one was moved to the other side of the arena. For NOR two identical objects (circles) were presented, and after 1 minute, a new object was presented in place of one of the old ones (circle and triangle). Crosses mark the mouse entry points.

### 3.2.5 Data Analysis

Data was recorded by hand during testing. This was entered into a Microsoft Excel spreadsheet. The discrimination index (DI) was calculated using the following formula:

$$\text{Discrimination index} = \frac{\text{time at novel object} - \text{time at familiar object}}{\text{total exploration time}}$$

This formula produces a value between -1 and 1. A positive score indicates preference for the novel object, while a negative score indicates preference for the familiar object. A score of zero indicates no preference.

Exploration times below 10 seconds during the test phase were excluded from analysis. If mice did not explore at all during the sample phase, their scores were also excluded. Bar graphs were produced in GraphPad Prism 5. Scatter Plots were produced in Microsoft Excel. Statistics were performed with IBM SPSS Statistics 21. Effect sizes were calculated with partial eta squared or Cohen's *d*. Power analysis was conducted with G\*Power (Faul et al., 2009, Faul et al., 2007).

### 3.3 Experiment 1: NPR Replication Study

This experiment aimed to replicate the impaired NPR DI previously reported in the HDH<sup>Q111+/-</sup> mouse (Chandler (2014) Chapter 1, Figure 1.4).

#### 3.3.1 Experimental Procedure

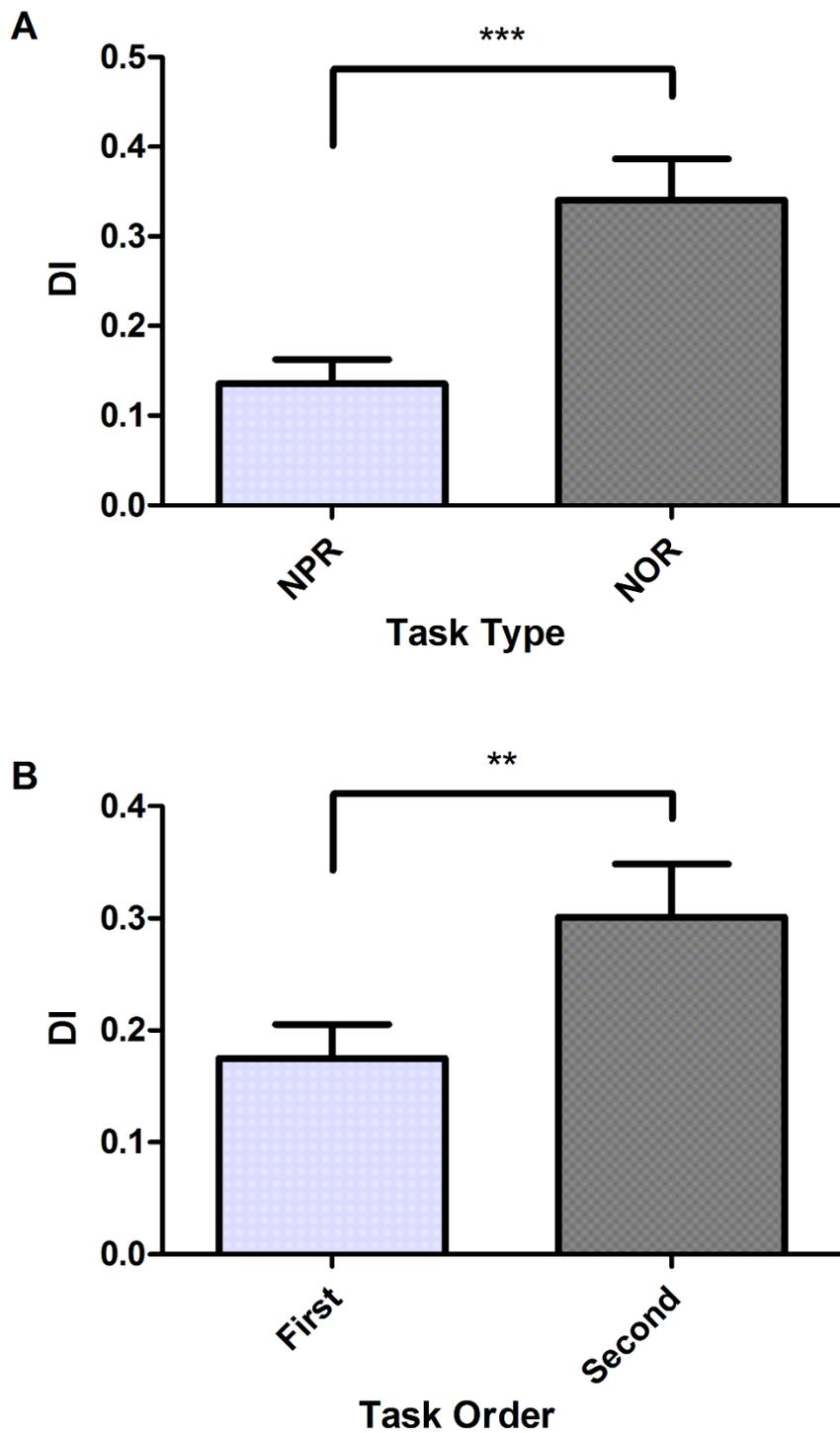
To increase power and to enable full-counterbalancing, this study was designed as repeated-measures. The intention was to use the (non-impaired) 1-minute NOR test as a comparison for the (impaired) NPR test in a subsequent drug study. Therefore, both the NPR and the control-NOR tests were performed, one immediately after the other, on four consecutive days. The possibility of task-order effects was controlled for by counterbalancing: on days 1 and 3 the NPR task immediately preceded the NOR task, while on days 2 and 4 the NOR task preceded the NPR task. The side of the novel place or object was counterbalanced across each day. DI data were analysed using a two-way analysis of variance (ANOVA). Significant ANOVA results were analysed further with post-hoc pairwise comparisons using a Bonferroni correction. Exploration data was analysed with a paired t-test.

#### 3.3.2 Results

The HDH<sup>Q111+/-</sup> mice showed a reduced preference for the novel place compared to the novel object (main effect of task,  $F_{(1,56)}=16.7$ ,  $p<0.001$ , partial eta squared = 0.23, Figure 3.4A) by having a lower DI in the 1-minute NPR task compared to the 1-minute NOR task. The task order also had an effect on DI

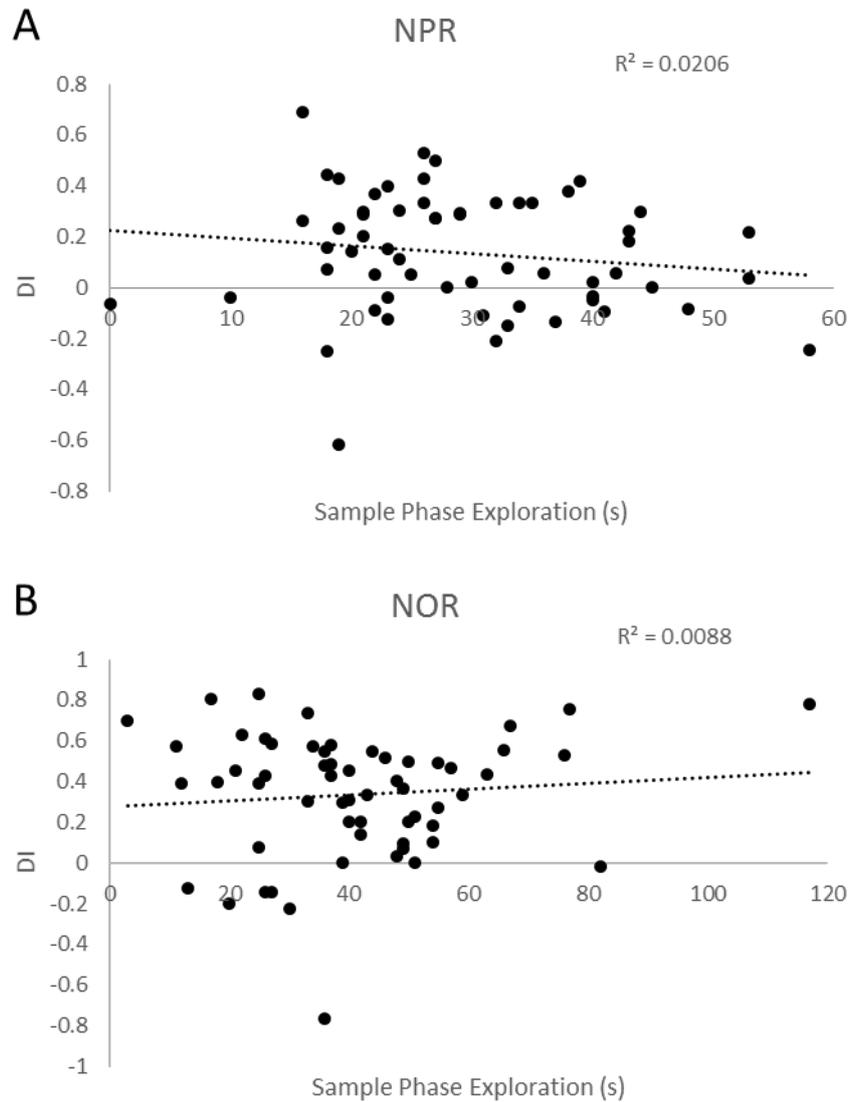
score, where the first task of the day had a significantly lower score than the second (main effect of order:  $F_{(1, 56)}=6.3$ ,  $p<0.01$ , partial eta squared = 0.10, Figure 3.4B). However, there was no interaction effect ( $F_{(1,56)}<1.0$ ,  $p>0.05$ , partial eta squared = 0.01), thereby showing that the NPR score is consistently lower independent of order, and likewise, the first task score is consistently lower independent of task. One DI value was excluded from analysis due to low exploration.

Additionally, the HDH<sup>Q111+/-</sup> mouse did not show a preference for the novel object in a 24-hour NOR task, where mice were given 24 hours in between the sample and test phases (data not illustrated; one-sample t-test,  $t=1.1$ ,  $df=14$ ,  $p>0.05$ , Cohen's  $d = 0.28$ ).



**Figure 3.4:** *HDH<sup>Q111+/-</sup>* mice had a lower DI for the first task of the day compared to the second, and the NPR task compared to the NOR task. On days 1 and 3 the NPR task preceded the NOR task, while on days 2 and 4 the NOR task preceded the NPR task. **(A)** The NPR score was lower compared to the NOR DI ( $p < 0.001$ ) **(B)** and the DI of the first task of the day was lower than the second ( $p < 0.01$ ). Error bars show SEM.

Total exploration times were lower for the NPR task than the NOR task in both the sample (paired t-test;  $t = 4.7$ ,  $df = 59$ ,  $p < 0.001$ , Cohen's  $d = -0.61$ ) and test (paired t-test;  $t = 6.4$ ,  $df = 59$ ,  $p < 0.001$ , Cohen's  $d = -0.81$ ) phases. However, sample phase exploration times do not correlate with DI scores in the test phase (Figure 3.5).



**Figure 3.5: Sample phase exploration does not correlate with DI score. (A)** The DI score plotted against the seconds of object exploration in the sample phase of the NPR task. **(B)** The DI score plotted against the seconds of object exploration in the sample phase of the NOR task.

### 3.4 Experiment 2: Org49209 Study

As the NPR impairment of the HDH<sup>Q111+/-</sup> at first inspection, before full analysis, appeared to be replicated, Org49209 was administered to test if it could increase the NPR DI score.

#### 3.4.1 Experimental Procedure

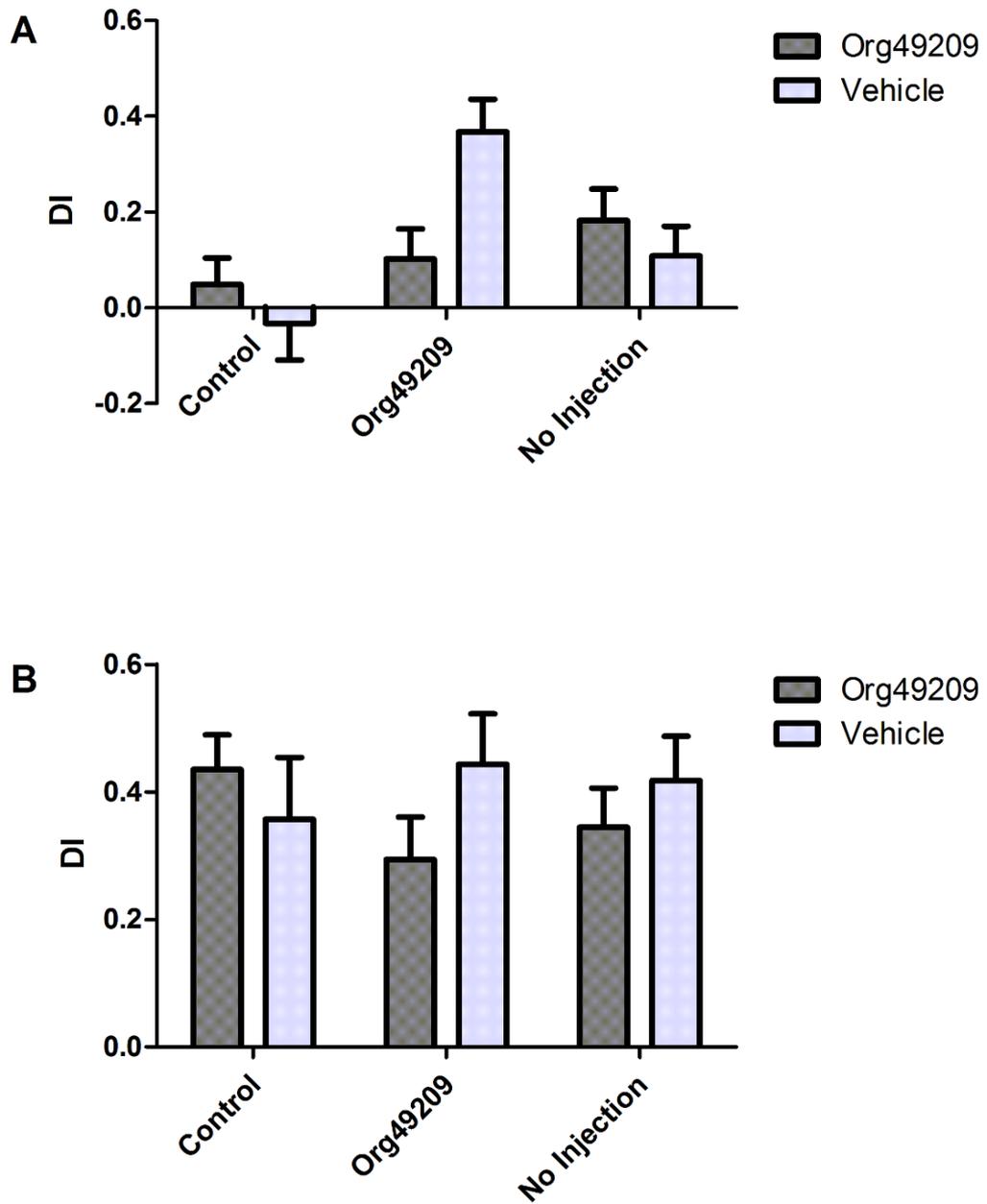
Mice were tested for five days on both the NOR and NPR task. As both the task and testing order had a significant effect on the DI (see results 3.3.2), NPR was always tested before NOR as this resulted in the lowest DI. On days 1, 3, and 5 mice were tested without injections. Day 1 was used as a baseline day. On days 2 and 4, eight mice received 10mg/kg Org49209 (intraperitoneal, i.p.); with injection volume not exceeding 10ml/kg), and seven received vehicle (20% hydroxypropyl beta-cyclodextrin, HPBCD, in sterile phosphate buffered saline, PBS) 30 minutes before testing. The experimenter was blind to the drug conditions. Days 3 and 5 were used to assess if performance returned to baseline after drug administration on the previous day. Data were analysed using a 2-way ANOVA.

#### 3.4.2 Results

As the HDH<sup>Q111+/-</sup> mice appeared to show no preference in the 1-minute NPR task, we tested them with Org49209 to see if this restored the preference. Five scores were excluded from analysis due to low exploration. Figure 3.6 shows that Org49209 changed neither novel place nor novel object preference DI scores compared to vehicle injections (2-way ANOVA, main effect drug:  $F_{(1,56)} = 1.40$ ,  $p > 0.05$ , partial eta squared = 0.03; task and drug interaction:  $F_{(1,56)} = 2.85$ ,  $df = 1$ ,  $P > 0.05$ , partial eta squared = 0.049). A sample size of 155 would be needed to see a significant interaction effect.

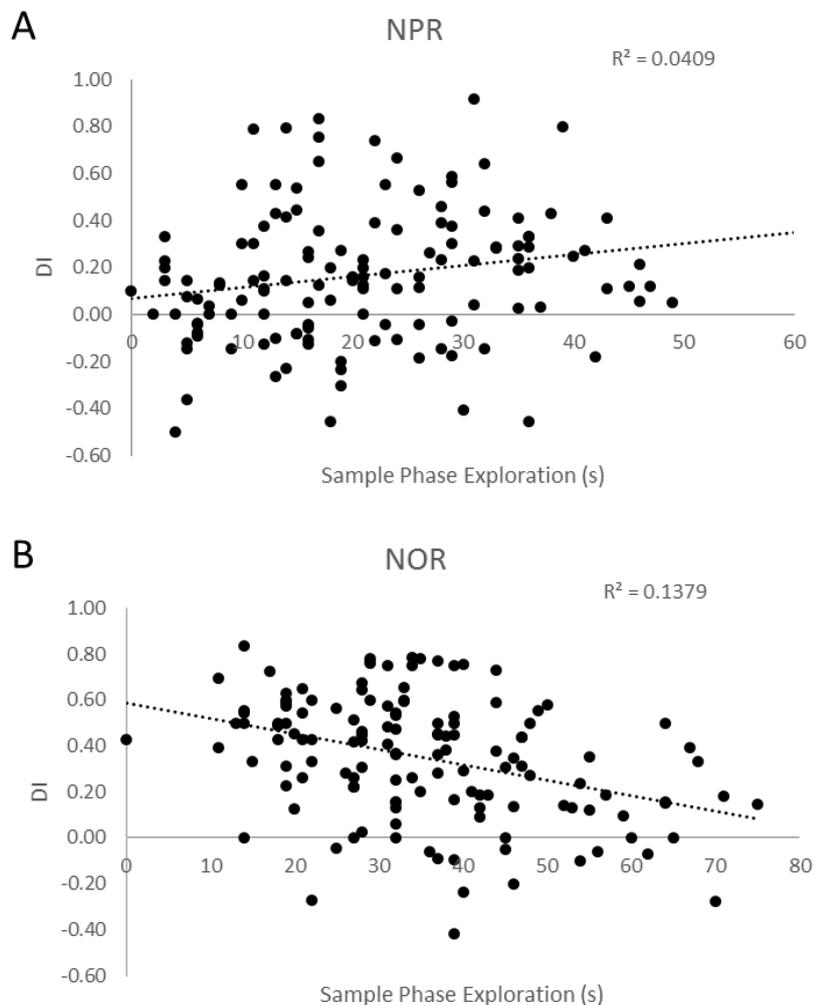
To rule out the effects of injection stress causing increased variability and thus masking main effects, we tested the mice following PBS injections (DI NPR =  $0.26 \pm 0.06$ ; NOR =  $0.30 \pm 0.08$ ) compared to no injections (DI NPR =  $0.10 \pm$

0.03; NOR=  $0.33 \pm 0.08$ ). There was no significant difference of DI between injection conditions (2-way ANOVA, main effect of injection:  $F_{(1,56)}=1.30$ ,  $p > 0.05$ , partial eta squared= 0.022). As expected there was a significantly lower score in the NPR than NOR task (main effect of task:  $F_{(1,56)}=6.37$ ,  $p < 0.05$ , partial eta squared= 0.10), but the task score did not change with injection condition (interaction:  $F_{(1,56)}=1.9$ ,  $p > 0.05$ , partial eta squared= 0.03). Three scores were excluded from this analysis due to low exploration.



**Figure 3.6: *Org49209* did not improve NPR DI scores of *HDH<sup>Q111+/-</sup>* mouse (5 months old).** Mice were tested in the NPR task (A) followed by the NOR task (B) over 5 days. On day one all mice (n=15) were tested in control conditions of no injections. This was followed by a drug day where 8 mice received 10 mg/kg *Org49209* and 7 mice received the vehicle (20% HPBCD in PBS). This was followed by a day of no injections, and on day 4 the mice received the same drug or vehicle treatments, followed again by a day of no injections. The bar graphs show the average score for each treatment. Error bars show SEM.

Mice explored less during the sample phase when in the Org49209 treatment compared to the control day (ANOVA main effect of condition:  $F_{(3,142)} = 3.9$ ,  $p < 0.05$ , Bonferroni pairwise comparison  $p < 0.05$ , partial eta squared = 0.08). As in experiment 1, they also explored less during the NPR task than the NOR task (main effect of task:  $F_{(1,142)} = 32.8$ ,  $p < 0.001$ , partial eta squared = 0.19). A significant interaction ( $F_{(3,142)} = 7.0$ ,  $p < 0.001$ , partial eta squared = 0.13) indicates that exploration is affected by both task and condition. Again, higher sample phase exploration does not predict higher DI scored during the test phase (Figure 3.7).



**Figure 3.7: Sample phase exploration during the Org49209 study does not correlate with DI scores.** (A) The DI score plotted against the seconds of object exploration in the sample phase of the NPR task. (B) The DI score plotted against the seconds of object exploration in the sample phase of the NOR task.

### 3.5 Experiment 3: Task Order and Practice Effect Study

After analysis of experiments 1 and 2, we hypothesised that providing mice sequentially with two tests would give them additional practice, causing an improvement of the DI score on the second test. To assess why task order has an effect of the DI, the NPR task was repeated with three sample phases instead of one.

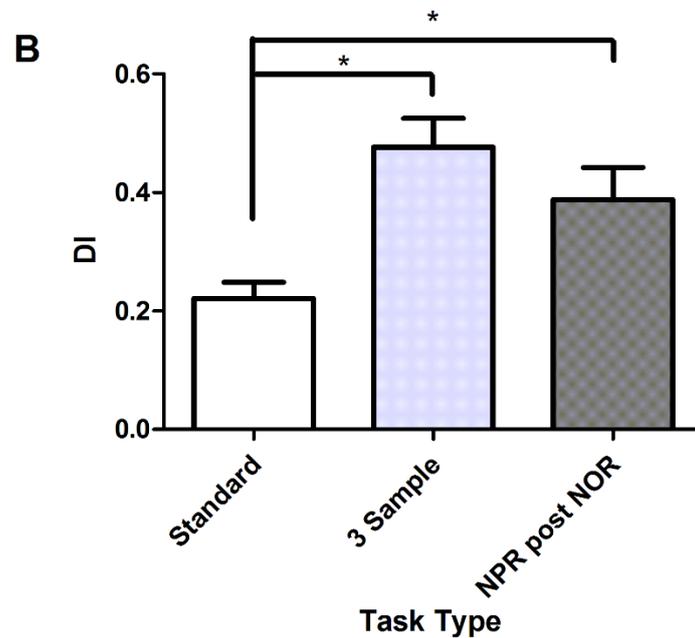
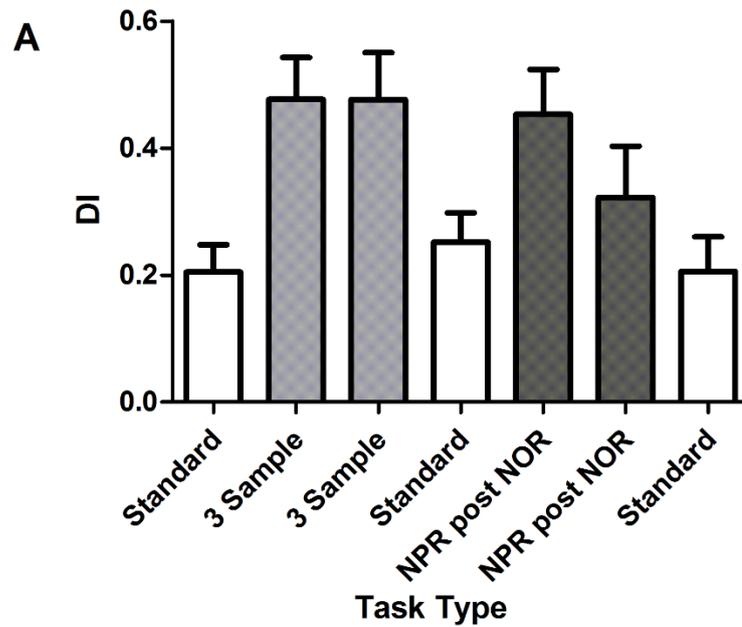
#### 3.5.1 Experimental procedure

This experiment was conducted over 7 days. For two days the NPR task was conducted with two additional sample phases, for a total of three sample phases before the test phase. To compare, the NPR post-NOR task was conducted for two days. The standard NPR task (section 3.2.4.2) was conducted once at the start of testing, once in between the two testing conditions above, and once at the end, to serve as baseline days for the altered testing conditions to see a return to baseline NPR scores. During this experiment, a new set of objects was given to each mouse and these rotated throughout the experiment. Therefore, all mice saw the same objects over the course of the experiment; however, different objects were used each day across the mice. This eliminated the potential for preferential exploration of any particular object(s) to influence the results. Repeated measures ANOVA was used for the analysis of the practice effect study. The practice effect was further analysed by presenting all the instances in which the NPR task was conducted either in isolation or as the first test of the day. A repeated measures ANOVA was used to assess if performance increased across days. Significant ANOVA results were analysed further with post-hoc pairwise comparisons using Bonferroni corrections.

#### 3.5.2 Results

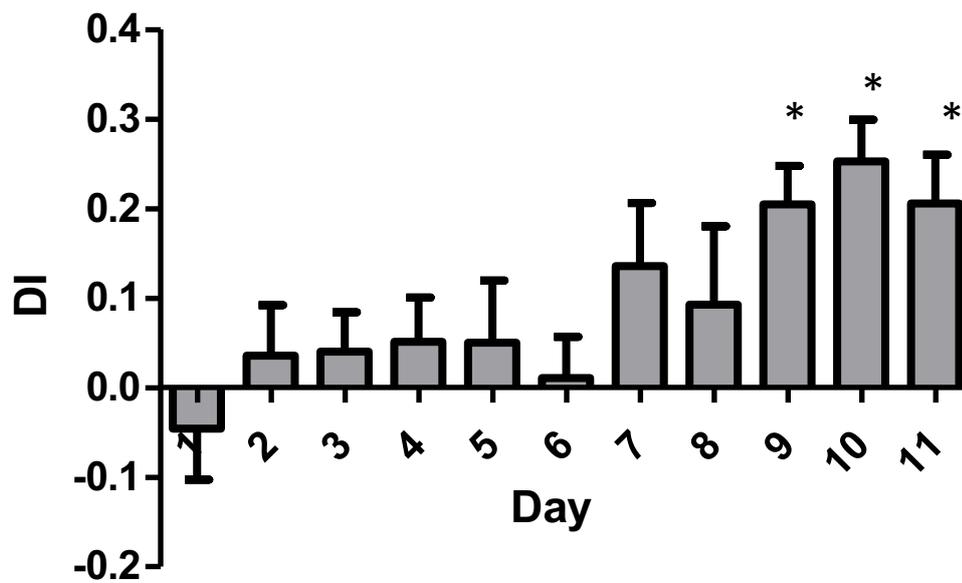
To explore why the second task produced a higher DI score than the first, we tested the hypothesis that the NOR task preceding the NPR task effectively provided two additional sample phases, allowing for more encoding time

before the final NPR test phase. Indeed, the testing conditions produced significantly different DI scores (repeated measures ANOVA: effect of task type  $F_{(2,50)} = 10.70$ ,  $p < 0.001$ , partial eta squared = 0.30), where both additional sample phases, and the NOR-preceding-NPR task increased NPR task performance (Figure 3.8B, pairwise post-hoc comparisons with Bonferroni corrections,  $p < 0.05$ ). Four scores were excluded from analysis due to low exploration.



**Figure 3.8: Additional sample phases improve NPR task performance in the  $HDH^{Q111+/-}$  mouse.** Mice (n=15) were tested over 7 days. **(A)** On day 1 the standard NPR task was conducted in isolation. On the following two days the NPR task was conducted with three sample phases instead of one. The standard NPR was conducted again, before two days of the NPR post NOR task. On the final day the standard NPR task was tested once more. **(B)** The averaged DI scores based on task type, where the score for the standard NPR task was significantly reduced compared to the 3 sample ( $p < 0.05$ ) and NPR post NOR task ( $p < 0.05$ ).

Finally, it was discovered that with repeated testing, the HDH<sup>Q111+/-</sup> mouse increased its DI score in the NPR task (repeated measure ANOVA,  $F_{(10,140)}=2.86$ ,  $p<0.05$ , partial eta squared = 0.17, Figure 3.9). When all the data was compared during which the NPR task was the only or first task of the day, performance during the 9-11<sup>th</sup> days were significantly better than on the first day of testing (pairwise comparisons with Bonferroni corrections,  $p<0.05$ ).



**Figure 3.9: Repeated testing of the NPR task enhanced task performance in the HDH<sup>Q111+/-</sup> mouse.** All testing days in which the NPR task was conducted in isolation or as the first task of the day are plotted in chronological order (days are not consecutive). Days 9, 10, and 11 are significantly different to the score on the first day of testing ( $p<0.05$ ).

### 3.6 Discussion

Experiment 1 (Figure 3.4) appeared to confirm a deficit in NPR, but not NOR, in HD mice and, as such, was consistent with the previous findings of Chandler (2014) (see Chapter 1, Figure 1.4) that there is no place preference in the 1-minute NPR test, but 1-minute NOR is normal and only at 24-hours is there no NOR effect (as seen by Mitchell (2012), see section 1.3.6). However, this experiment has also demonstrated that the behavioural deficits may not have a simple explanation. As the observed effect was that which was expected (i.e., no DI in the NPR task), the assumption was that this was the same impairment. However, a wild-type control group was not available due to breeding, and therefore was not tested and the only control was 'task'. Clearly, only a wild-type control group would have enabled the strong conclusion to be drawn regarding the presence or otherwise of an NPR deficit in these mice.

As a difference in exploration at the sample phase could have resulted in an explanation for a difference in DI scores during the test phase, this was also investigated. Although mice did explore more during the NOR sample phases than the NPR sample phases, the higher exploration times did not predict higher DI scores (Figure 3.6). Therefore, it seems that as long as mice explored the objects, they were able to encode them, independent of how long they actively spent exploring. Nevertheless, the difference in sample phase exploration at the NPR and NOR test could have been avoided by using the same objects for both tests, in a counterbalanced fashion, instead of using separate objects for the NPR and NOR tests.

It is possible to conclude from this set of experiments that exposure to the testing arena and stimuli has an effect on performance. This was manifest as the task-order effect in Experiments 1 and 2 and confirmed by the manipulation in Experiment 3. In addition to the task-order, or practice effect, the NPR task overall resulted in a lower DI score than the NOR task.

The assumption was that the deficit in NPR task performance might be ameliorated by the NMDA receptor PAM and putative cognitive enhancer, Org49209, prior to testing. However, there was no effect on the DI scores compared to vehicle in either task (Figure 3.5). The possibility of injection stress influencing the results was ruled out by showing that PBS injections did not change DI scores. However, injection stress may effect exploration times, as there was a significant effect of treatment condition for sample phase exploration times (section 3.4.2). As higher exploration times do not predict higher DI scores see (Figure 3.7), this difference in exploration should not affect the DI scores.

Experiment 3 investigated the reason why task order might influence the DI scores. It was hypothesised that mice have a higher DI score in the second test than the first, because in the case of the NOR-preceding-NPR task, the NOR task could provide “extra” sample phases. The NOR task could act as these “extra” sample phases because the locations of the objects throughout the NOR task are the same as the locations in the sample phase of the NPR task. This reasoning could not work for increasing the NOR DI score, as the object identity changes between the NPR and NOR tasks; therefore, the NPR cannot provide “extra” sample phases for the NOR task. Figure 3.8 shows that additional NPR sample phases (where the same objects and locations are presented 3 times before the test phase) increase the DI score, just as the NOR task which immediately preceded the NPR task might increase the DI score. A possible explanation for the increased NOR score after exposure to the NPR task is that the NPR task allows for a higher familiarity with the testing arena. It is well documented that familiarity with the environment enhances exploration of the novel object, which is also why mice are habituated to the testing arena prior to testing (Besheer and Bevins, 2000).

Finally, additional analysis shows that the NPR task DI score increases with practice over days. This analysis took all the DI scores from the NPR tasks that were performed as the only or first task of the day. Figure 3.9 shows that, by

day 9, the DI is significantly higher compared to the first day when there was no evidence of discrimination of novelty.

Collectively, these data show that not only does repeated exposure to testing increase the NPR task DI score within a day, but there is a cumulative effect of testing over days, where the DI score of the NPR task increases even when tested first in the day. There are two possible explanations for this increase in scores: first, it could be the mice improve memory for the familiar place with practice; second, the salience of the novel place could have increased with repeated testing.

If the NPR score increases with repeated testing, this could be a reflection of improved spatial memory with practice. Just as increased sample phases increased the DI score during the test phase within one test, repeated exposure to the objects in the testing arena could increase the encoding time of those object locations. However, as no specific behaviour is reinforced (due to the spontaneous nature of the task), it cannot be that the mice learn the task, *per se*, with practice. Nevertheless, repeated exposure to the objects in the same locations could allow for more experience and familiarity with the object locations.

It is possible that the spatial memory could be improved through repeated testing due to the environmental enrichment provided by the testing experience itself. Environmental enrichment increased the spatial memory of R6/1 mice in the Barnes maze (Nithianantharajah et al., 2008). These authors also attempted to test the effects of environmental enrichment in the NPR task, however, WT performance was confounded and therefore no conclusions could be made to R6/1 mouse performance.

Future experiments would not only benefit from a WT control group, but it would also be worthwhile investigating sub-chronic administration of Org49209. Past studies have shown that sub-chronic administration of pregnenolone ameliorates NOR deficits in dopamine transporter knockout

mice, which display a schizophrenic – like phenotype (Wong et al., 2012). Additionally, sub-chronic administration of the NDMA receptor co-agonist D-cycloserine was able to improve memory deficits, including impaired NOR, in a 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD rat model (Ho et al., 2011). If a genotype difference is found in the rate of learning in the NPR task, sub-chronic steroid administration could be compared across administration, between HETs and WTs.

The novel place recognition task is established as a test of spatial memory. However, as this is a spontaneous task, there is no reinforced motivation to explore the object in the new location; the task relies on the mouse naturally wanting to explore the new location. This makes the task very susceptible to stress effects and environmental or procedural changes. Therefore, a DI score that does not show a preference for the novel location does not automatically mean that the mouse has no memory for the old object location: it could just be that the same object in a different location is not salient or interesting enough to warrant exploring. In contrast to the NOR task, where a completely new object may be much more interesting than the same object in a different location, resulting in a preferred exploration of the novel object but not the novel location, as seen in this study. It could be useful to be able to measure exploration both at the object in the novel location, as well as the location where the object was previously- as both of these locations are theoretically novel (i.e., an object is somewhere it has not been before, and an object is no longer somewhere it was previously). However, we cannot measure exploration of empty space, although the use of tracking software may be able to give an idea of time spent in the vicinity of the old object location. To help with these questions, having a control group of WT animals to compare to the HDH<sup>Q111+/-</sup> animals would be very useful. The next chapter will look at a spatial memory task which uses reinforcement to attempt a different approach to

test the spatial abilities of the HDH<sup>Q111+/-</sup> mouse, and also has a WT control group.

## Chapter 4

# HDH<sup>Q111+/-</sup> mice are Unimpaired in Spatial and Reversal Learning in the Morris Water Maze

“I see that you have a little swimming mouse” -David Sedaris



Illustration by C. Brazaitis

## 4.1 Introduction

The Morris water maze (hereafter referred to as water maze) was developed by Richard Morris at the University of St Andrews (Morris, 1984). It is a classic apparatus for measuring spatial learning in the context of motivated escape and therefore differs from other discrimination or spatial learning tasks based on appetitive reinforcement or spontaneous behaviours such as exploration. The above paper describes a variety of spatial tasks that can be used in the water maze, as well as a method for testing visual discrimination learning. This chapter consists of two studies, both utilising the water maze: spatial memory and spatial re-location, and two-choice cue discrimination and reversal learning.

### 4.1.1 Spatial Learning

The primary reason for choosing the water maze task to test for spatial learning is to provide a motivational drive to complete the task without the need for dietary restrictions, as the HDH<sup>Q111+/-</sup> has an altered metabolism (Carroll et al., 2015). Chapter 2 showed my first attempt to measure spatial memory in the HDH<sup>Q111+/-</sup> mice using the NPR task. Although the mice initially showed no preference for the novel place, repeated testing revealed that they had spatial memory. Therefore, we decided that a task was needed which did not rely on the unconditioned responses of the mice. The water maze was chosen as the mice would be motivated to locate the platform. This would also ensure that performance was due to memory rather than other factors such as attention or salience. However, the possible confound to a motivational task is a difference in motivation to perform the task. Indeed, HD patients often have decreased motivation (van Duijn et al., 2014), and the HDH<sup>Q111+/-</sup> mouse has previously shown lower motivation in the progressive ratio task (Yhnell et al., 2016b).

Both HD patients and rodent models have deficits in spatial memory (Brandt et al. (2005), Lione et al. (1999), and sections 1.3.5 and 1.3.6). We propose

Org49209 would have the same beneficial effects on spatial memory impairments found in the water maze as in NPR task (see Chapter 2): briefly, enhancing NMDA receptor function could lead to enhanced hippocampal LTP which may improve spatial memory.

#### 4.1.2 Reversal Learning

Reversal learning is a test of behavioural flexibility (see section 1.4.4), where both HD patients and rodent models show impairments (Lawrence et al. (1999), Farrar et al. (2014), and section 1.4.6). Org49209 has previously ameliorated reversal learning deficits of aged rats (Chase (2013), see Chapter 1, Figure 1.11); therefore, if a reversal deficit were also found in this study with HDH<sup>Q111+/-</sup> mice, we would predict it to have a similar effect.

#### 4.1.3 Study Aims

To assess the spatial memory function and reversal abilities of the HDH<sup>Q111+/-</sup> mouse in the water maze, with the aim to use Org49209 in future studies to reverse any deficits that may arise.

### 4.2 Methods

#### 4.2.1 Subjects

The mouse model used is described in detail in section 3.2.1. All water maze experiments were conducted with 16 experimentally naïve male and female mice consisting of 8 HDH<sup>Q111+/-</sup> (HET) and 8 littermate control wild types (WT), 3-4 months of age at the start of testing. Mice were kept in standard housing conditions on a 12 hour light/dark cycle from 7 AM to 7 PM. Food and water was available *ad libitum*. Discrimination learning started when the mice were 6 months old and they had reached 8.5 months by the end of testing. Mice were weighed weekly during testing. Two WT mice failed to learn the initial discrimination and so were excluded from the remainder of testing.

#### 4.2.2 Apparatus

The water maze was 2 meters in diameter and had a raising platform (CNS Technologies, Edinburgh). The platform can be held down with electromagnets that are remotely switched off to release the platform back to just under the water's surface. When filled, the water was at  $21 \pm 1$  °C and the surface is 0.5 cm above the platform. A CCTV camera was mounted on the ceiling above the centre of the pool and the image was sent to both the computer recording the data and a separate monitor. The CCTV set up recorded a back-up copy of the testing.

For the spatial learning experiments extra-maze cues were hung around the water maze; these included brightly coloured and patterned curtains, decorative hanging objects, as well as normal essential laboratory furniture such as lamps, shelves, etc. Around these cues two sets of white curtains were drawn to separate the maze from the rest of the room, and the experimenter. This was to ensure the experimenter did not act as a spatial cue.

For the discrimination and reversal learning experiments, the extra-maze cues were removed, and white curtains were drawn directly around the maze to minimize visual extra-maze cues; however, spatial cues due to smells and sounds could not be eliminated. Therefore, although spatial learning was discouraged during the visual discrimination task, it was still possible for mice to have a representation of their location in space due to sounds from the computer and smells from their cage mates. A barrier was introduced to the water maze, which extended from the outer edge into the middle of the pool. On either side of the barrier, two cues were hung directly over the pool by transparent nylon string attached to the ceiling with Velcro strips or curtain hangers: one centrally, above the platform location, in the target quadrant; and the other in the equivalent location in the neighbouring quadrant.

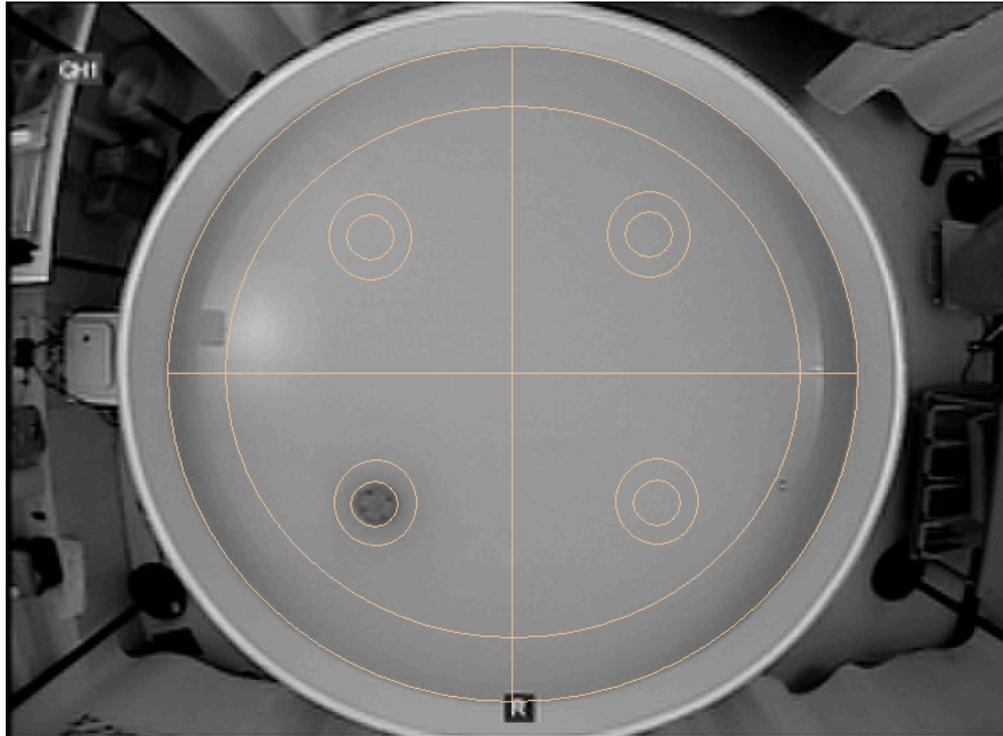
### 4.2.3 Data Collection

Data was collected using ANYmaze (Version 4.99m). Within the software, regions of interest (or “zones”) were marked both for the spatial learning task (Figure 4.1) and the reversal learning task (Figures 4.2 and 4.3). ANYmaze tracked the mouse in the maze, and recorded the time and path the mouse took during each trial. Data was collected in Microsoft Excel and pivot tables were used to organise the data.

### 4.2.4 Spatial and Spatial Relocation Learning

Mice were handled for one week prior to testing. During this time, they were transported to the water maze room daily and handled for two minutes each. Each testing day consisted of 6 trials. The mice were run in two cohorts of 8, with equal numbers of genotype and sex in each cohort. Cohort A was run first, followed by cohort B. Each trial was started by selecting one of the 4 potential start locations, spaced equally around the maze, pseudo-randomly (using random.org). The mouse was gently lowered into the water, keeping its head above the water and positioned facing the side of the pool, away from the centre. Each mouse had an inter trial interval of 10 minutes, during which it was placed under a heat lamp to stay warm and get dry. Each mouse was housed in a separate labelled cage, lined with absorbent tissue for the duration of testing.

Each trial had a maximum duration of 90 seconds, after which the mouse was guided to the platform. The mouse was left on the platform for 30 seconds to allow for additional spatial encoding of the environment. The mouse was only removed when on the platform, to train it to remain on the platform for the duration of the waiting time, and to make sure that locating the platform was associated with the only means of escape.



**Figure 4.1: The set-up of the water maze for spatial learning.** The pool was split into zones. These consisted of the thigmotaxis, four quadrants, the platform zone and a zone immediately around the platform.

The spatial learning experiment consisted of 3 stages:

#### **4.2.4.1 Visual Platform**

On the first day of testing, the hidden platform was made visible with a black and white striped cue card placed directly on top of the platform. White curtains were drawn directly around the water maze to eliminate extra-maze cues as described above for the discrimination learning task. Visual platform training served two purposes: firstly, so that the mice would become habituated to the pool and training procedure; secondly, so that the visual ability of the mice was tested. Differences in duration or distance to find the visual platform could be due to impaired vision, and differences in duration could be due to different swimming speeds. The visual platform stage was used to check for visual ability, where genotype difference would make the water maze an inappropriate task for examining learning and memory in this mouse model.

#### **4.2.4.2 Spatial Learning**

During spatial learning mice were required to use extra-maze cues to locate the platform. The platform was in a different quadrant location for each cohort and different to the visual platform location. Mice were tested until their duration and distance had reached a plateau, and the swim paths to the platform were direct.

#### **4.2.4.3 Spatial Relocation**

Once the mice had learned the location of the platform, the platform was moved to the opposite quadrant location. Testing was stopped once performance reached the same level as for the initial spatial learning. Spatial re-location learning is a test of behavioural flexibility, and the ability to learn a new spatial location.

#### **4.2.4.4 Probe Trials**

Probe trials (or transfer tests), in which the hidden platform is removed entirely from the pool, are a further test of the spatial memory and confirm that the mouse is not using a non-spatial search strategy. If the mouse has learned the spatial location of the platform, it is expected to spend more time in the proximity of the (now absent) platform location. Probe trials were conducted on the last day of spatial learning and the last day of spatial re-location learning, one hour after the last trial was completed. During the trial, the platform was held down by electromagnets for 60 seconds, after which the magnets were switched off, raising the platform. The mouse was then allowed to locate the platform and remain on it for the usual 30 seconds. This ensured that no extinction occurred during the probe trial which could impact future learning.

#### **4.2.4.5 Data Analysis**

ANYmaze recorded the distance and duration taken for each mouse to reach the platform on each trial. During probe trials ANYmaze provided data on the

distance and duration spent in the quadrants. Below is a summary of the zones and measurements used by ANYmaze.

#### *Zones Used*

*Thigmotaxis*- is the area around the edge of the water maze. Mice spending time in this area are trying to find an escape from the edge of the pool.

*Quadrant*- The pool is split into 4 equal quadrants: NE, NW, SE, SW. The platform is located in the centre of one quadrant.

*Platform*- denotes the exact location of the platform within each quadrant.

*Platform proximity*- a zone drawn just around the platform, to measure the instances when the mouse is close to the platform.

#### *Factors Measured*

*Distance to find platform (in meters)*- the length of the swim path from the entry point to the platform location.

*Duration to find platform (in seconds)*- the time from entry to arriving at the platform location.

*Speed (in meters per second)*- Distance / Duration gives the speed of the mice for each trial.

*Length of swim path and duration spent in quadrant*- used in analysis of the probe trials to measure the distance of swim path and time spent in the relevant quadrants.

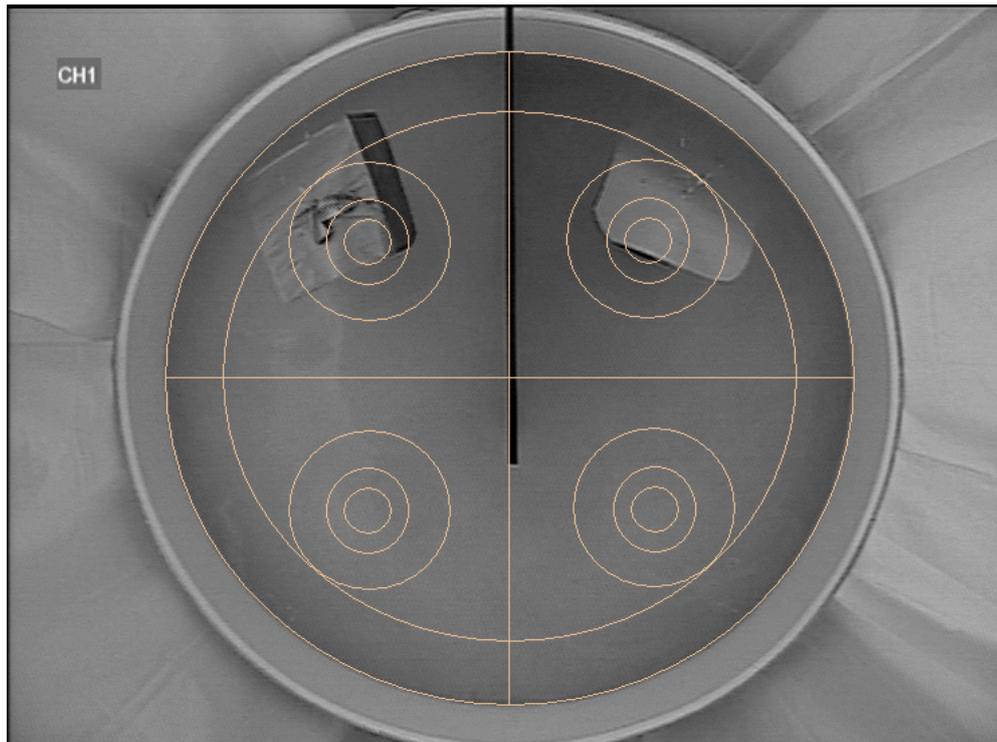
ANYmaze also records the actual path taken by the mouse, which can be used to compare swim paths and assess if the mouse is using a direct route to find the platform. These traces were used to show representative swim paths at different stages of the water maze tasks.

The distance to reach the platform was assessed across trials for each genotype with a repeated measures ANOVA. Probe trials were analysed by averaging the time spent in the target quadrant for each genotype. A 2-way ANOVA with pairwise comparisons using Bonferroni corrections was used to see if mice spent more time in the target quadrant compared to other quadrants. Effect sizes were calculated with partial eta squared or Cohen's *d*. Power analysis was conducted with G\*Power (Faul et al., 2009, Faul et al., 2007).

#### 4.2.5 Visual Cue Reversal Learning

Two-choice discrimination stimulus-response (S-R) reversal learning is often used as a measure of behavioural flexibility. Spatial re-location learning is sometimes referred to as reversal learning, although it is not a S-R reversal. For a S-R reversal task in the water-maze, the mouse learns which of two stimuli indicates the platform location (discrimination learning). The correct choice is reinforced by the platform providing a means of escape from the maze. Once the discrimination has been learned the previously reinforced stimulus is no longer reinforced, instead the unreinforced stimulus becomes the new predictor of successful escape.

Trial number was increased from 6 to 8 trials per animal per day. The platform location was pseudo-randomly determined (using random.org) on every trial. Only the north and south entry points were used for this experiment. Entry points were alternated daily, and both entry points were used between the two cohorts each day.



**Figure 4.2: The set-up of the water maze for reversal learning.** White curtains were drawn directly around the maze. A barrier was placed in half of the pool to separate the quadrants which contain different cues. Only one of the cues predict the platform location. The pool was split into the same zones as in Figure 4.1 of spatial learning, with the addition of the cue proximity zone, which is a larger circle around the platform locations in each quadrant. These zones are used as a threshold for the mouse making a choice between the two cues.

#### **4.2.5.1 Cue Training**

In the first stage of the task the mice were trained to associate the platform location with a cue hanging directly above the platform. The cue was a large 5L blue bottle. Each trial used only one cue to ensure the mice were using the cue as a predictor for platform location rather than spatial or egocentric memory. A threshold of 80% first choice accuracy for the genotype group as a whole was used as a criterion for learning each stage of the task.

#### **4.2.5.2 Discrimination Learning**

Once the cue training was complete, two new cues (owl lunch box, dustpan and brush set) were introduced to the water maze at the same time. Only one cue predicted the platform location. The owl lunch box predicted platform location for cohort A, while the dustpan and brush predicted platform location

for cohort B. On the last day of discrimination learning, a probe trial was conducted before the usual 8 trials to test for memory of the correct cue 24 hours after the last trial. The cues were set up as for discrimination learning; however, the platform was held down for 60 seconds, as in the probe trial during spatial learning (described in section 4.2.4.4). More time spent at the correct cue compared to the incorrect cue indicates a learned discrimination.

This stage of the task was attempted first without the presence of a barrier (as described in Bannerman et al. (2012)); however, after two weeks of testing the mice were not showing any learning. Therefore, the barrier was introduced to increase the cost of making a wrong choice. This was sufficient to motivate the mice to learn the discrimination.

#### **4.2.5.3 Reversal Learning**

Once discrimination learning was complete, the stimulus-outcome contingency was reversed. The cue previously predicting the platform location no longer did so, and the other cue became the new predictor of the platform location.

#### **4.2.5.4 Data Analysis**

The distance and time were measured as in the spatial learning tasks above. To measure discrimination and reversal learning, first choice accuracy was used.

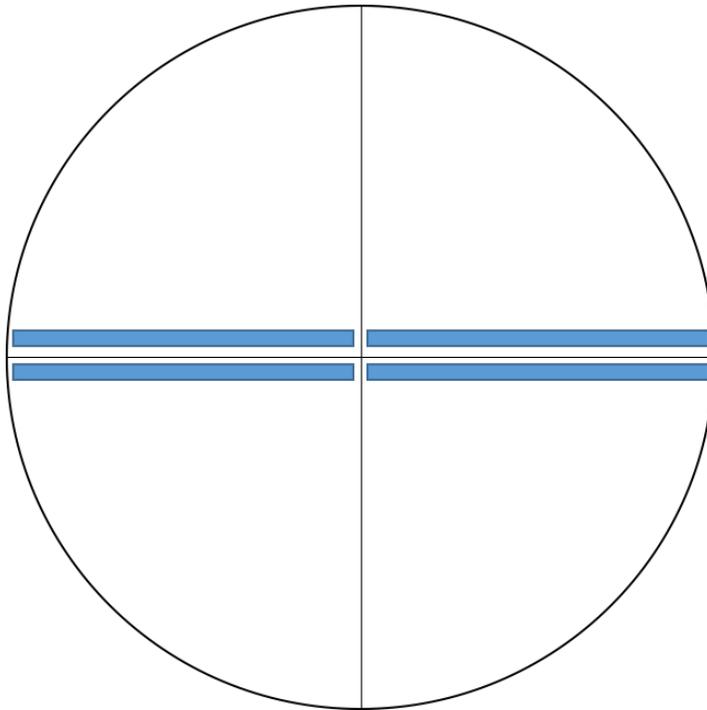
Below is a full description of the zones used and dependent variables measured:

##### *Zones Used*

The same zones were used as in the spatial learning task, as well as:

*Cue proximity*- A wider circle around the platform used as a threshold to calculate first choice (see Figure 4.2). If the mouse crosses into this zone, it is recorded as having made a choice between the two cues.

*Quadrant entry zones (Figure 4.3)*- Used as a measure of indecision. During this task ANYmaze was prone to track the cues instead of the mouse, which would inflate the number of quadrant entries; therefore, the side of the quadrant zone that is used on mouse entry is given an additional zone, to ensure any crossings are due to mouse behaviour rather than an error of the software to track the mouse.



**Figure 4.3: Zone locations for quadrant entry areas.** These zones were used to measure indecision of choosing between the two cues.

#### *Factors Measured*

The same factors were measured as in the spatial learning task, as well as:

*Distance to first entry of Cue proximity zone*- The distance travelled to enter a cue proximity zone for the first time.

*Distance to first entry of quadrant*- The distance travelled to enter a quadrant for the first time.

*Number of quadrant entries*- Number of times the mouse crossed the zones shown in Figure 4.3.

First choice accuracy was calculated by comparing the distance to reach the cue proximity zone of each of the cues using a program written in Perl (written by Gordon McCormick, see Appendix 1 for program code). The shorter distance was scored as the cue first approached (first choice). If the first choice was at the correct cue, the trial was scored as 1; if the first choice was at the incorrect cue, the trial was scored as 0.

The processed data were analysed in Microsoft Excel using pivot tables. The average was taken for each genotype for each trial. The average across each day (8 trials) was then taken to make the final graph in Microsoft Excel. A repeated measures ANOVA was used to compare the percent first choice accuracy across 28 days for each genotype. Individual data for each mouse were used to exclude outliers and was also averaged across each day. Mice were excluded if they did not learn the initial discrimination after 28 days. These individual data were also used for regression analysis. A trend line was fitted for each mouse and the slopes were compared between genotypes with a t-test.

Probe trial data was collected manually as the ANYmaze software occasionally had trouble tracking the mouse due to tracking the cues instead of the mouse. Therefore, correct and incorrect quadrant entries and cue proximity zone entries were counted manually during testing. A 2-way ANOVA was used to see if mice differed in frequency of quadrant or cue proximity zone entries.

The initial reversal stage was analysed by calculating the number of days taken to reach chance performance for each mouse. Chance level is the correct first choice made in 4/8 trials. The days to reach chance level were compared between genotypes using a t-test. The whole reversal stage was analysed with a repeated measures ANOVA to compare the percent first choice accuracy across 27 days for each genotype. Regression lines were also fit for the individual mice during the reversal stage and slopes were compared with a t-test between genotypes. Effect sizes were calculated with partial eta squared

or Cohen's *d*. Power analysis was conducted with G\*Power (Faul et al., 2009, Faul et al., 2007).

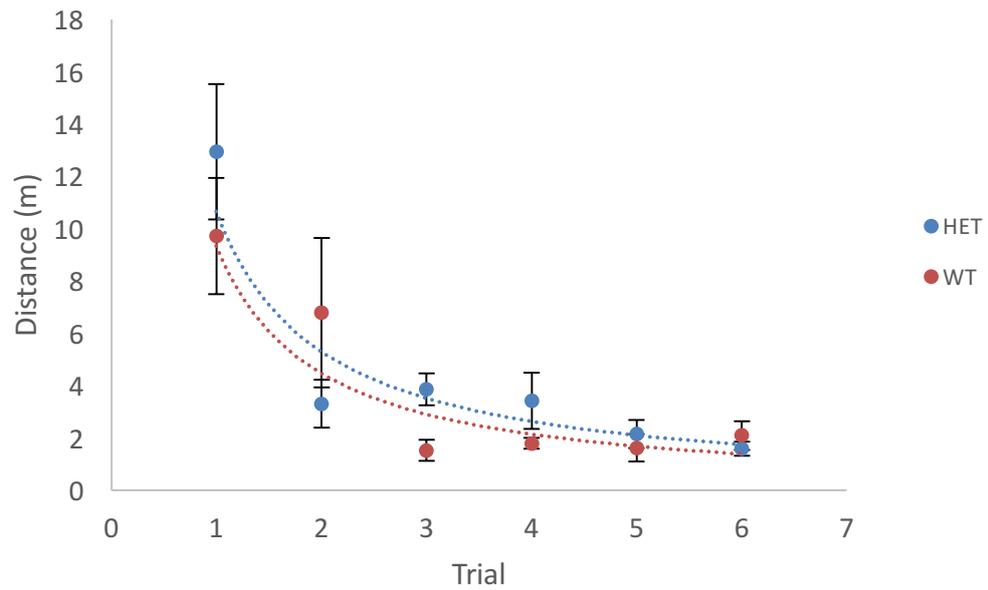
## 4.3 Results

### 4.3.1 Spatial Learning and Spatial Relocation Learning

The first water maze experiment conducted was spatial learning and spatial relocation learning. Both WT (n=8) and HDH<sup>Q111+/-</sup> (n=8) littermates were required to search for a hidden platform using extra maze cues. First the mice were habituated in the visual platform task, followed by the spatial learning task, and finished with spatial relocation learning.

#### 4.3.1.1 Visual Platform

During the visual platform task mice are required to locate the platform based on a visual cue placed directly on top of the platform. This test served to habituate the mice to the pool and testing procedure and ensured that all subjects had sufficient visual and motor ability. All subjects located the platform within one day, consisting of 6 trials (ANOVA, main effect of trial:  $F_{(5,65)}=13.7$ ,  $p<0.001$ , partial eta squared= 0.51), with no difference between genotypes (no main effect of genotype:  $F_{(1,13)}<1$ ,  $df=1$ , partial eta squared= 0.04, and no interaction effect:  $F_{(5,65)}<1$ , partial eta squared= 0.08, Figure 4.4).

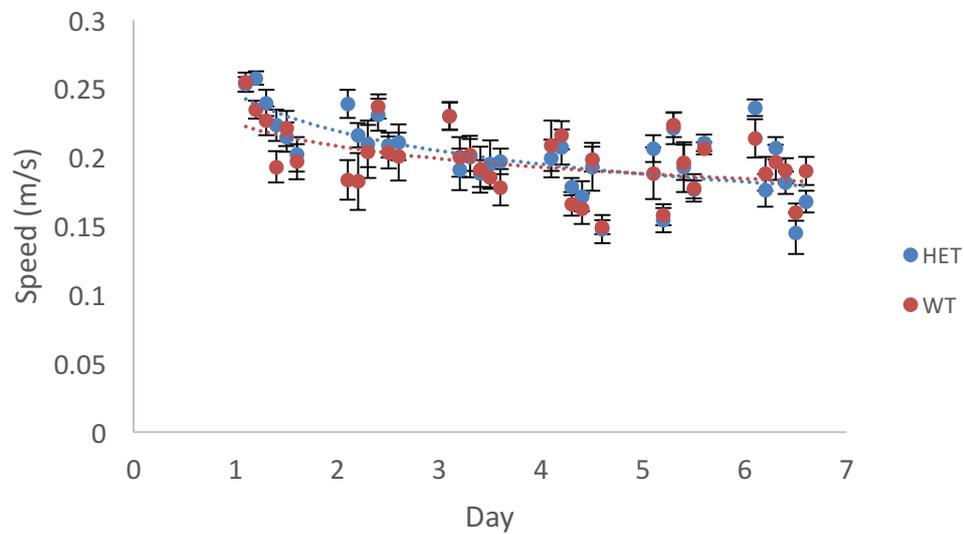


**Figure 4.4:  $HDH^{Q111+/-}$  (HET) and littermate controls (WT) learned the visual platform task equally quickly.** The platform was hidden just under the water surface with a black and white striped card sitting on top to mark the platform location. Both genotypes learned the platform location equally quickly in one day, consisting of 6 trials. Fit line parameters: HET:  $y = 10.664x^{-1.009}$ ,  $R^2 = 0.86586$ ; WT:  $y = 9.3467x^{-1.064}$ ,  $R^2 = 0.75779$ . Error bars show SEM.

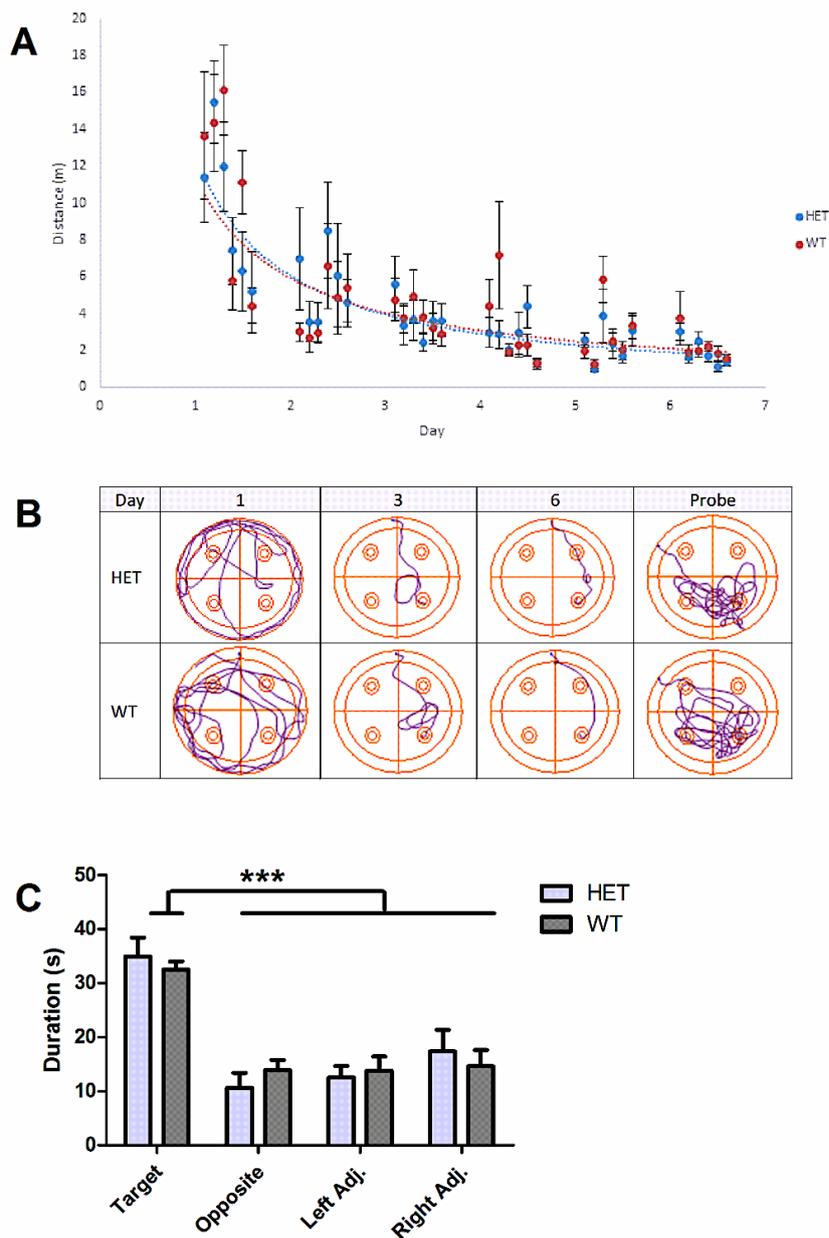
#### 4.3.1.2 Spatial Learning

The spatial learning task assesses the mouse's ability to locate a hidden platform with the use of visual extra maze cues. This is a test of spatial learning, as the mouse must orient itself in space, before swimming to the correct platform location. First, it is crucial to confirm that the  $HDH^{Q111+/-}$  mouse is able to swim equally well as its littermate controls. This is achieved by measuring the swim speed, which does not differ between the genotypes (repeated measures ANOVA: main effect genotype:  $F_{(1,14)} < 1$ , partial eta squared = 0.04). The mice do get slightly slower during spatial learning (main effect of trial:  $F_{(35,490)} = 7.2$ ,  $p < 0.001$ , partial eta squared = 0.34, Figure 4.5). Figure 4.6A shows each group's mean distance (length of swim path) for each of the 6 trials over the 6 days of spatial learning. It is evident that the mean distance swum decreases even within the first day (ANOVA, main effect of trial:  $F_{(35,490)} = 11.1$ ,  $p < 0.001$ , partial eta squared = 0.44). Furthermore, there is no difference between groups in the slope of the decrease: both WT and

HDH<sup>Q111+/-</sup> mice learned the platform location equally well over 6 days of testing (no main effect of genotype:  $F_{(1,14)} < 1$ , partial eta squared = 0.03, no interaction effect:  $F_{(35,490)} < 1$ , partial eta squared = 0.05). The tracings of a typical swim path (Figure 4.6B) indicate that the swim paths are increasingly direct to the platform. Tracings from the probe trial (Figure 4.6B) show that the mice have a memory for the platform location; even when the platform was absent, they remained in the vicinity of the former platform location. WT and HDH<sup>Q111+/-</sup> did not differ in the spatial learning probe trial (ANOVA main effect of genotype:  $F_{(1,56)} < 1$ , partial eta squared = 0.00), where mice spent significantly more time in the target quadrant (main effect of quadrant:  $F_{(3,56)} = 26.7$ ,  $p < 0.001$ , partial eta squared = 0.59; Figure 4.6C).



**Figure 4.5:** HDH<sup>Q111+/-</sup> (HET) and littermate controls (WT) do not differ in swim speeds. Both genotypes become slightly slower across spatial learning days ( $p < 0.001$ ). Fit line parameters: HET:  $y = 0.2464x^{-0.168}$ ,  $R^2 = 0.4226$ ; WT:  $y = 0.2249x^{-0.11}$ ,  $R^2 = 0.24445$ . Error bars show SEM.



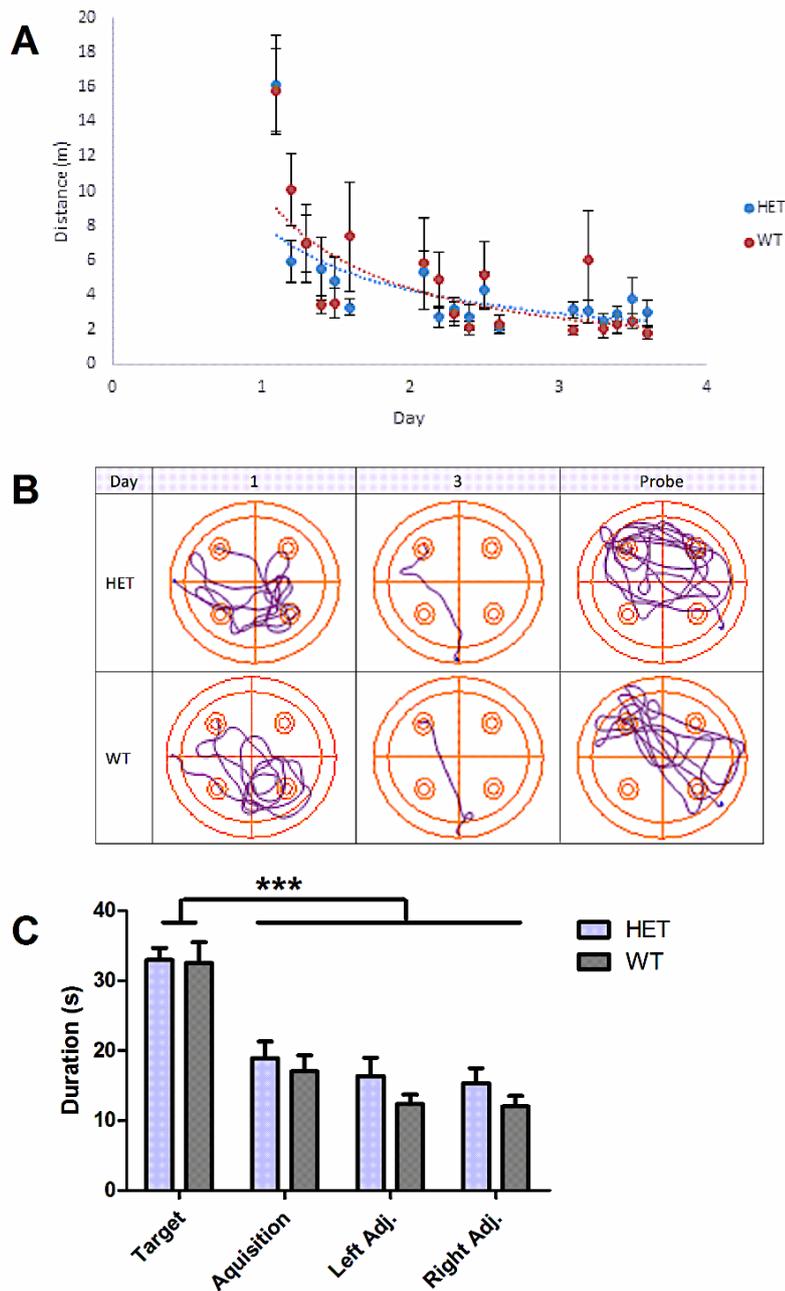
**Figure 4.6:** *HDH<sup>Q111+/-</sup>* mice were unimpaired in the spatial water maze task at 3 months.

Following the visual platform task, mice were tested in the spatial learning task, where the platform was under the water's surface and not visible. On each trial the mouse was released from a different entry site at random, but the platform location always remained constant. **(A)** Over 6 days of testing, with 6 trials per day, the *HDH<sup>Q111+/-</sup>* (HET) mouse and littermate controls (WT) learned to locate the platform equally quickly. Fit line parameters: HET:  $y=12.611x^{-1.056}$ ,  $R^2 = 0.73064$ ; WT:  $y = 11.398x^{-0.941}$ ,  $R^2 = 0.59342$ . **(B)** Representative path traces for each genotype during days 1, 3, and 6 of spatial learning and the spatial probe trial. **(C)** One hour after the last spatial learning trial on day 5 the platform was submerged so that the mouse could not reach it. In this probe trial, both genotypes spent more time in the target quadrant (Target) than all other quadrants (Opposite, Left Adjacent, Right Adjacent) ( $p < 0.001$ ). Error bars show SEM.

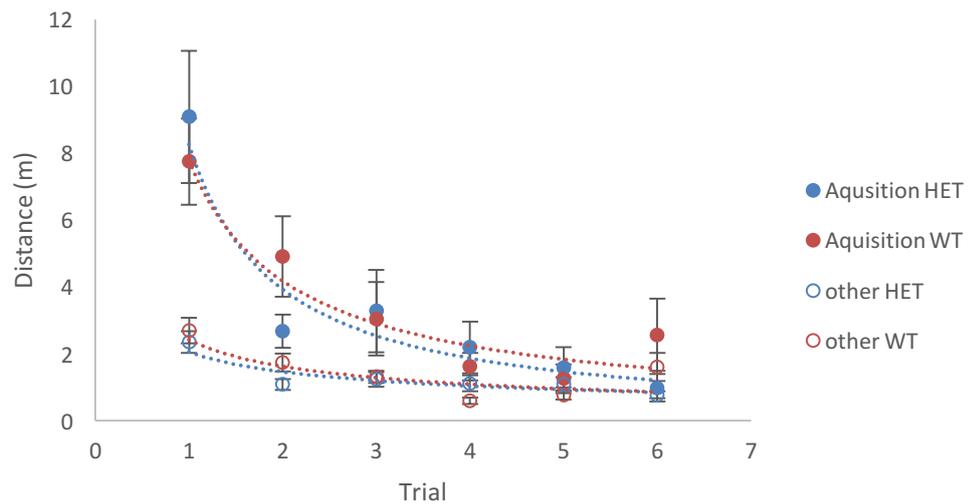
#### 4.3.1.3 Spatial Relocation

The spatial relocation task is intended to measure behavioural flexibility and the ability to learn a new platform location. After the mice have learned the location of the platform during spatial learning (section 4.3.1.2), the platform was moved to the opposite quadrant. Both genotypes learned the new platform location equally quickly (Repeated ANOVA main effect of genotype:  $F_{(1,14)} < 1$ , partial eta squared = 0.01; Figure 4.7A). A sample size of 710 would be needed to see a significant genotype effect. The mice rapidly learn the new platform location over 3 days, shown by a decrease in distance swum to reach the platform (main effect of trial:  $F_{(17,238)} = 11.7$ ,  $p < 0.001$ , partial eta squared = 0.45). The traces show that although the mice explore the old-location thoroughly, they then swim in all 4 quadrants until they find the new platform location. By the third day, they swim directly to the new location (Figure 4.7B). The probe trial shows that the mice have a memory for the new platform location as they preferentially remain in the vicinity of the current target platform location, rather than the acquisition location learned during the spatial learning task (Figure 4.7C). There is no genotype difference in the time spent in each quadrant (ANOVA no main effect of genotype:  $F_{(1,56)} = 2.3$ ,  $p > 0.05$ , partial eta squared = 0.04). The mice spent more time in the target quadrant than in all other quadrants (main effect of quadrant,  $F_{(3,56)} = 33.2$ ,  $p < 0.001$ , partial eta squared = 0.64, where Bonferroni corrected pairwise comparisons show the target quadrant is different to all other quadrants,  $p < 0.001$ ). The increased path length to reach the platform at the beginning of the relocation task was due to increased time spent in the former correct platform location (Figure 4.8). This is in accordance with the spatial learning probe trial data (Figure 4.6C). After the first trial the mice quickly learned the new platform location, and after the first day they had already almost reached asymptotic path length to the platform. Accordingly, the time spent in the former correct platform location quickly dropped after the first trial (Figure 4.8, repeated measures ANOVA: main effect of quadrant:  $F_{(3,45)} = 21.1$ ,  $p < 0.001$ ,

partial eta squared= 0.58; main effect of trial:  $F_{(5,75)}=23.5$ ,  $p<0.001$ , partial eta squared= 0.61; and a significant trial by quadrant interaction ( $F_{(15,225)}=6.7$ ,  $P<0.001$ , partial eta squared= 0.31), where Bonferroni corrected pairwise comparisons show the acquisition quadrant is significantly different to all other quadrants ( $p<0.001$ ).



**Figure 4.7:** *HDH<sup>Q111+/-</sup> mice were unimpaired in water maze spatial relocation task.* After mice had established the location of the hidden platform during the initial spatial learning phase, the platform was moved to the opposite quadrant. **(A)** Over 3 days of testing, with 6 trials per day, the *HDH<sup>Q111+/-</sup>* (HET) mouse and littermate controls (WT) learned to locate the new platform location equally quickly. Fit line parameters: HET:  $y = 8.1475x^{-0.925}$ ,  $R^2 = 0.58589$ ; WT:  $y = 10.122x^{-1.204}$ ,  $R^2 = 0.57308$ . **(B)** Representative path traces for each genotype during days 1 and 3 of spatial relocation learning and the spatial relocation probe trial. **(C)** One hour after the last spatial learning trial on day 5 the platform was submerged so that the mouse could not reach it. In this probe trial, both genotypes spent more time in the target quadrant than all other quadrants ( $P < 0.001$ ). The x-axis labels the quadrant: Target, Acquisition, and Left and Right Adjacent (to the target). Error bars show SEM.



**Figure 4.8: Analysis of the distance travelled in the acquisition quadrant compared to the average of all other quadrants across 6 trials during the first day of spatial relocation learning.** During the first trial both HDH<sup>Q111+/-</sup> (HET) and littermate controls (WT) spent more time in the acquisition quadrant than in all other quadrants, but this time quickly dropped with the learning of the new platform location. Mice spent more time in the acquisition quadrant during trial 1 compared to other quadrants ( $p < 0.001$ ). Fit line parameters: Acquisition Het:  $y = 8.2691x^{-1.074}$ ,  $R^2 = 0.89464$ ; Acquisition WT:  $y = 7.7894x^{-0.899}$ ,  $R^2 = 0.77027$ ; Other HET:  $y = 2.4178x^{-0.575}$ ,  $R^2 = 0.47628$ ; Other WT:  $y = 2.0508x^{-0.49}$ ,  $R^2 = 0.77875$ . Error bars show SEM.

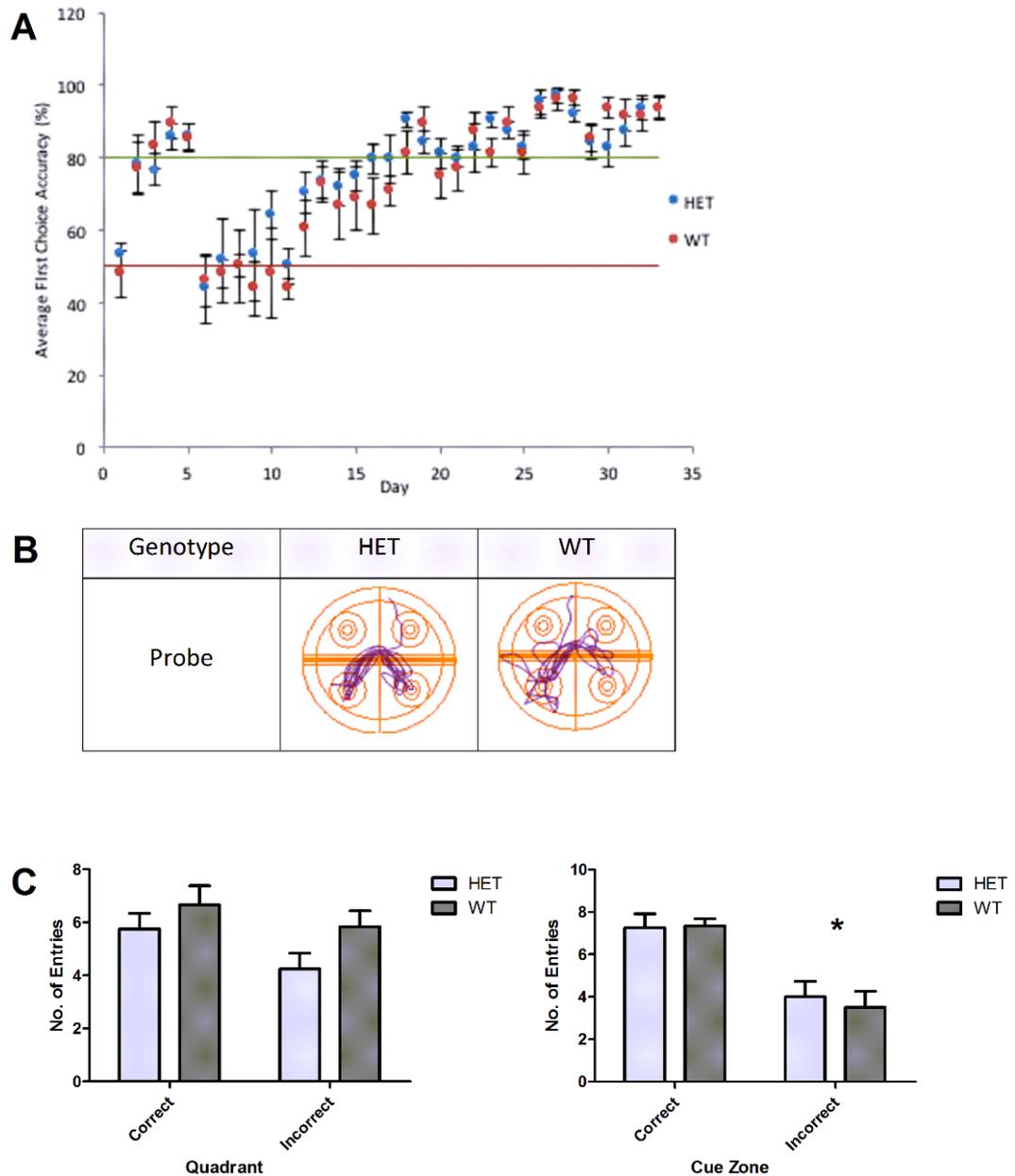
### 4.3.2 Visual Cue Reversal Learning

To assess the executive functions of the HDH<sup>Q111+/-</sup> mouse the water maze was used to perform a two-choice visual discrimination learning task followed by a reversal.

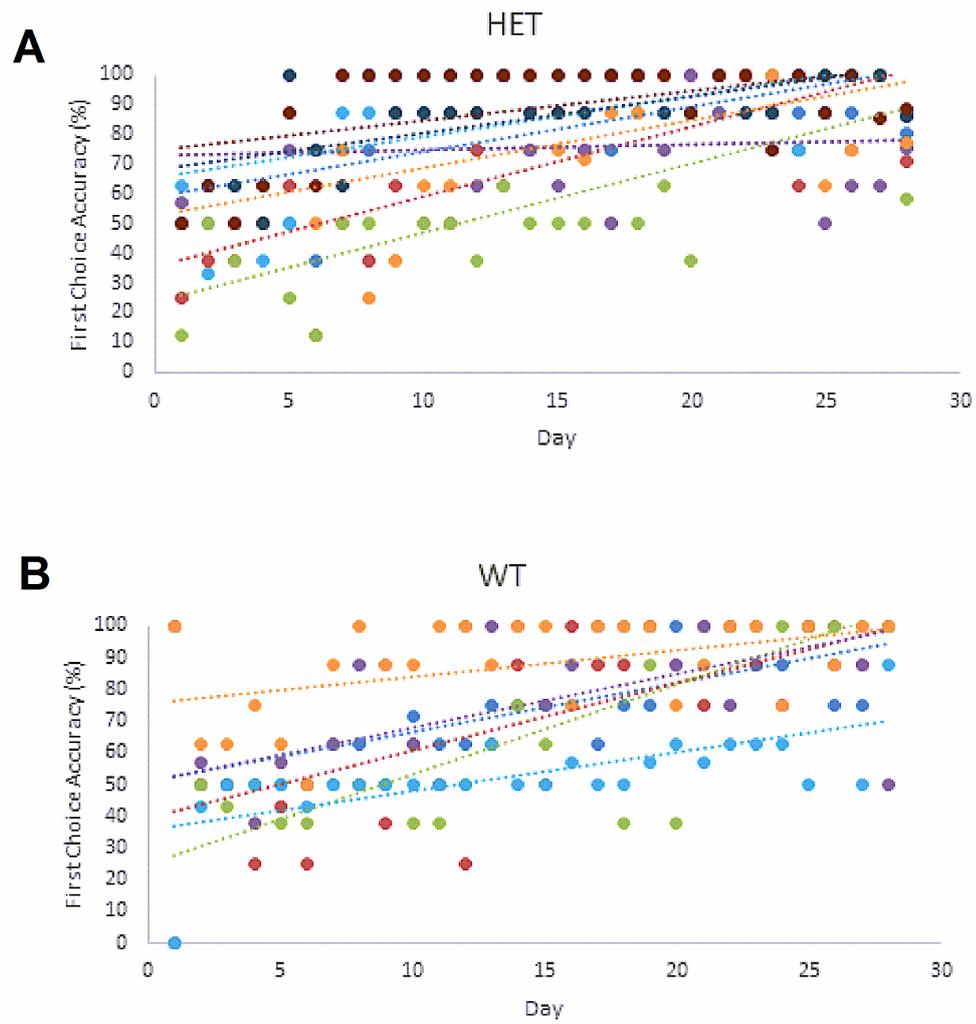
#### 4.3.2.1 Cue Training and Discrimination Learning

Mice were trained, over five days, to locate the platform using a single cue hanging directly above the platform location. Both genotypes learned the cue association equally quickly (repeated measures ANOVA, no main effect of genotype:  $F_{(1,12)} < 1$ , partial eta squared = 0.00, main effect of day:  $F_{(4,48)} = 13.2$ ,  $p < 0.001$ , partial eta squared = 0.53, no interaction effect:  $F_{(4,48)} < 1$ , partial eta squared = 0.03; Figure 4.9A).

Following cue training, two new cues were hung next to each other and the mice had to discriminate between the cues and learn which one indicated the platform location. Over 28 days both genotypes reached the discrimination criterion of 80% first choice accuracy (repeated measures ANOVA, main effect of day:  $F_{(27,324)}=14.0$ ,  $p<0.001$ , partial eta squared= 0.54; no main effect of genotype:  $F_{(1,12)}=1.5$ ,  $p>0.05$ , partial eta squared= 0.11; no interaction effect:  $F_{(27,324)}<1$ , partial eta squared= 0.06; Figure 4.9A). A probe trial was conducted on the last day of discrimination learning. The number of quadrant entries, as well as the number of entries into the cue proximity zones, were compared for the incorrect and correct cues for each genotype. There was no genotype difference during the probe trial both in quadrant entries (ANOVA main effect of genotype:  $F_{(1,24)}=3.9$ ,  $p>0.05$ , partial eta squared= 0.14) and in cue proximity zone entries (ANOVA main effect of genotype:  $F_{(1,24)}<1$ , partial eta squared= 0.00). All mice entered into the correct cue proximity zone more frequently than the incorrect cue zone, but this was not the case for quadrant entries (main effect of cue zone:  $F_{(1,24)}=27.0$ ,  $p<0.001$ , partial eta squared= 0.53; main effect of quadrant:  $F_{(1,24)}=3.4$ ,  $p>0.05$ , partial eta squared= 0.13; Figure 4.9C). Regression lines were fitted for individual mice for discrimination learning; the average slopes for each genotype were not different ( $t= 0.7$ ,  $df= 12$ ,  $p> 0.05$ , Cohen's  $d= 0.19$ ; Figure 4.10).



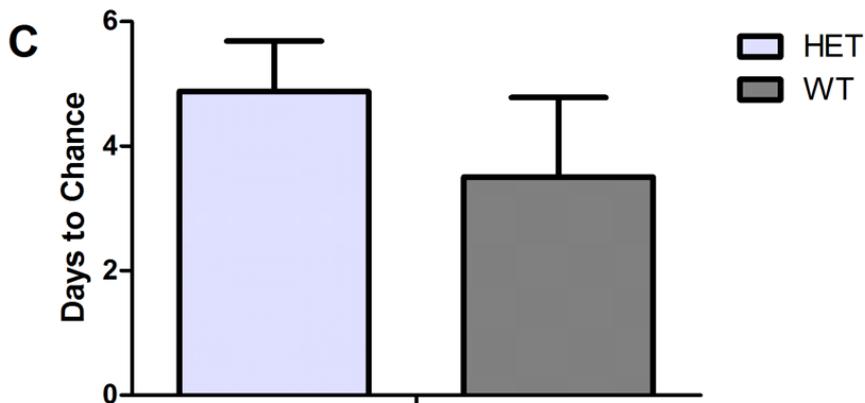
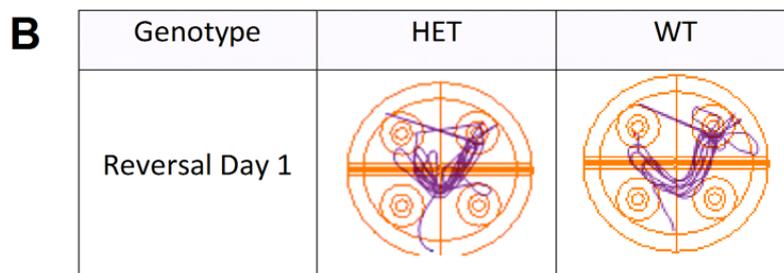
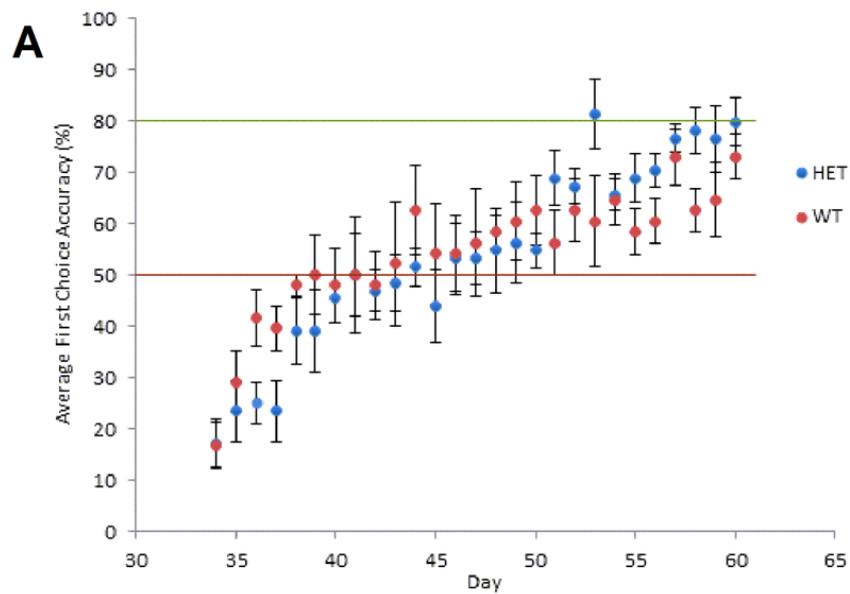
**Figure 4.9: The  $HDH^{Q111+/-}$  (HET) mouse learns to discriminate between two objects in the water maze equally quickly as the littermate controls (WT).** (A) 8 HETs and 6 WT mice learned to locate the platform during cue training within five days, where only one cue was used to associate the platform location with a cue. On the sixth day two different cues were used; however, only one predicted the platform location. Both genotypes learned this discrimination equally quickly within 28 days. The red line shows 50% first choice accuracy (the average percent per day to choose the correct cue first). 50% represents chance level. The green line shows the learning criterion of 80% first choice accuracy. (B) Representative track plots from each genotype during the probe trial. (C) Probe trial 24 hours after discrimination learning on day 28. The left hand graph shows the quadrant entries for both the correct and incorrect quadrants for HETs and WT; the right hand graph shows the same data for the cue proximity zones. Both genotypes had significantly less entries to the incorrect cue zone than the correct one ( $p < 0.001$ ). Error bars show SEM.



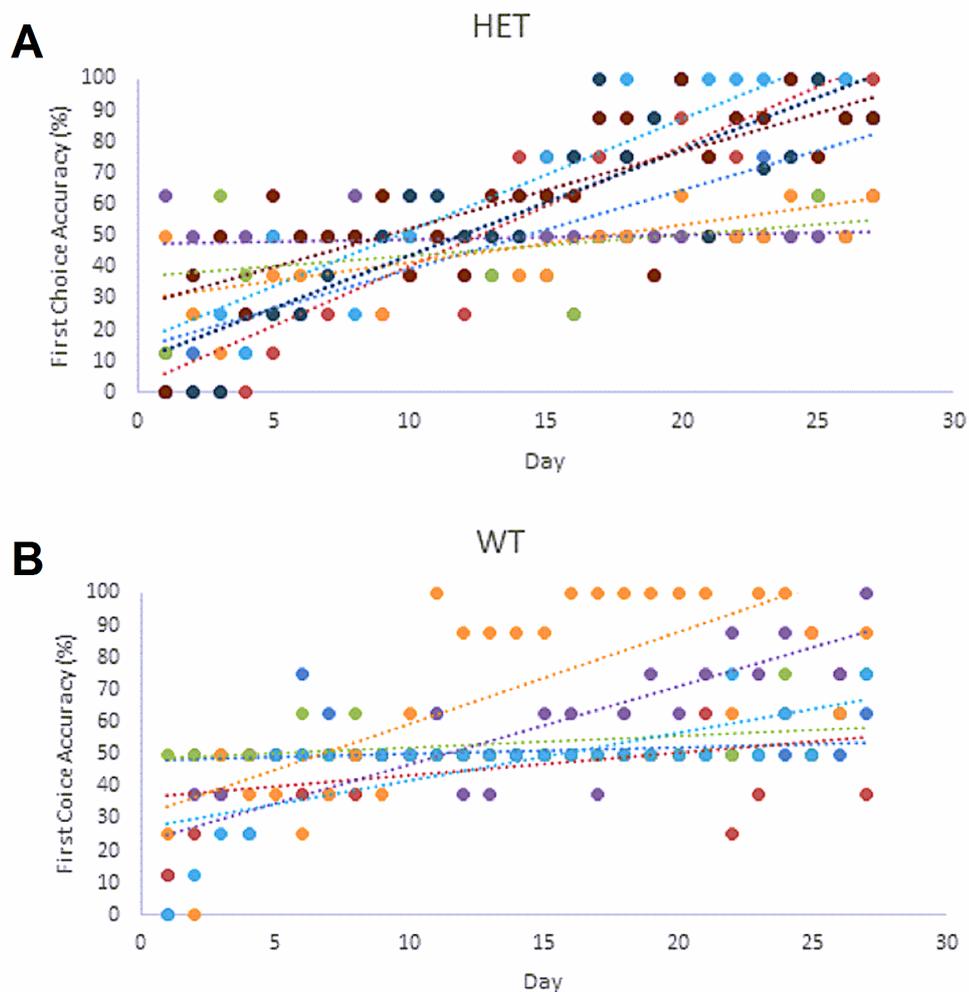
**Figure 4.10: Individual data shown with a regression line for each mouse during the discrimination learning stage.** The average of the slopes of the HDH<sup>Q111+/-</sup> group (HET) was not different to the littermate control group (WT) ( $p > 0.05$ ). **(A)** The regression lines of the 8 individual HET mice and **(B)** the regression lines of the 6 WT mice. Each colour represents one mouse.

#### 4.3.2.2 Reversal Learning

The cue association was reversed once discrimination learning was complete. The mice initially persisted at the formerly correct cue for about four days, at which point they reached chance performance (Figure 4.11A). They slowly learned the new association (ANOVA effect of day,  $F_{(26,312)}=12.2$ ,  $p<0.001$ , partial eta squared= 0.50); however, testing was stopped at just under 80% first choice accuracy after 27 days of testing (Figure 4.11A). It was expected that the mice would learn the reversal faster than the initial discrimination, and when this did not occur, due to time and cost restraints, testing was stopped. There was no difference between genotypes (main effect genotype,  $F_{(1,12)}<1$ , partial eta squared= 0.00). As can be seen from the individual data (Figure 4.12), there was also no difference in the rate of learning between HDH<sup>Q111+/-</sup> and WT mice ( $t= 1.3$ ,  $df= 12$ ,  $p>0.05$ , Cohen's  $d= 0.36$ ). A sample size of 246 would be needed to show a significant difference in rate of learning. The first four days of reversal looked as though there might be a small trend where HDH<sup>Q111+/-</sup> mice took slightly longer to reach chance performance, but Figure 4.11C shows there is no difference in the days to reach chance between the genotypes ( $t= 0.95$ ,  $df= 12$ ,  $p>0.05$ , Cohen's  $d= 0.26$ ). Figure 4.11B shows that the mice of both genotypes were repeatedly visiting the formerly correct cue during the first day of reversal learning, as expected.



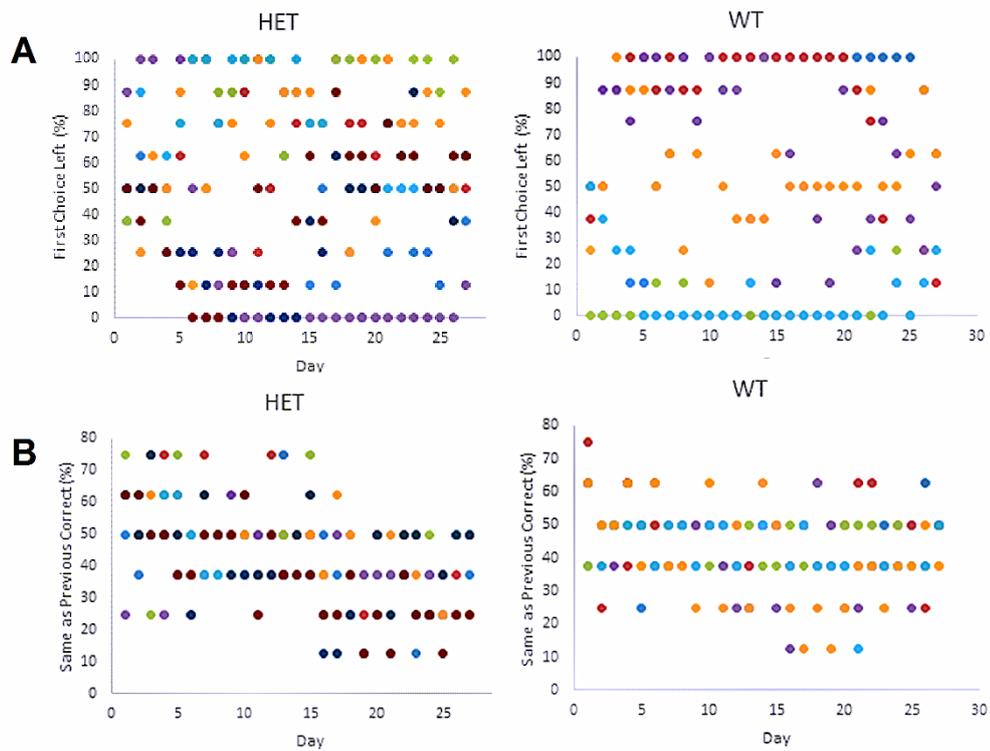
**Figure 4.11: The  $HDH^{Q111+/-}$  (HET) mouse is equally fast at reversal learning as the littermate controls (WT).** (A) Reversal learning was conducted for 27 days. The reversal stage consisted of reversing the cue and platform association previously established at the discrimination stage. The red line shows 50% first choice accuracy (the average percent per day to choose the correct cue first). 50% represents chance level. The green line shows the learning criterion of 80% first choice accuracy. (B) Representative track plots from each genotype during the first day of reversal learning. (C) The average days for each genotype to reach chance performance. Error bars show SEM.



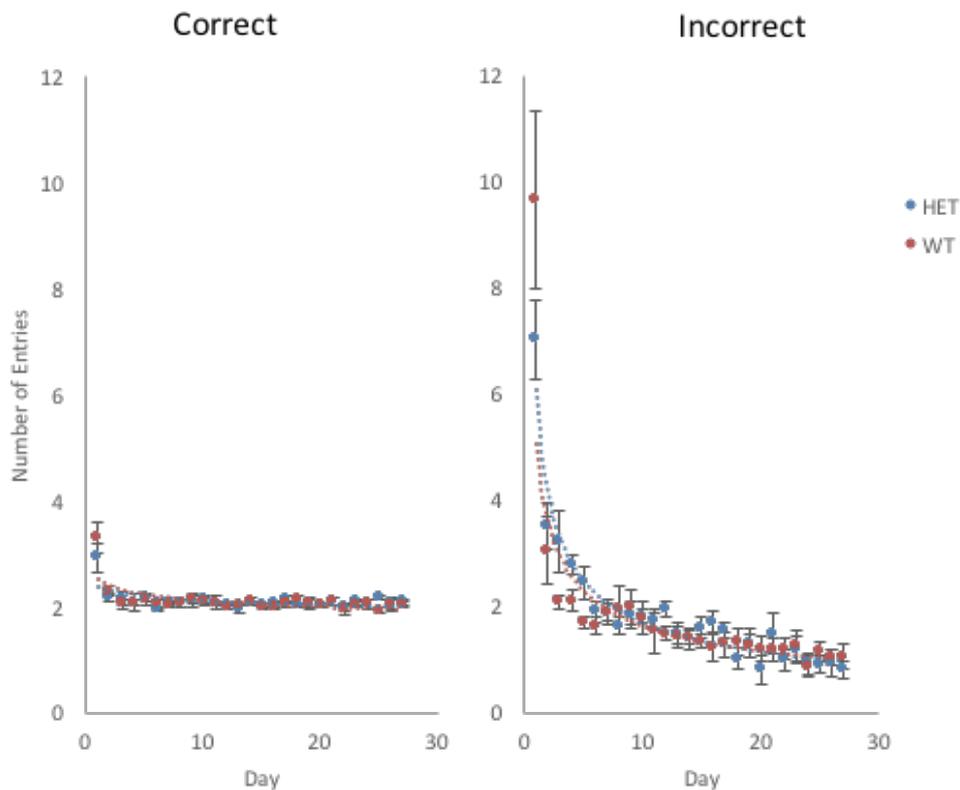
**Figure 4.12: The Individual data shown with a regression line for each mouse during the reversal learning stage.** The average of the slopes of the HDH<sup>Q111+/-</sup> group (HET) was not different to the littermate control group (WT) ( $p > 0.05$ ). **(A)** The regression lines of the 8 individual HET mice and **(B)** the regression lines of the 6 WT mice. Each colour represents one mouse.

A range of strategies were investigated that the mice could have tried to use to locate the platform during the reversal stage of the task, other than cue identity. A few mice were biased towards either the left or right side of the barrier (Figure 4.13A) but there is no pattern indicating the use of a win-stay strategy by visiting the location correct on the former trial (Figure 4.13B). To assess if there was a difference in the decision making between the genotypes the number of entries into the correct or incorrect platform were compared

during reversal learning. Figure 4.14 shows a rapid drop in the number of entries made to the incorrect quadrant (repeated measures ANOVA, main effect of day:  $F_{(26,312)}=37.5$ ,  $P<0.001$ , partial eta squared= 0.76). There is no genotype difference in the number of entries made to either the correct (repeated measures ANOVA, main effect of genotype:  $F_{(1,12)}<1$ , partial eta squared= 0.00) or incorrect (main effect of genotype:  $F_{(1,12)}<1$ , partial eta squared= 0.00) quadrants.



**Figure 4.13: Alternative strategies used by both  $HDH^{Q111+/-}$  (HET) and control (WT) to attempt locating the platform during the reversal stage.** The average percent over 8 trials for each day is plotted for the individual subject. **(A)** Side preference: the percent that left was chosen first at any given trial. Points near 100% or 0% indicate preference for one side. **(B)** Win-stay analysis: the percent that the chosen side was the previously correct side.



**Figure 4.14:** *HDH<sup>Q111+/-</sup> (HET) and control (WT) mice show no difference in indecision in cue choice.* This was measured by the number of entries into the quadrant with the correct and incorrect cues during reversal learning. Fit line parameters: Correct HET:  $y = 2.3867x^{-0.053}$ ,  $R^2 = 0.38126$ ; Correct WT:  $y = 2.538x^{-0.079}$ ,  $R^2 = 0.47722$ ; Incorrect HET:  $y = 6.089x^{-0.555}$ ,  $R^2 = 0.89373$ ; Incorrect WT:  $y = 5.0659x^{-0.49}$ ,  $R^2 = 0.831$ . Error bars show SEM.

## 4.4 Discussion

This chapter utilised the water maze to assess spatial memory and reversal learning in the *HDH<sup>Q111+/-</sup>* mouse. As a pre-requisite, all mice had to pass the visual platform stage, where the platform is clearly marked with a black and white striped marker. All mice could locate the platform using direct swim paths after one day of training (Figure 4.4); showing that mice have no visual impairments.

The spatial learning task showed that both genotypes could find the hidden platform equally quickly using only the extra-maze cues to locate the platform location in space (Figure 4.6). As there was no genotype difference in swim

speed, it can be assumed that there are no motor impairments which could interfere with measurements of cognition (Figure 4.5). Spatial re-location learning measures the ability for the mice to learn a new platform location when the previous learned location is no longer rewarded. This is a measure of behavioural flexibility, as the mice must learn to inhibit swimming to the old platform location to search for the new one. Both genotypes also learned the spatial re-location equally quickly, indicating that the HDH<sup>Q111+/-</sup> mouse is not impaired in spatial learning in the water maze (Figure 4.7). This contrasts with the HDH<sup>(CAG)150</sup> and R6/2 mouse which showed increased distance to find both the initial platform location, and the platform during spatial re-location (Brooks et al., 2012b, Lione et al., 1999, Murphy et al., 2000).

Moving from hippocampal to frontal cortex mediated tasks, we next tested the 2-choice discrimination reversal learning in the HDH<sup>Q111+/-</sup> mouse. Both genotypes learned the simple 2-choice discrimination equally quickly within 28 days (Figure 4.9). Learning of the initial discrimination took many more trials than recorded in literature to reach the same criterion; Bannerman et al. (2012) showed that WT C57BL6 mice were able to learn between two cues in the water maze within 144 trials, compared to the mice in this study, which took 224. Differences here could be due to the experimental set up, where Bannerman's group used cues sitting on poles above the water's surface, rather than hanging cues. The poles and close vicinity of Bannerman's cues to the platform may be able to create a stronger association between the platform and cue, than a cue hanging above the platform. Upon reversal of the stimuli, there was no genotype difference in the days to reach chance level performance (or the unlearning of the previously learned S-R contingency) indicating that the HDH<sup>Q111+/-</sup> mice did not persevere at the old stimulus (Figure 4.11). This is unlike the literature which shows reversal learning impairments in both BACHD mice and HD patients (Lawrence et al., 1999, Farrar et al., 2014). Both genotypes also learned the new discrimination at the same rate (Figure 4.12).

Differences in our HDH<sup>Q111+/-</sup> model to other HD mouse models mentioned above, and to patient data, indicate that at this age and using the water maze protocol, HDH<sup>Q111+/-</sup> mice do not show the typical spatial or behavioural flexibility impairments common in HD.

There has been some criticism for using the water maze in mice, including increased levels of stress due to swimming. It is possible for stress to alter behaviour (Harrison et al., 2009), perhaps making the task less sensitive to finding a genotype difference. As mice originate from arid habitats, swimming is a behaviour that they can engage in when necessary, but they do not enjoy it. This is in contrast to rats, which are much more comfortable with water, and will even dive under the surface, which mice do not. Harrison et al. (2009) have measured plasma corticosterone levels in C57/BL6J mice (the same strain as the HDH<sup>Q111+/-</sup> background) after testing in the water maze. Corticosterone is released in response to stressful stimuli. They observed a negative correlation in task performance with increasing corticosterone levels. In comparison, rats show a robust increase in corticosterone on the first day of testing, which attenuates with testing progression over 5 days (Aguilar-Valles et al., 2005). This indicates that the experimental procedure is stressful enough to produce motivation to escape, but is not as stressful in general as for mice. As stress can influence learning and memory, it was important to make sure that our HDH<sup>Q111+/-</sup> mice do not show an anxious phenotype before testing in the water maze. Past work from the Lambert/Langston laboratory reported that although homozygous mice showed an increase freezing response in the activity box, heterozygous mice did not differ from WT (Mitchell, 2012).

In summary, this chapter shows that the HDH<sup>Q111+/-</sup> mice are not impaired in spatial learning or reversal learning when tested in the water maze. However, other measures of behavioural flexibility which are impaired in HD, like attentional set-shifting (see section 1.4.1), may still be good candidates for treatment with Org49209.



## Chapter 5

# Inhibition of the mPFC Impairs Attentional Set-Shifting: Can This be Ameliorated by Org49209?

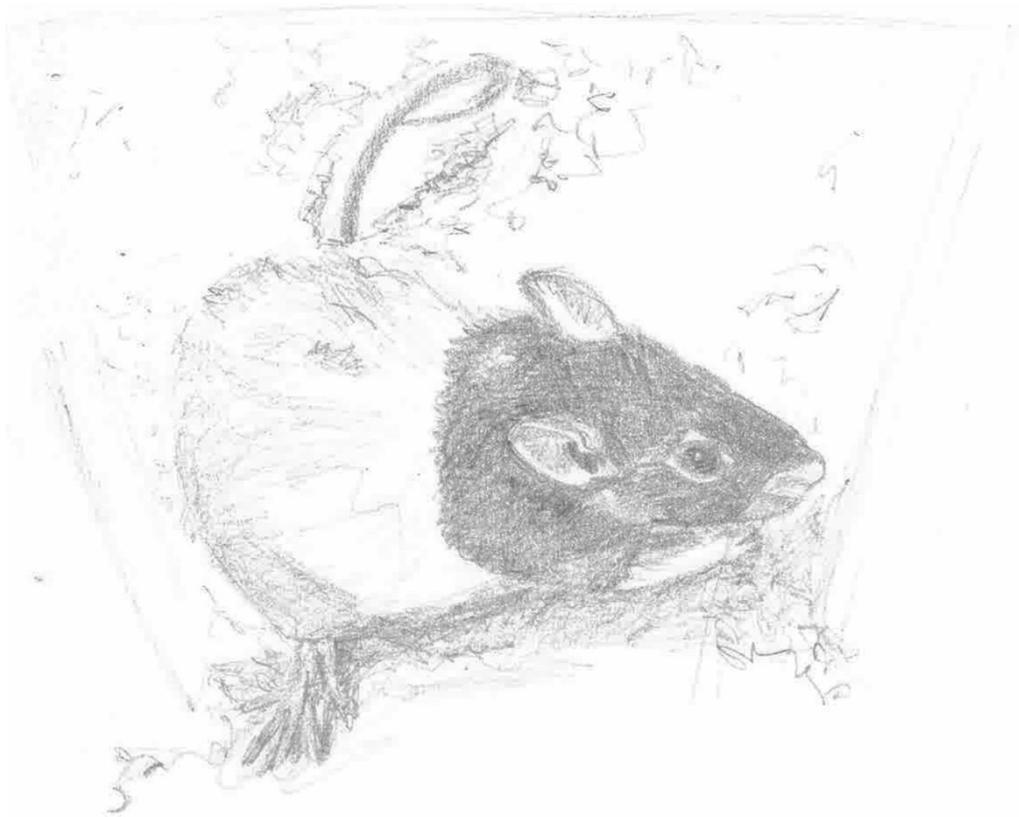


Illustration by C. Brazaitis

## 5.1 Introduction

Due to the hypothesised cognitive enhancing potential of Org49209 through its modulation of the NMDA receptor (see section 1.6.5), this chapter sets out to explore this potential in further aspects of executive control through an archetypal test of behavioural flexibility- the attentional set-shifting task (see section 1.4.1).

NMDA receptors play a major role in set-shifting (see section 1.4.2) and HD patients have impaired set-shifting ability, likely due to disruption to the frontal cortex and/or corticostriatal loops (see section 1.4.3). To date, there have been no convincing reports of set-shifting impairments in rat or mice HD models. Therefore, the approach taken here was to test a group of rats, in which impaired set-shifting ability had been demonstrated, to test the hypothesis that Org49209 could ameliorate the deficit. The set-shifting impairment had been induced by inactivation of the medial prefrontal cortex (mPFC) and confirmed behaviourally by A. Whyte. The same rats were then tested with Org49209 and vehicle. This chapter does not aim to validate mPFC inactivation as a model for HD, but rather, aims to investigate an alternative method for Org49209 application in ameliorating cognitive deficits (which may have a future application to HD).

### 5.1.1 DREADDs

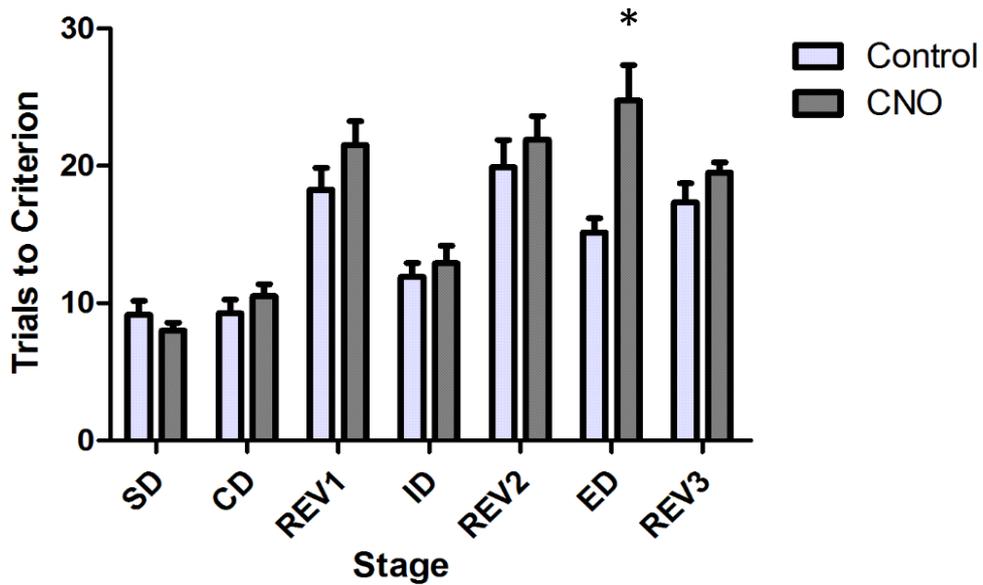
The mPFC was inactivated using DREADDs (Designer Receptors Exclusively Activated by Designer Drugs), which are G-protein coupled receptors (GPCRs) that have been mutated to no longer be targets for their endogenous ligands, and instead, are activated solely by a synthetic ligand. This ligand is otherwise completely inert. Strader et al. (1991) mutated the first receptor to be exclusively activated by a synthetic ligand; the first use of this technology to attempt to control cell signalling was published by Coward et al. (1998). The DREADDs used in this experiment were developed by Armbruster et al. (2007) using muscarinic acetylcholine receptors (mAChRs). The authors developed

these DREADDs specifically to reduce the prevalence of synthetic ligand binding to native receptors, which previously limited their application *in vivo*.

DREADDs are available for the three major GPCR pathways;  $G_s$ ,  $G_i$ , and  $G_q$ . DREADDs have a large variety of uses, such as in developmental studies of tissue growth and engineering, or in excitable cells of the heart, where they can be used to control heart rate (Conklin et al., 2008). For this thesis, the most exciting application is the ability to change behaviour by targeting specific cell types in key brain areas. Just as  $G_s$  and  $G_i$  pathway stimulation can increase and decrease heart rate,  $G_q$  and  $G_i$  can excite or silence neuronal cells, respectively. A viral vector allows for the expression of the designer receptors in the target cells. Specific cell types are targeted through the use of promoter regions that are used in those cell types. This experiment uses DREADDs which target glutamatergic cortical pyramidal cells using the CamKII promoter. The DREADD is the inhibitory hM4Di receptor which is a  $G_i$ -GPCR. This GPCR causes temporary neuronal silencing via a sustained membrane hyperpolarisation through decreased cyclic AMP (cAMP) signalling and increased activation of inward rectifying potassium channels (Armbruster et al., 2007, Rogan and Roth, 2011). Various viruses can be used to administer the DREADD; here, adeno-associated virus 5 (AAV5) was used due to its relative low anatomical spread, to keep expression localised around the injection sites. The synthetic ligand used for activation of the DREADDs is clozapine-N-oxide (CNO).

### 5.1.2 Study Aims

Using the inhibitory hM4Di DREADDs in the mPFC of rats, A. Whyte has shown that CNO caused an increase in TTC in the ED stage of the task compared to control (Figure 5.1). This study builds on A. Whyte's findings, using the same experimental cohort and experimental design, to assess the effect of Org49209 on set-shifting behaviour at the ED stage.



**Figure 5.1: Results from the ID/ED task** (SD, simple discrimination; CD, compound discrimination, REV1, reversal of CD; ID, intradimensional acquisition; REV2, reversal of ID; ED, extradimensional acquisition; REV3, reversal of ED). Rats (N=12) were tested in a within-subjects design where they received both CNO or vehicle. CNO activation of the mPFC DREADDs resulted in a set-shifting deficit (greater trials to criterion at the ED stage,  $p < 0.05$ ). Data collected by A. Whyte.

## 5.2 Methods

### 5.2.1 Subjects

The same twelve male Lister Hooded rats (Charles River, UK), infected with inhibitory DREADDs, that had previously been tested by A. Whyte, were used in this study. Rats were pair-housed throughout the study in standard housing conditions. Prior to this study, the rats had been tested in the standard 7-stage ASST or a variation of the standard task 9 times. Surgery and previous testing was performed by D. Tait and A. Whyte, respectively. Surgery used the Paxinos and Watson (1998) co-ordinate system ( 1: Anterior/Posterior + 3.9, Medial/Lateral  $\pm$  0.5, Dorsal/Ventral -3.1; 2: Anterior/Posterior + 2.9, Medial/Lateral  $\pm$  0.5, Dorsal/Ventral -3.1) to administer bilateral microinjections on 1  $\mu$ l AAV5-CamKII-hM4Di. At the time of testing rats were approximately 7-9 months old, weighing 448-518 g.

### 5.2.2 Apparatus

**Box:** The testing was conducted in an in-house modified plastic housing-cage (69.5 x 40.5 x 18.5 cm) with two separate compartments taking up one third of the box (Figure 5.2). The compartments were separated from the rest of the testing box using a removable Plexiglas panel. A further, smaller, panel was used to be able to close one compartment at a time. The bowls were placed in two different compartments in the testing box. The holding area of the box contained sawdust and a bowl of water.

**Bowls:** The digging bowls were ceramic pet food bowls (8 cm diameter, 4 cm deep, Mason Cash, Liverpool, UK).

**Media and odours:** Spices and herbs were bought from local stores, usually Tesco own brand, or Schwarz brand. The fine shavings were produced in-house from coarse shavings (Golden Flakes, DBM), using a food processor until they are fine enough using visual inspection. Additionally, sand (Children's Play Sand from Argos), grit (Cage Proud Mixed Bird Grit), coarse tea (Darjeeling, Waitrose own brand), and the fine tea (local store bought) were used as media. Odour was added to the digging media until a difference in smell was clearly discernable. All media and odours used are listed in Table 5.1.

**Reward:** One third of a Honey loop (Kellogg's) was used as a reward per trial.

Data was recorded on a laptop in software developed by A. Whyte.

**Table 5.1: The media and odours used in the 7-stage ASST.**

Medium	M1	M2	M3	M4	M5	M6
	Coarse tea	Fine tea	Gravel	Sand	Coarse wood shavings	Fine wood shavings
Odour	O1	O2	O3	O4	O5	O6
	Paprika	Sage	Cinnamon	Ginger	Turmeric	Cloves



**Figure 5.2: The ASST testing box.** One third of the box was divided with a removable Plexiglas panel. This section was again split in two sections, in which each bowl was placed. The rat must choose to dig in one of the two bowls for a food reward. Image taken by A. Chase.

### 5.2.3 Behaviour

The effect of Org49209 was tested in the standard 7-stage ASST, as previous results have shown that rats with active DREADDs were impaired at the ED stage (see Figure 5.1).

#### 5.2.3.1 7-Stage ASST with CNO and Org49209

Due to previous testing, rats were already on mild food control prior to this study, which was maintained at 15-20 g per day. Water was available *ad libitum*. As rats were already trained to the task (digging for reward, exposure to testing stimuli, and habituation to apparatus) due to prior testing, it was not necessary to repeat it for this experiment. A. Whyte tested half of the rats, at the same time and the same room, using the same experimental criteria.

Org49209 was freshly prepared each day, where the suspension was sonicated for 30 minutes prior to use. CNO was suspended in a 'jelly' mix (60ml, Robinsons concentrate; 6 g, Dr. Oetker Gelatine Sachet; stirred for 15 minutes). 30 minutes before testing, rats received either 10mg/kg Org49209 or vehicle (10% HPBCD in saline) i.p. injection, followed immediately by a jelly containing 10mg/kg CNO. To ensure CNO activated the DREADDs for the duration of the task, a concentration of 10 mg/kg was used, which falls in the high end of the effective range (Roth, 2016). A. Whyte previously compared the behavioural effects in the 7-stage task of CNO administration, showing no significant differences between i.p. injections and oral administration. Others have also found oral administration of CNO to be successful (Jain et al., 2013). Org49209 was still shown to be present in mouse plasma 3 hours after i.p. administration (Paul et al., 2013), which should allow sufficient time for task completion.

Each trial was initiated by raising the Perspex panel which allowed the rats to access the compartments containing the food bowls. A dig was recorded if more than two paws are seen and/or if the nose is buried deep enough that the food reward could be reached. A correct or incorrect choice is recorded, as

well as whether the rat had investigated both bowls before making a choice. A choice which is made correctly after investigating both bowls makes a stronger argument for a learned discrimination, as the incorrect bowl was rejected. This means that a rat is less likely to have passed the stage by choosing the correct bowl by chance. The first four trials of each stage were exploratory trials, where the rat is allowed to retrieve the food reward from the correct bowl even if an incorrect choice was made. If an incorrect choice was made after the first four trials, the barrier was lowered to the chamber containing the correct bowl to prevent the rat from correcting the error. Rats had to reach the criterion of 6 six consecutive correct responses, which is above chance performance (if each response has probability of 0.5,  $p=0.0156$ ). The stimuli were always presented in pairs, where M1/M2 was presented with O1/O2, M3/M4 was presented with O3/O4 and M5/M6 was presented with O5/O6. The stimuli used were counterbalanced with regard to a set-shift from odour to medium, and from the odd to even stimuli. The order the stimulus pairs were presented in was also counterbalanced.

The task consisted of 7 stages:

**Simple (SD):** Either odour in cage bedding, or medium with no added odour.

**Compound (CD):** Both odour and medium are present, but only one dimension is relevant.

**Reversal (REV1):** Reversal of the CD. Previously rewarded stimulus is now unrewarded, previously unrewarded stimuli is now rewarded.

**Intradimensional acquisition (ID):** Both medium and odour are changed, but the relevant dimension stays the same.

**Reversal (REV2):** Reversal of the ID. Previously rewarded stimulus is now unrewarded, previously unrewarded stimuli is now rewarded.

**Extradimensional shift acquisition (ED):** Both odour and medium are changed, and relevant dimension is switched.

**Reversal (REV3):** Reversal of the ED. Previously rewarded stimulus is now unrewarded, previously unrewarded stimulus is now rewarded.

#### 5.2.3.2 Data Analysis

Data were collected in a csv file from the software, and analysed in Microsoft Excel. The number of trials to criterion for each stage were averaged for vehicle and drug groups. The data were plotted in a histogram using GraphPad Prism 5 with SEM. A two-way ANOVA was used to assess the effect of Org49209, and to compare the CNO data with previously collected control data (from A. Whyte, shown in Figure 5.1).

#### 5.2.4 Histology

Once behavioural testing was complete, rats were deeply anaesthetised with pentobarbital (0.8ml, i.p.; Pharmasol, Ltd, UK) and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The whole brain was dissected out and stored in a 20% sucrose solution for 24 hours at 4°C. The cerebellum and brainstem were removed, washed with distilled water, dried, placed in a well of a 24-well plate, covered in egg yolk and placed in a formaldehyde bath for 72 hours. Brains were then sectioned at 50 µm on a freezing stage microtome (Jung HistoSlide 2000, Reichert-Jung, Cambridge Instruments GmbH). The sections were stored in glycerol solution at -20°C until use. D. Tait and A. Whyte assisted with perfusions.

The DREADD contains a fluorescent reporter, mCherry, which can be used to verify receptor expression and location. Every 4<sup>th</sup> section of the frontal cortex was used for mCherry immunoreactivity detection. The whole immunohistochemistry protocol described here was performed at room temperature. Sections were placed in 9-hole netwells and petri dishes and washed four times (5 minutes each) in PBS (158 M NaCl in 0.2 M PB) on a

shaker. Next the netwells were incubated in a blocking solution (1:5 normal goat serum, 1:100 10% Triton, in PBS) on the shaker for one hour. Sections were again washed as above, and the placed in histology pots with 5 ml of the primary antibody (rabbit anti-mCherry 1:2000, Abcam) in antibody diluting solution (ADS; 1:100 normal goat serum, 1:100 10% triton, in PBS) overnight. Sections were again washed (as above), and incubated in 5 ml biotinylated secondary antibody (goat anti-rabbit, 1:500, Vectorlabs, in ADS) for one hour. After a fourth wash, the sections were incubated in 5 ml of biotinylation solution (Vectastain ABC KIT, Vectorlabs) and after an hour, were washed for a fifth time. Finally, sections were stained with 3,3'-Diaminobenzidine (DAB; one tablet per 20 ml, Sigma, in distilled H<sub>2</sub>O) until landmark anatomical structures were visible, followed by a final wash. Sections were stored at 4°C until they were mounted on glass slides and cover-slipped with DPX mountant. A. Whyte assisted with immunohistochemistry.

Frontal cortex sections from three brains were used for fluorescent mCherry detection in order to compare mCherry expression in the cell bodies and axons. The procedure was identical to the above DAB immunoreactivity protocol, except that after the primary antibody, the sections were washed five times for 5 minutes, incubated in the secondary antibody (goat anti-rabbit IgG H&L, Alexa Flour 594, Abcam) for one hour, and covered in aluminium foil. Sections were then washed three times for 5 minutes in PBS and mounted on slides with Vectashield anti-fade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectorlabs). Finally, slides were cover-slipped and sealed with nail polish.

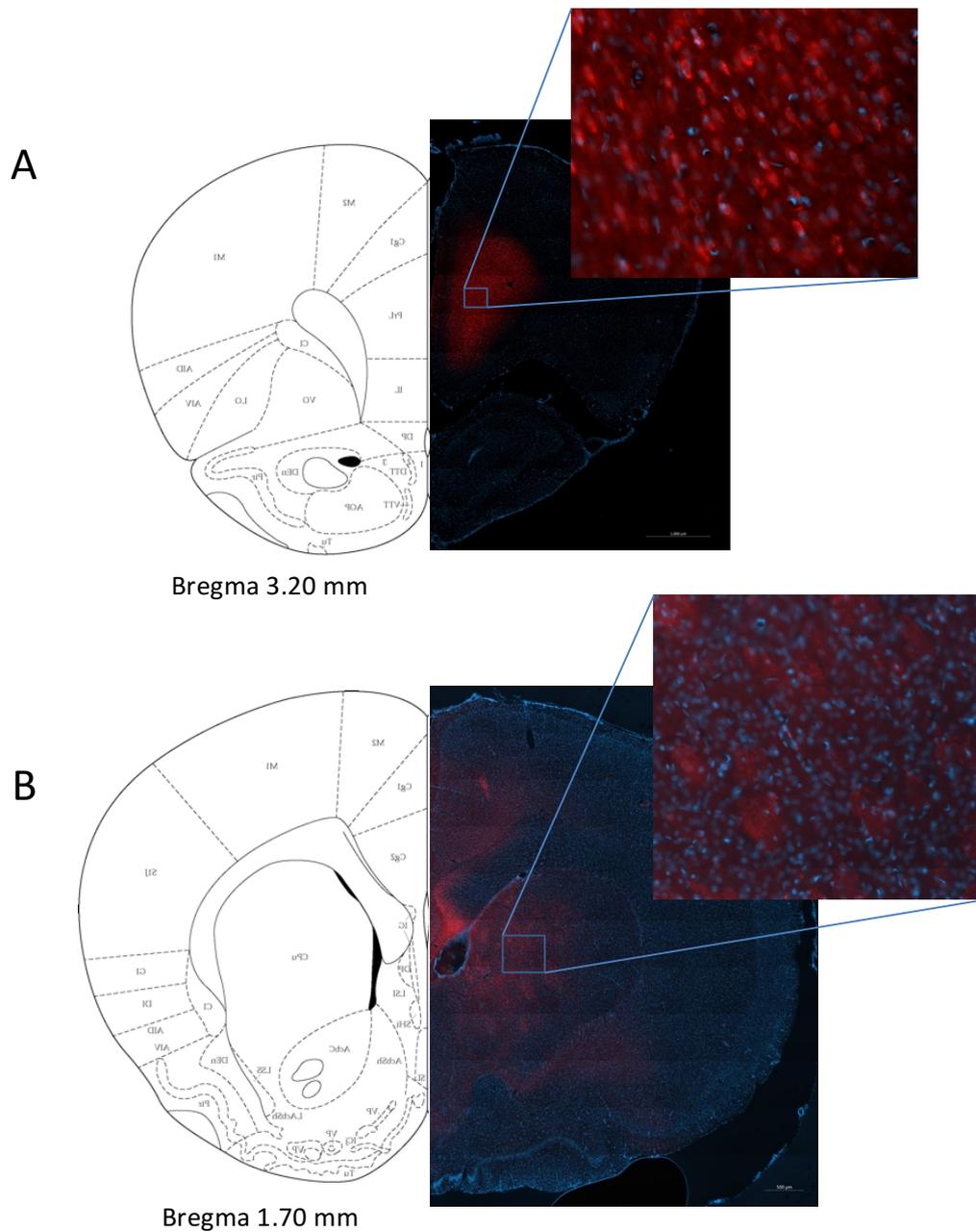
Sections were imaged on a Zeiss Axio Imager M2 using Zen software.

## 5.3 Results

### 5.3.1 Histology

DAB staining showed DREADDs were present in all brains.

Immunofluorescence showed a precise and punctate spread of the DREADDs in the mPFC (Figure 5.3). It was also interesting to note that co-localisation of DAPI, a nuclear stain, and mCherry was seen in mPFC regions (Figure 5.3A); however, in projection areas of the mPFC mCherry was also present, but was not co-localised with the cell bodies, indicating DREADDs were transported along axons to mPFC projection sites, as shown in the striatum in Figure 5.3B. The histology showed no indications of disruptions caused by using the AAV as a vector.

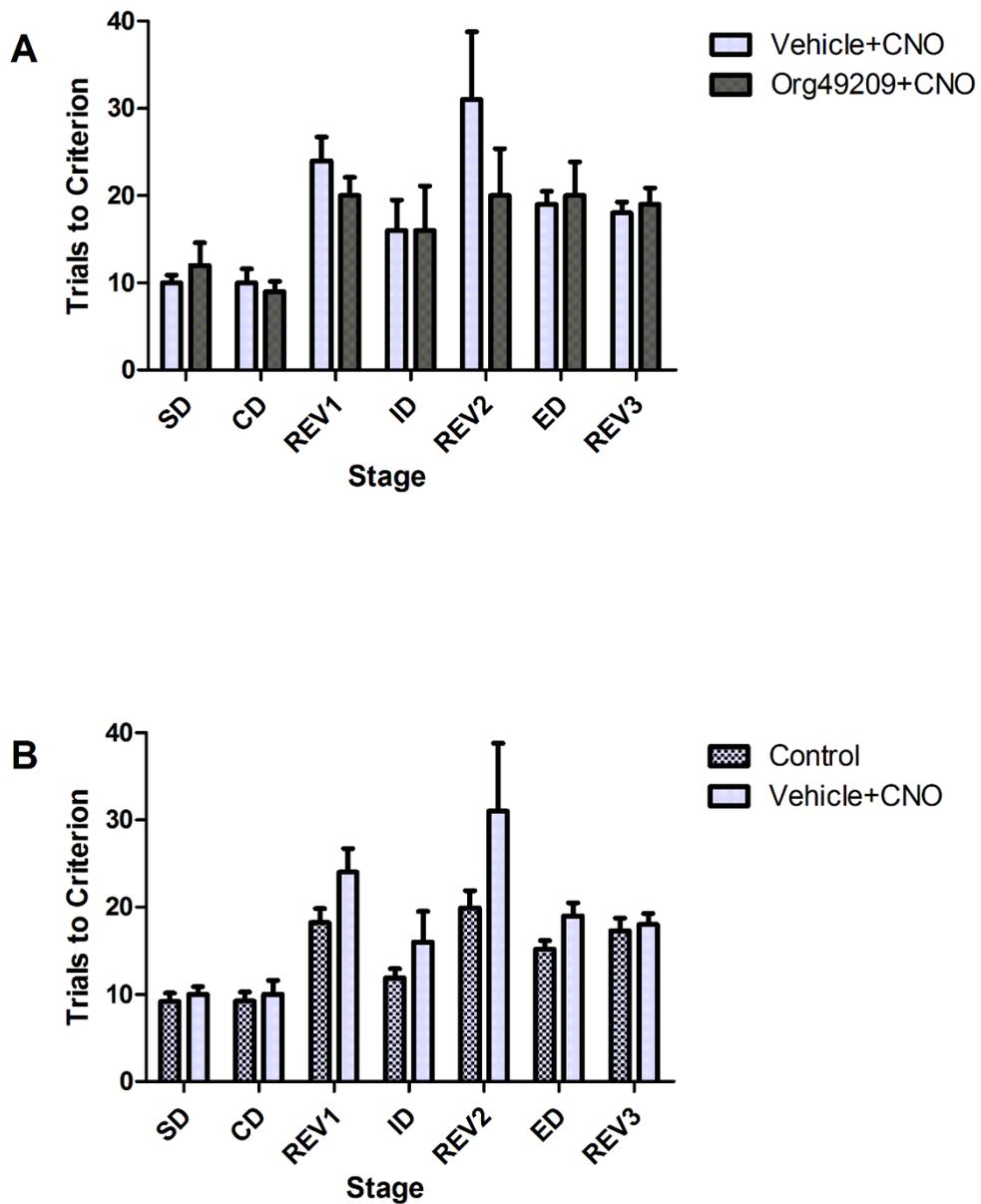


**Figure 5.3: DREADDs were tagged with mCherry to visualise viral spread. (A)** mCherry (red) can be seen in the mPFC region. The cut out shows co-localisation of mCherry with DAPI (blue) to show DREADD expression at the cell bodies. **(B)** mCherry seen in the striatum, but not co-localised with DAPI. Schematics from Paxinos and Watson (1998).

### 5.3.2 Standard 7-Stage ASST with Org49209

Org49209 had no effect on the trials to criterion in the standard 7-stage ASST (main effect of Drug,  $F_{(1,70)} < 1.0$ ,  $p > 0.05$ , partial eta squared = 0.01; interaction,  $F_{(6,70)} < 1.0$ ,  $p > 0.05$ , partial eta squared = 0.07; Figure 5.4A). The different stages were not equal, as expected (main effect of stage  $F_{(6,70)} = 5.04$ ,  $p < 0.001$ , partial eta squared = 0.30); however, there also did not appear to be an ID/ED difference (planned contrast,  $p > 0.05$ ).

The graph seemed to indicate that the previously seen impairment at the ED stage after CNO was not present. To confirm this, the CNO + vehicle was compared directly with the previously acquired control data. CNO-treated rats required more TTC than the controls overall (main effect of Group:  $F_{(1,112)} = 11.33$ ,  $p < 0.01$ , partial eta squared = 0.09); however this did not vary by stage (group by stage interaction:  $F_{(6,112)} = 1.39$ ,  $p < 0.05$ , partial eta squared = 0.07), confirming that there was no selective impairment at the ED stage (see Figure 5.4B). Again, as expected, different stages were not equal (main effect of stage:  $F_{(6,112)} = 14.04$ ,  $p < 0.001$ , partial eta squared = 0.43), but there was an ID/ED difference (planned contrasts,  $p < 0.01$ ).



**Figure 5.4: *Org49209* does not have an effect on the 7-stage ASST for rats with CNO-activated mPFC DREADDs. (A)** All rats were given 10mg/kg CNO via a jelly half an hour before testing. At the same time, they also received *Org49209* (n=6) or vehicle (10% HPBCD, n=6) via i.p. injection. There was no difference in TTC between *Org49209* and vehicle treated rats ( $p>0.05$ ). **(B)** Vehicle treated rats (with CNO) were compared to previous data of control jelly (i.e. no CNO). There was no difference between the CNO and no CNO conditions ( $p>0.05$ ).

The graphs suggest that CNO may actually increase TTC at the second reversal stage; however, on closer examination of those data it was noted that these increased values are due to two very high values (rat 4 & 6) and not representative of the rat's performance as a whole (Table 5.2).

**Table 5.2: TTC for each rat during reversal 2.** CNO is present, with either control or Org49209 injections.

Rat	Condition	TTC Rev 2
1	Control	12
2	Control	20
3	Control	14
4	Control	50
5	Control	31
6	Control	57
7	Org49209	10
8	Org49209	12
9	Org49209	9
10	Org49209	21
11	Org49209	23
12	Org49209	44

## 5.4 Discussion

To assess the expression of DREADDs, mCherry immunofluorescence and the nuclear stain DAPI stain were used to show co-localisation of the DREADDs with the cell bodies. This co-localisation can be seen in the mPFC region (Figure 5.3A), while mCherry in the absence of DAPI co-localisation in non-targeted brain areas show the projections from the target area (i.e. striatum, Figure 5.3B). This can be due to the axonal transport of the DREADD receptors, which has been shown to occur after around 4-9 weeks post-infection (Smith et al., 2016).

At first the successful use of Org49209 to reverse ED deficits caused by activated inhibitory-DREADDs was questioned due to the mechanism of action of the DREADDs. If glutamatergic neurons are being silenced due to increased activation of inward rectifying potassium channels, would a PAM of the NMDA receptor be able to overcome the consequences of this? It is likely that due to the strong hyperpolarisation, Org49209 will have no direct effect on the DREADD activated cells, as the NMDA receptor requires membrane depolarisation for activation. However, Org49209 may be able to have an effect for two reasons. Firstly, DREADDs are not able to infect all target neurons; Mahler et al. (2014) showed a 60% suppression of activity in ventral pallidum, with 79% of cells affected. This would suggest that there is room for improvement with Org49209 of the remaining neurons. Smith et al. (2016) also suggest that incomplete activity suppression/activation from DREADDs may be a more realistic manipulation as the changes are made by the neuron's own downstream signalling- the same mechanisms that are used if endogenous receptors were activated by their natural ligands. Secondly, the DREADD is expressed in a specifically targeted brain area, whereas Org49209 is given systemically. This allows for Org49209 to enhance NMDA receptor function in possible downstream regions where DREADD activation has no direct influence. On the other hand, even though NMDA receptors mediate excitatory post synaptic potentials (EPSPs), NMDA receptor antagonists have been shown to cause cortical excitation in humans and rodents *in vivo* (Lahti et al., 1995, Jackson et al., 2004, Suzuki et al., 2002). Homayoun and Moghaddam (2007) intriguingly found that NMDA receptor hypofunction could increase pyramidal neuronal firing. They propose that this mechanism is through the disinhibition of the pyramidal neurons by the decreased activity of GABA-ergic interneurons. Therefore, it is possible that increased NMDA receptor function actually increases the inhibitory control of GABA-ergic neurons on pyramidal cells, which would result in further silencing of the pyramidal neurons.

One of the issues in this study is that CNO did not result in the impairments at the ED stage of the task that had been seen in the same rats in previous tests. Therefore, we cannot confidently say whether Org49209 can or cannot recover DREADD induced set-shifting impairments. There may be a number of reasons for this lack of CNO effect, which are explored below.

A possible disadvantage of using DREADDs is that it cannot be excluded that insertion of additional synthetic receptors on the neuronal membranes would not interfere with the remaining receptors on the cell, or cause signalling changes. Indeed, early DREADDs, or Receptors Activates Solely by Synthetic Ligands (RASSLs) did show constitutive activity (activation in the absence of the synthetic ligand) (Conklin et al., 2008); however, Roth (2016) claim that this has not yet been observed with the known and now widely used DREADDs. As DREADDs can have a more widespread effect than simply increasing or decreasing the activity of the target neurons, Smith et al. (2016) also suggest that the physiological impact of the DREADDs should be measured in the brain, either through electrical recordings, imaging or by staining for immunohistochemical markers of activity (e.g. cFos). Although we investigated cFos expression in these rats, results did not show a difference in CNO vs. vehicle treated rats in the mPFC. However, in A. Whyte's behavioural experiments, there was a very clear effect of CNO on set-shifting behaviour. Although cFos has been used successfully to show physiological changes of hM4Di DREADDs before (Michaelides et al., 2013), it would be interesting if the other methods suggested by Smith et al. would reveal any physiological differences consistent with the behavioural change. Ideally, we would have stained for mCherry and cFos in the same slices, to assess if the neurons expressing DREADDs show reduced activity/cFos expression.

Other possible explanations for the lack of a CNO effect at the ED stage could be: a practice effect due to previously performed tasks, or the repeated administration of CNO. As rats were tested nine times previously in either the 7-stage task, or a related task, it is possible that they could have improved

performance over time. Similar effects due to repeated testing have been seen before, improving either TTC at all stages (Xia, 2014) or the reversal stages (Chase, 2013). However, Tait et al. (2014) also describe that they have very good experience with re-testing the 7-stage task up to 9 times.

Regarding repeated CNO administration, it has been shown that GPCRs are sensitive to desensitisation and subsequent internalisation (DeWire et al., 2007). It is possible that repeated DREADD activation through CNO administration during the repeated testing could cause this desensitisation. Roth (2016) argues, however, that this tends to not be an issue unless DREADD expression is very low. If expression is sufficiently high, the maximum CNO response can be achieved without full receptor occupancy; and more importantly, if some receptors are internalised or inactive, this will not change the maximal response (Roth, 2016). An example of successful repeated CNO administration twice daily over 4 days showed a robust behavioural effect throughout (Roman et al., 2016). As immunohistochemistry showed a robust expression of DREADDs, it is likely that repeated CNO administration was not the cause of a reduced behavioural phenotype.

A. Whyte's discovery of the DREADD induced set-shifting deficit nevertheless shows a promising model for testing therapeutics for behavioural flexibility. To assess the effect of Org49209 in this model in the future, an increased sample size would be advisable, as 6 animals per group were not enough to allow for abnormally high scores from a couple of rats. Further, if running a fully controlled study, rather than a pilot, four experimental groups would have been used: CNO  $\pm$  Org49209 and no-CNO/vehicle  $\pm$  Org49209. The pilot used previous data that demonstrated the behavioural effect of CNO; therefore, this pilot study assumed this behavioural effect would continue to be present and the effect of Org49209 was assessed compared to the vehicle injection. The no-CNO/vehicle  $\pm$  Org49209 condition would be needed in a full study to support previous data for the CNO behavioural deficit, as well as assessing if Org49209 has any effect on normally behaving rats. The animals would be

experimentally naïve, and DREADD activation would be confirmed through physiological measurements such as electrophysiological slice recordings. Indeed, it would be interesting to assess Org49209 application in neurons of DREADD activated slices, to answer some of the questions about the suitability of Org49209 as a treatment for DREADD induced behaviour.



# Chapter 6

## General Discussion

“What we know is a drop, what we don't know is an ocean.” — Isaac Newton

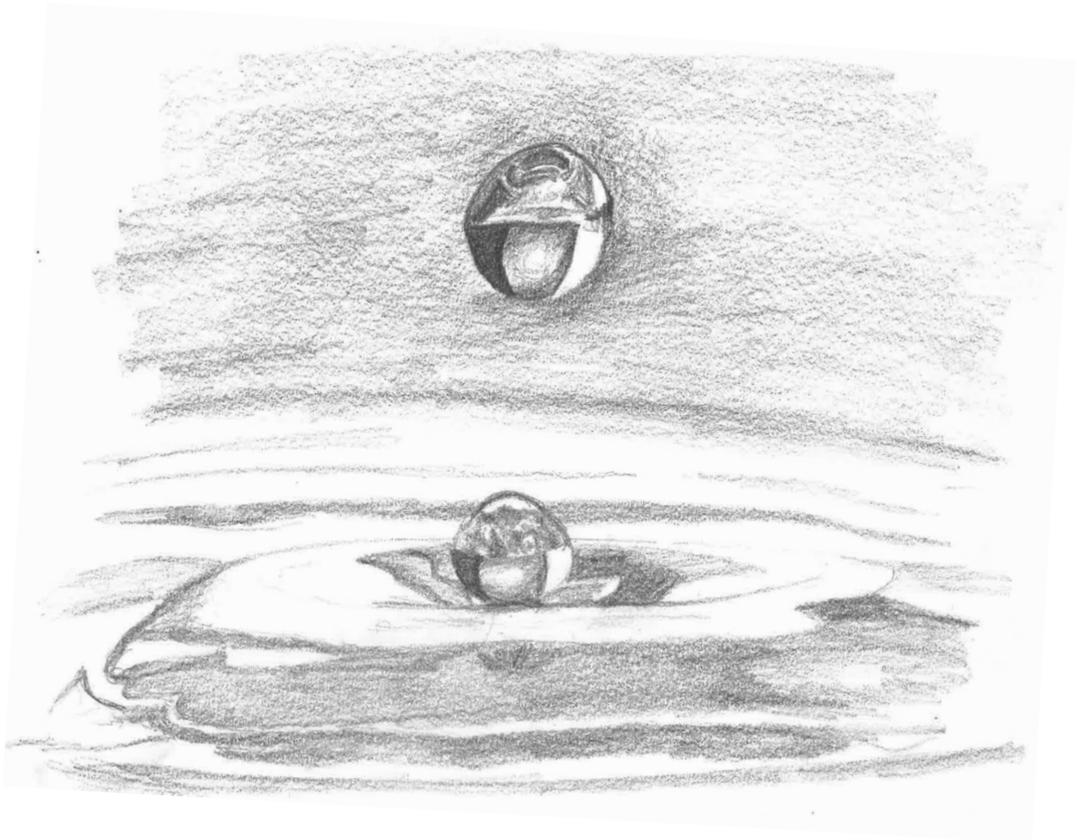


Illustration by C. Brazaitis

In this thesis, I have reported the results of a series of studies investigating the action of the synthetic steroid Org49209 and the endogenous neurosteroid, cerebrosterol, on the NMDA receptor. The studies presented in this thesis can be split into two categories: *in vitro* and *in vivo* experiments. We first set out to validate the mechanism of action of the steroids on the NMDA receptor *in vitro*, before attempting to assess the relevance of Org49209 *in vivo*. Chapter 2 used calcium imaging in primary cell cultures derived from mice to elucidate the action of these steroids directly on the receptor. Moving from the molecular to the functional and cognitive level, the following 2 chapters used behavioural tasks in mice with a genetic mutation modelling HD: the ‘novel place recognition’ test of spatial memory, and a water maze test of spatial learning and a form of spatial-reversal (new place) learning and cue-reversal learning. The limitation when using the HD mouse model was the restriction to non-appetitive (escape) positive reinforcement, rather than using an appetitive (food reward) positive reinforcement. Therefore, in the final experimental chapter, food-restricted rats were used in the attentional set-shifting task. This task includes reversal learning but also measures the formation and shifting of attentional set, which are impaired in HD patients and therefore, might reasonably be expected to be amenable to ‘treatment’ with the steroid compound. The range of techniques and model systems was intentionally selected to be broad to maximise the possibility that the functional effects of Org49209 would be apparent. As, ultimately, the study of Org49209 administration in rodent models of disease could give an insight both into treatment opportunities and disease aetiology, this approach is an important step in drug discovery and the study of neurological disorders.

Thus, the overall goal, and the potential impact, of this research is the identification of models and assays sensitive to deficits in an animal model in which putative treatments could be validated. In particular, the identification a potent and selective PAM of the NMDA receptor – a modulator that has no effect alone, but enhances the effect of the endogenous NMDA ligand – opens

the possibility for treatments targeting the cognitive deficits seen in many neurodegenerative diseases and psychiatric disorders, not just HD. The dysfunction of the NMDA receptor could very likely under-pin the cognitive deficits of all these disorders. As NMDA receptors also play a role in excitotoxic cell death when over-activated (Hardingham and Bading, 2010), the identification of a PAM is crucial, so as to limit activation outwith physiological activity. This may help avoid excitotoxicity associated with neurodegenerative disease progression (Parsons and Raymond, 2014). Although it is still possible that PAMs could lead to NMDA receptor over-activation, it is less likely to cause harm than NMDA receptor agonists.

These cognitive deficits are life-changing impairments; loss of cognitive abilities required for everyday functioning, such as time management and organisation of thoughts and activities, compromises independence for patients and reduces life-quality (Ghosh and Tabrizi, 2015). This puts strain on the wellbeing of the patients themselves, as well as society and relatives. Finding a treatment for the early-onset cognitive symptoms, as well as those accompanying later motor symptoms, would result in a large unburdening of society.

#### *In Vitro Studies*

Chapter 2 showed that both cerebrosterol and Org49209 potentiate NMDA-evoked responses in cultured neurons. This is consistent with data from HEK cells (Paul et al., 2013) and oocytes (Lambert laboratory unpublished observations, Chapter 1, Table 1.1) expressing NMDA receptors; both studies reported an increased NMDA-evoked current with application of both cerebrosterol and Org49209. While previous work showed enhancement in hippocampal cultures (Paul et al., 2013), I have demonstrated the same effect in cultured cerebellar granule neurons using calcium imaging techniques (see Chapter 2, Figures 2.7 and 2.9). As cerebrosterol is produced by neurons (Ramirez et al., 2008), and the present results have shown that it is also able to modulate neuronal NMDA receptors, it is possible that cerebrosterol has

effects outwith the role of a modulator of cholesterol homeostasis in the brain. Although the enhanced efficacy of Org49230 compared to the unsulphated Org49209 has been shown in oocytes (unpublished data from the Lambert laboratory, see Chapter 1, Table 1.1), I have shown here that this is also the case in neurons at the concentrations used (see Chapter 2, Figure 2.8). I also demonstrated that the endogenous cerebrosterol sulphate has a similar potentiating effect on the NMDA receptor as Org49230 (see Chapter 2, Figure 2.10). Unlike the known NMDA receptor PAM pregnenolone sulphate, which only enhances NMDA receptor activity when given in concentrations that far exceed endogenous levels (Schumacher et al., 2008), cerebrosterol is present in the brain in concentrations well above those which enhance NMDA receptor activation (Lutjohann et al., 1996). As SULT enzymes are able to sulphate cerebrosterol in the brain (Fuda et al., 2007), the enhanced effect of cerebrosterol sulphate compared to cerebrosterol raises the exciting possibility that the strength of NMDA receptor enhancement is modulated by the sulphation and desulphation of cerebrosterol. Linsenhardt et al. (2014) have already investigated the potential binding site of Org49209, suggesting a location within the cell membrane or extracellularly. Although beyond the scope and time available for this thesis, in future studies, it would be valuable to investigate the effect of sulphation on the properties of the binding of these neuroactive steroids. Patch-clamp recordings comparing the kinetics of cerebrosterol sulphate to cerebrosterol would give insight into the binding properties of the neurosteroid. Additionally, competition studies and removal of steroid with cyclodextrin application would show whether the sulphate group changes affinity for the binding site.

As indicated in the discussion above, the reason for optimism about NMDA PAMs is that they can operate within physiological parameters, and cause facilitation of relevant signalling, unlike agonists that could be neurotoxic at high doses. It has been suggested that NMDA excitotoxicity plays a major role in the neurodegeneration seen in HD (Parsons and Raymond, 2014).

Therefore, simply enhancing NMDA receptor function with the aim of treatment of cognitive symptoms is fraught with danger. The toxicity of cerebrosterol alone on neurons in culture has been demonstrated (Yamanaka et al., 2011); however, whether non-toxic levels of cerebrosterol in the presence of non-toxic levels of NMDA would become toxic has not been determined. It would also be important to establish whether the sulphated steroid would be more toxic than the non-sulphated steroid. This would provide important information about the safety of what could be long-term treatments, as well as an insight into the significance of sulphation and also the role of cerebrosterol in disease progression. As cerebrosterol and Org49209 have a relatively low efficacy, and as PAMs, only modulate NMDA receptor activity when the receptor is activated by an endogenous ligand, it is possible for modulation to occur without a high risk of excitotoxicity or overstimulation. It is also possible that cerebrosterol has beneficial effects in this finely balanced system (Noguchi et al., 2015, Boussicault et al., 2016). The benefits of endogenous cerebrosterol could be investigated using the 24-hydroxylase inhibitor, voriconazole, to stop cerebrosterol production in cultures; if cerebrosterol supports cell survival, inhibiting cerebrosterol would result in an increase in cell death that could be reversible with cerebrosterol application.

The absence of endogenous cerebrosterol has been investigated by others with the use of a transgenic CYP46A1 knockout mouse (Kotti et al., 2006). This mouse had reduced hippocampal LTP as well as spatial memory impairments (Kotti et al., 2006, Kotti et al., 2008). Additionally, data from the Lambert laboratory has shown a LTP deficit in the HDH<sup>Q111+/-</sup> mouse (Mitchell, 2012). As cerebrosterol has now been well validated as a PAM of the NMDA receptor, the next steps for *in vitro* work, besides investigating the above mentioned binding properties and toxicity, would be to further assess its role in synaptic plasticity. The above mentioned models could be used to test this by applying cerebrosterol to the hippocampal slices in attempt to recover the LTP deficits.

Org49209 has already been shown to be able to enhance low LTP due to lowered stimulation (Etherington (2012), unpublished observations, see Chapter 1, Figure 1.10). Alternatively, hippocampal slices could be incubated with voriconazole (a 24-hydroxylase inhibitor) before induction of LTP. If this resulted in reduced LTP due to a reduction in cerebrosterol, exogenous cerebrosterol application could be used to attempt to recover this effect. The effect of sulphation of the steroids on LTP could also be investigated, as a more efficacious PAM may be able to potentiate LTP further, or at lower concentrations.

Long-term depression (LTD), in contrast to LTP, is the weakening of synaptic strength and both are involved in the healthy functioning of synaptic plasticity (Collingridge et al., 2010). LTD is usually dependent on activation of either NMDA receptors (like LTP) or metabotropic glutamate receptors (Collingridge et al., 2010). There is evidence for NMDA receptor-mediated LTD being necessary for hippocampal-dependent learning and memory (Brigman et al., 2010) as well as for forebrain-dependent reversal learning and behavioural flexibility (Courtney et al., 1990, Ma et al., 2015). Additionally, there is some evidence that disrupted LTD may be involved in HD; both the R6/2 and R6/1 HD mouse model showed hippocampal CA1 LTD in response to low frequency stimulation, while WTs did not (Murphy et al., 2000, Milnerwood et al., 2006). Dallerac et al. (2016) provided evidence that this aberrant LTD induction is not due to dopamine dysregulation, leaving a possibility that abnormal NMDA receptor signalling may play a role. To further explore the phenotype of the HDH<sup>Q111+/-</sup> mouse, hippocampal and cortical LTD could be explored in addition to the already present LTP deficit. Application of cerebrosterol to slices of mice with aberrant LTD could give insight to the role of cerebrosterol in synaptic plasticity beyond LTP.

#### *In Vivo Studies*

As one of the main aims of the development of a NMDA receptor PAM is to treat the cognitive deficits associated with neurodegenerative diseases,

including HD, the effect of Org49209 needs to be investigated *in vivo*. To this end, a variety of behavioural tasks were selected for study. The particular tasks in murine and rat model systems were selected on the basis that there was prior evidence suggesting there were relevant cognitive impairments. First, the HDH<sup>Q111+/-</sup> mouse was used as it was previously shown to have spatial memory impairments (Mitchell, 2012, Chandler, 2014). The behavioural results reported by Chandler, to the extent to which the methodology matched, were replicated in the present study. However, the purpose of this study was to replicate and extend; this resulted in a rather dramatically different conclusion being drawn. Specifically, Chandler concluded that the novel-object recognition of the HDH<sup>Q111+/-</sup> mouse was intact, while novel-place recognition was impaired (Chapter 1, Figure 1.4). However, although this seemed to be the case when NPR was the first test of the day, when NPR followed NOR testing, they were significantly improved in the NPR task (see Chapter 3, Figure 3.4). When the HDH<sup>Q111+/-</sup> mice were then tested repeatedly, with each test, the DI score increased until the HDH<sup>Q111+/-</sup> mice eventually showed a preference for the novel place (see Chapter 3, Figure 3.9). This would indicate that the HDH<sup>Q111+/-</sup> mouse does not have a spatial memory impairment as previously thought, because they can demonstrate memory for object location. As the present design did not include a WT control group, it is not known whether the apparently lower DIs in NPR compared to NOR are a result of an inherently lower exploration motivation when a familiar object moves, compared to a novel object that appears. Org49209 was without effect in this study (see Chapter 3, Figure 3.6); however, in the absence of a behavioural impairment and without a WT control, the significance of this is not known.

A key property of NOR / NPR tasks is that they are spontaneous demonstrations of an intrinsic perception of novelty. Exploration is a spontaneous response to novelty and is no more 'reinforced' by novelty than shivering is by cold air. For this reason, it is possible that memory impairments

will only become apparent in tasks where there is learning by reinforcement. The water maze was selected as it is also an exploratory task, with inherent motivation (to escape) and yet the learning of the platform location is positively reinforced (i.e., the behaviour increases with reinforcement) by the removal of a negative stimulus (i.e., drowning threat). This study showed that HDH<sup>Q111+/-</sup> mice and WT mice performed equally well in both initial learning of the platform location and in spatial re-location learning, where the platform was moved to the opposite quadrant (see Chapter 4, Figures 4.6 and 4.7). Again, this indicated that the HDH<sup>Q111+/-</sup> mouse does not have a spatial memory impairment. This result was somewhat surprising as deficits have been reported in another HD model; the R6/2 line is impaired at platform acquisition and the probe trial compared to WT controls (Murphy et al., 2000, Lione et al., 1999).

Although the NPR task and water maze task were both used to probe spatial memory in the HDH<sup>Q111+/-</sup> mouse, they may be looking at different levels of hippocampal cellular function as Danielson et al. (2016) have recently argued. In their study, goal orientated activity was more associated with deep layer pyramidal neurons of the CA1, in contrast to spontaneous exploratory behaviour, which was associated with superficial pyramidal neurons. As NPR and water maze protocols are both exploratory, but only the water maze is goal-orientated, it could be speculated that HDH<sup>Q111+/-</sup> mice could be differently impaired due to different populations of neurons being responsible for different types of learning in the hippocampus. In addition, Whishaw and Tomie (1996) found that, in contrast to rats, mice performed less well in spatial tasks when tested in the water maze than when tested on dry land. This could be due to increased stress, which has been known to affect learning (Harrison et al., 2009). Therefore, it may be possible that different results would be found if the spatial learning task used food rewards and was land-based.

Although HD patients show clear executive function disturbances, including a reversal learning deficit (Lawrence et al., 1999), this had not been extensively studied in HD mouse models. The R6/2 line of mice do show spatial memory deficits, but we did not find any deficits in the HDH<sup>Q111+/-</sup> model. It is possible that using spontaneous exploratory behaviour to test spatial memory was not testing executive functions, and this was why the HDH<sup>Q111+/-</sup> mouse did not seem to be impaired and yet, an impairment might be revealed in an explicit learning task. The water maze was used again, because escape-reinforcement removes the need to control food or water intake, and the mice were presented with a simple visual discrimination task followed by a reversal of the stimulus-outcome contingency. As in the spatial learning task, HDH<sup>Q111+/-</sup> mice were no different to WT controls in either the rate or efficiency of initial discrimination or reversal learning (see Chapter 4, Figures 4.9 and 4.11). As mentioned above, the R6/2 mouse had a reversal learning impairment when required to discriminate between a bright and dimmed light in the water maze (Lione et al., 1999). It was, therefore, surprising that the HDH<sup>Q111+/-</sup> mouse did not seem to have a reversal learning impairment. The HDH<sup>Q111+/-</sup> mouse may have a milder phenotype than other models such as the R6/2 line, which shows more prominent cognitive dysfunctions (Li et al., 2005, Lione et al., 1999, Murphy et al., 2000). The disadvantages of the R6/2 line, however, are that motor impairments are present at a very early age and the total life span of the animals is short, resulting in complications of measuring cognitive function using behavioural tasks (Li et al., 2005).

The mice were surprisingly slow to learn the initial discrimination and even slower to acquire the reversal; performance remained poor even after 27 days of reversal learning when testing was terminated due to a lack of group difference. It is likely, given the above report, that land-based protocols, such as a T-maze or the 7-stage set-shifting task, would be learned more rapidly. It is also possible that, with a more rapidly learned discrimination, that subtle behavioural impairments might be apparent. Results from studies using mice

have suggested that they are less reliable in their performance of the set-shifting task and may require additional discriminations to form attentional set compared to rats (Garner et al., 2006). For this reason, rats were used to explore the potential value of the attentional set-shifting task for testing cognitive benefits of Org49209.

A cohort of rats was available that had inhibitory DREADDs in the mPFC resulting in impaired set-shifting. HD patients also have impairments in set-shifting, thought to arise from corticostriatal dysfunction (Lawrence et al., 1996). Therefore, it was of interest to ask whether the cognitive impairments arising from inactivation of the mPFC could be reversed with Org49209. Set-shifting is an executive function and, along with reversal learning, is a form of behavioural flexibility. Unfortunately, although the rats had been tested previously and showed normal acquisition of attentional set, when tested in the present study, the rats did not form set (see Chapter 5, Figure 5.4). Not only was the previously observed set-shifting deficit not replicated (in the absence of attentional set, there cannot be a set-shifting deficit), there was also no effect of Org49209 (which is not surprising, given that there was no deficit to be ameliorated). This means that conclusions could not be drawn from Org49209 administration, although no differences in the drug condition were observed during any stage of the standard 7-stage set-shifting task (see Chapter 5, Figure 5.4).

Although the original goal of the behavioural testing was to establish the effect of Org49209 administration on the behaviours of animal models with impaired cognition, this was not possible to explore due to all the assays tested here not showing the expected cognitive deficits. More aggressive models of HD such as the R6/2 model which has good published evidence of cognitive deficits (Lione et al., 1999, Murphy et al., 2000) could be more fruitful to the investigation of cerebrosterol. Indeed, Boussicault et al. (2016) have already suggested that cerebrosterol dysfunction contributes to HD pathology using the R6/2 line; however, they only investigated striatal

neurodegeneration and motor abilities. Investigating the effect of CYP46A1 restoration on the cognitive impairments of the CYP46A1 knockout, and the R6/2 model may give insight to the role of cerebrosterol in cognition and for the potential benefit of exogenous Org49209 administration. As sulphation of both Org49209 and cerebrosterol results in a PAM of the NMDA receptor of much higher efficacy, it would be interesting to investigate the effect of cerebrosterol sulphate on behaviour compared to cerebrosterol. Although cerebrosterol and Org49209 easily cross the BBB (Paul et al., 2013), assessing the function of steroid sulphation *in vivo* would require the direct administration to the brain as the sulphated steroid cannot cross the BBB. However, due to the presence of enzymes in the brain that can sulphate and desulphate cerebrosterol, it may not even be possible to use direct delivery to the brain as the administered cerebrosterol sulphate could be rapidly desulphated. Finally, rodent models other than those attempting to model HD, which also show cognitive impairments, could be investigated as NMDA receptor mediated cognitive impairments are implicated in a wide range of disorders (for instance, a developmental rat PCP model of schizophrenia as described by Broberg et al. (2013)).

#### *The Bigger Picture and Future Directions*

This thesis focused on the cognitive impairments associated with HD disease; however, other disorders where NMDA receptor dysfunction is observed may also respond well to PAM treatments. The ketamine model of schizophrenia (Frohlich and Van Horn, 2014), for example, is gaining support, especially with regard to understanding the cognitive symptoms of the disorder (see section 1.2.1). Currently treatments for schizophrenia focus on the positive psychotic symptoms, but not the negative or cognitive symptoms (Balu, 2016). The ketamine model suggests that specifically NMDA receptor hypofunction could be at the root of cognitive impairments; therefore, positive allosteric modulation is a prime candidate for a treatment avenue for schizophrenia. Specifically, NMDA receptor hypofunction of the cortical GABAergic

interneurons, which consequentially cause disinhibition of the cortical pyramidal cells, causes general increased cortical excitation (Jadi et al., 2016, Homayoun and Moghaddam, 2007). Therefore, using the ability of DREADDs to silence specific neuronal populations, specifically targeting hyperactive cortical pyramidal cells may alleviate the symptoms seen in models of schizophrenia. Alternatively, NMDA receptor PAMs such as Org49209 may be able to increase GABAergic interneuron firing to regulate the circuit in a similar fashion. As Org49209 has no direct effect on GABA receptors, this would be accomplished through the increase in NMDA receptor mediated activity in GABAergic interneurons, which cause inhibition of glutamatergic (excitatory) pyramidal cells.

Examples of other diseases which may benefit from NMDA receptor modulation are AD (see section 1.7.1) and anti-NMDA encephalitis. Regarding the former, as with other neurodegenerative diseases such as HD or Parkinson's disease, dysfunctional cholesterol metabolism may in fact imply a direct involvement of cerebrosterol in disease aetiology (Brown et al., 2004, Popp et al., 2012). Investigation of the role of cerebrosterol in behavioural models would give greater insight to cerebrosterol activity, beyond the use as a potential biomarker for neurodegenerative disease. In contrast, anti-NMDA encephalitis is an autoimmune disease which causes the internalisation of NMDA receptors resulting in psychotic, motor, and cognitive dysfunction (Miya et al., 2014). Consequently, it may be beneficial to enhance the activity of the remaining NMDA receptors in attempt to slow and alleviate some of the cognitive disorders present in this disease. The extent of possible applications for a potent and selective NMDA receptor PAM show how exciting and important the discovery of these neuroactive steroids is.

The use of Org49209 as a treatment for cognitive impairments presents some practical challenges. Although Org49209 has good brain permeability when administered i.p. (Paul et al., 2013), the oral availability remains unstudied. Due to its lipophilic structure, however, it is likely to have poor oral availability

(pers. comm. N. Hamilton). Therefore, in its current form, Org49209 is a good tool for investigation of the endogenous role of cerebrosterol, and for elucidating the potential for exogenous neuroactive steroid treatments, but structural modifications to improve drug viability remain to be investigated.

Returning to the endogenous role of cerebrosterol, if cerebrosterol is needed for normal cognitive function in the healthy brain, the ubiquitous use of statins may affect this interaction. Korinek et al. (2015) found that cholesterol depletion almost abolished the NMDA induced current responses in cultured rat CGNs, which was reversible with cholesterol repletion. The authors claim this is a direct effect of cholesterol on the open probability of the NMDA receptor. A part of the loss of response could also be due to decreased cerebrosterol levels due to cholesterol depletion. There has also been some evidence that statins impaired memory from case studies (Wagstaff et al., 2003), and randomised control trials (Santanello et al., 1997). However, more recent randomised control trials, systematic reviews, and meta-analyses have found no association between statin use and memory function (Richardson et al., 2013, Ott et al., 2015, Bitzur, 2016). Finally, Thelen et al. (2006) showed that treatment with high doses of statin either did not alter or slightly increased the cerebrosterol to cholesterol ratio in plasma. This ratio is a marker for brain homeostasis, indicating that although overall plasma cholesterol levels decrease, brain cholesterol remains largely unchanged. It seems that there is not sufficient evidence to believe statin treatment has a negative impact on cognition.

#### *Summary and Conclusions*

To summarise, the extent to which the aims set in the introduction (section 1.8) will be assessed. The calcium imaging experiments clearly demonstrated the NMDA receptor-enhancing effects of cerebrosterol and Org49209 in neurons. The effect of the sulphation of these neuroactive steroids was also shown, where an increased potentiation was seen at equivalent concentrations of unsulphated steroid. Thus, it can be concluded that

sulphation creates a more potent steroid; however, conclusions about efficacy could not be made as an asymptotic maximum response to the unsulphated steroids was not measured. With regard to recovering behavioural phenotypes of the HDHQ<sup>111+/-</sup> mouse with Org 49209, no conclusions could be made as the phenotype seen previously in the lab was not replicated. Additional behavioural tests were tried to find a deficit both in spatial learning and discrimination and reversal learning, but showed that the HDHQ<sup>111+/-</sup> mouse was not impaired compared to WT mice. Finally, the effect of Org49209 on the set-shifting impairment of mPFC inactivated rats could also not be assessed as the set-shifting deficit was also not replicated. Therefore, the basic mechanism of these steroids on the enhancing effect of NMDA receptor activity was supported with the calcium imaging experiments, and more experiments are required with models displaying more robust cognitive impairments to assess the application of these neuroactive steroids as cognitive enhancers.

To end on a hopeful note, methodological progress, such as the development of DREADDs, is providing useful tools that will allow a more thorough investigation toward the origins and treatments of cognitive impairments. Until successful treatments are found, however, lifestyle changes may be able to alleviate some of the pressure of neurodegenerative and neuropsychiatric disease. For example, dietary restriction may indeed be beneficial to Huntington's disease treatment, as shown by Duan et al. (2003) who demonstrated delayed disease onset and motor dysfunction, prolonged lifespan, reduced brain atrophy, reduced huntingtin aggregation, and reduced caspase activity in a mouse model for HD on dietary restriction. Additionally, environmental enrichment improved motor symptoms and peristriatal cerebral volume loss in the R6/2 mouse (Hockly et al., 2002), indicating that environmental changes may be able to slow disease progress and improve quality of life for HD patients.

The results from this thesis, together with the literature of cerebrosterol in disorders such as HD, and the wide range of disorders associated with NMDA

receptor dysfunction such as schizophrenia, it would be valuable to focus further research on the interaction of cerebrosterol with the NMDA receptor with the aim to ameliorate the cognitive deficits associated with these disorders. Not only would this contribute to a better understanding of a wide range of disorder aetiologies, but also provide the opportunity for drug development (such as Org49209) to aid in the treatment of the detrimental cognitive impairment associated with these disorders. The synthetic Org49209 may prove useful not only in the possible treatment of cognitive deficits, but can be used to elucidate the role of the NMDA receptor in cognitive mechanisms.

# Appendix 1

## Code for Discrimination Learning Analysis

```
#!/usr/bin/perl

use strict; # This line just makes sure that errors are more noticable
use Data::Dumper;

my $randomiser_file = shift; # This takes the first argument to the program as
randomiser_file
my $randtype = `file $randomiser_file`;

my $casmira_data_file = shift; # This takes the second argument to the
program as casmira_data_file
my $datatype = `file $casmira_data_file`;

my $/ = "\r";
if($randtype =~ /CRLF/) {
    open(RFILE,'<:unix',$randomiser_file) or die("Can't open
$randomiser_file $!\n"); # this opens the file for reading
}
else {
    open(RFILE,'<:unix:crlf',$randomiser_file) or die("Can't open
$randomiser_file $!\n"); # this opens the file for reading
}
# define a hash (dictionaries in python speak)

my %random_data;

while(my $line = <RFILE>) { # This line says we will loop through the file one
line at a time
    chomp $line; # remove the newline at the end of the line
    my @line = split /,/, $line; # This takes each line and splits it into an
array (list in python)

    my $day = $line[0];

    if($day =~ /\d+/) { # This says - if the first entry is a number
```

```

my $a = 10; # column K
my $b = 20; # column U
for(my $i = 1;$i <= 8;$i++) {

    my $a_part = $a+$i;
    my $b_part = $b+$i;

    $random_data{'A'}->{$day}->{$i} = $line[$a_part];

    $random_data{'B'}->{$day}->{$i} = $line[$b_part];

}
}
}

```

```
close(RFILE);
```

```

#die Dumper(%random_data);
# We need to set the initial state variables
my $day = 0;
my $cohort = 'A';
my $init;
my $trial;

```

```

my %distance_rows;
my %latency_rows;
my %quad_distance_rows;
my %quad_latency_rows;

```

```

my %platform_distance;
my %platform_latency;
my %quad_platform_distance;
my %quad_platform_latency;

```

```

my %previous_platform;
my %previous_first_choice;
my $count_of_lines = 0;

```

```

my %other_platform = (
    1 => 4,
    2 => 3,
    3 => 2,
    4 => 1,

```

```

);

my %quad2cue = (
    'NE' => 1,
    'SE' => 2,
    'SW' => 3,
    'NW' => 4,
);

print "Test Number,Cohort,Correct Platform,Wrong Platform,Platform
distance correct platform,Platform distance wrong platform,Same As Prev
Correct Platform,Same as Prev First Choice,Latency Success,Success,Quad
Latency Success,Quad Success\n";

$/ = "\r";
open(DFILE,'<:unix:crlf',$casmira_data_file) or die("Can't open
$casmira_data_file $!\n");
while(my $line = <DFILE>) {

    $count_of_lines++;
    chomp $line;
    my @line = split /,/, $line;

    my $test = $line[0];
    my $mouse = $line[1];
    my $stage = $line[2];

    if($test eq 'Test') {
        foreach my $i (0..100) {
            if($line[$i] =~
/Cue\s(\d)\s:\s*distance\sto\sfirst\sentry/) {
                $distance_rows{$i} = $i;
            }
            if($line[$i] =~ /Cue\s(\d)\s:\s*latency\sto\sfirst\sentry/)
{
                $latency_rows{$i} = $i;
            }
            if($line[$i] =~
/(\w\w)\sQuad\s:\s*latency\sto\sfirst\sentry/) {
                $quad_latency_rows{$quad2cue{$i}} = $i;
            }
            if($line[$i] =~
/(\w\w)\sQuad\s:\s*distance\sto\sfirst\sentry/) {
                $quad_distance_rows{$quad2cue{$i}} = $i;
            }
        }
    }
}

```

```

    }

    warn "Distance Columns:
$distance_rows{1},$distance_rows{2},$distance_rows{3},$distance_rows{4}\n
";

    warn "Latency Columns:
$latency_rows{1},$latency_rows{2},$latency_rows{3},$latency_rows{4}\n";
    warn "Quad Distance Columns:
$quad_distance_rows{1},$quad_distance_rows{2},$quad_distance_rows{3},$q
quad_distance_rows{4}\n";
    warn "Quad Latency Columns:
$quad_latency_rows{1},$quad_latency_rows{2},$quad_latency_rows{3},$qua
d_latency_rows{4}\n";

    next;
}

$platform_distance{1} = $line[$distance_rows{1}];
$platform_distance{2} = $line[$distance_rows{2}];
$platform_distance{3} = $line[$distance_rows{3}];
$platform_distance{4} = $line[$distance_rows{4}];

$platform_latency{1} = $line[$latency_rows{1}];
$platform_latency{2} = $line[$latency_rows{2}];
$platform_latency{3} = $line[$latency_rows{3}];
$platform_latency{4} = $line[$latency_rows{4}];

$quad_platform_distance{1} = $line[$quad_distance_rows{1}];
$quad_platform_distance{2} = $line[$quad_distance_rows{2}];
$quad_platform_distance{3} = $line[$quad_distance_rows{3}];
$quad_platform_distance{4} = $line[$quad_distance_rows{4}];

$quad_platform_latency{1} = $line[$quad_latency_rows{1}];
$quad_platform_latency{2} = $line[$quad_latency_rows{2}];
$quad_platform_latency{3} = $line[$quad_latency_rows{3}];
$quad_platform_latency{4} = $line[$quad_latency_rows{4}];

if( ($trial == 8) and ($line[3] == 1) ) {
    if($cohort eq 'A') { $cohort = 'B'; } else { $cohort = 'A'; }
}
$trial = $line[3];

if($stage =~ /\(d+\)/) {
    $day = $1;
}

```

```

my $correct_platform = $random_data{$cohort}->{$day}->{$trial};
my $wrong_platform = $other_platform{$correct_platform};

my $success =
did_it_win(\%platform_distance,$correct_platform,$wrong_platform);
my $latency_success =
did_it_win(\%platform_latency,$correct_platform,$wrong_platform);
my $quad_success =
did_it_win(\%quad_platform_distance,$correct_platform,$wrong_platform);
my $quad_latency_success =
did_it_win(\%quad_platform_latency,$correct_platform,$wrong_platform);

my $which_platform_did_mouse_go_to_first;

if($success eq '1') {
    $which_platform_did_mouse_go_to_first = $correct_platform;
}
else {
    $which_platform_did_mouse_go_to_first = $wrong_platform;
}

my $was_prev_platform = '0';
my $was_prev_first_choice = '0';
if($which_platform_did_mouse_go_to_first eq
$previous_platform{$mouse}) {
    $was_prev_platform = '1';
}
if($which_platform_did_mouse_go_to_first eq
$previous_first_choice{$mouse}) {
    $was_prev_first_choice = '1';
}

print
"$test,$cohort,$correct_platform,$wrong_platform,$platform_distance{$corr
ect_platform},$platform_distance{$wrong_platform},$was_prev_platform,$w
as_prev_first_choice,$latency_success,$success,$quad_latency_success,$qua
d_success\n";

$previous_platform{$mouse} = $correct_platform;
$previous_first_choice{$mouse} =
$which_platform_did_mouse_go_to_first;
}

```

```
warn "Analysed lines: $count_of_lines (including header)\n";
```

```
sub did_it_win {  
    my $hashref = shift;  
    my $correct_platform = shift;  
    my $wrong_platform = shift;  
  
    my $success;  
  
    if($hashref->{$correct_platform} > 0) {  
        if($hashref->{$wrong_platform} > 0) {  
            if($hashref->{$correct_platform} < $hashref->{$wrong_platform}) {  
                $success = '1';  
            }  
            else {  
                $success = '0';  
            }  
        }  
        else {  
            $success = '1';  
        }  
    }  
  
    return $success;  
  
}
```

# Appendix 2

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