WHAT NEUROBIOLOGICAL MECHANISMS IN HIPPOCAMPUS SUPPORT RAPID SPATIAL LEARNING WITH FAMILIAR INFORMATION IN THE MORRIS WATER TASK?

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DEDICATION

To Ivonne Diaz, Juan Jose Rodriguez and Naty.

ABSTRACT

Long-term potentiation (LTP) is proposed as a molecular mechanism for learning and memory. N-methyl-D-aspartate receptors (NMDARs) are implicated during LTP and new learning. However, if rats are pre-trained prior to new learning NMDARs are not needed. Rather the activation of voltage gated calcium channels (VGCCs) and associated calcium influx might be responsible for LTP independent of NMDARs, and new learning with familiar information. The hypothesis was that to impair new learning with familiar information both NMDARs and VGCCs would need to be blocked. Rats were trained in a version of the Morris water task, consisting of a pre-training, mass-training, and a probe test. Before mass-training NMDARs and VGCCs were blocked in the hippocampus using AP5 and Verapamil, which are receptor antagonists. All rats were able to learn regardless of their experimental condition, which means that activation of NMDARs and VGCCs is not crucial for new spatial learning with familiar information.

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LIST OF ABBREVIATIONS

HPC	Hippocampus
CA1	Cornu Ammonis-1
MMS	Multiple Memory Systems
LTP	Long-term potentiation
LTD	Long-term depression
NMDARs	N-methyl-D-aspartate receptors
AP5	2-amino-5-phosphonopentanoate
CPP	3-(2-Carboxypiperazin-4-yl) propyl-1-phosphoric acid
BAPTA	1,2-bis (o-amino phenoxy) ethane – N, N, N', N'- tetra acetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) - N, N, N', N' – tetra acetic acid
VGCCs	Voltage-gated calcium channels
mGluRs	Metabotropic glutamate receptors
PKA	Protein kinase A
CaMKII	Calcium calmodulin – dependent kinase 2
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ERK	Extracellular signal–regulated kinase
MAPK	Mitogen activated protein kinases
CREB	cAMP-response element binding protein
MWT	Morris water task

Chapter 1

General Introduction

Learning is the act of acquiring information, which leads to alterations in an individual's knowledge and behaviour. Memory is the ability to recall previously learned information. Learning and memory are processes that engage specific areas of the brain and depend on unique patterns of neural activity resulting in biological changes in brain cells that outlast the learning experience. These processes are based on two theories. Firstly, learning and memory have a physical component in the brain, which allows neurons to effectively communicate (Hebb, 1949). Synaptic plasticity is a general term used to describe the strengthening and weakening of synapses. It is thought that synaptic plasticity is the molecular mechanism by which learning and memory occurs. Secondly, learning and memory is supported by different parts of the brain that are functionally dissociable (Kandel et al., 2014). Multiple Parallel Memory Systems is a theory, which posits that information is processed in parallel throughout multiple brain regions depending on the type of information and the processing style of the learning system (Gruber & McDonald, 2012; White & McDonald, 2002). Empirical research indicates that the hippocampus (HPC) is the central structure in a learning and memory system responsible for spatial representations and the formation of a cognitive map (O'Keefe & Nadel, 1978). The aforementioned functions and theories involved in learning and memory range from a molecular to a systems level of analysis and this is key to furthering our understanding of complex higher cognitive functions like learning and memory.

The experiments in this thesis are focused on the hippocampal learning and memory system and the effects of differences in pre-training trials, as well as the blockade of calcium ions

control systems in spatial learning and memory. The general introduction discusses current understanding of learning and memory at an organism, organ, system, cellular, and molecular level of analysis. These subjects provide a foundation to investigate the role of various calcium ion channel control systems in spatial learning and memory.

Organism Level - General localization of learning and memory

A good starting point is to ask, how is learning and memory organized in the brain? The difficulty in answering this question arises from the complexity of the human brain, harbouring billions of neurons in no more than three pounds of grey matter (Dekaban & Sadowsky, 1978). A way to simplify and begin to tackle this question is to answer the question at different levels of analysis. Beginning with the organism, then the organ, the system, the cellular and ending at the molecular level. Asking the question "How is learning and memory organized in the brain" from each level provides the best framework to finding answers.

At the organism level, learning and memory originates in the brain. Our understanding that higher order cognitive functions such as, learning and memory originate in the brain can be attributed to human case studies. Beginning in the mid 19th century with the account of Phineas Gage, whom displayed a significant change in behaviour after a railroad accident punctured his left frontal lobe. Another important case study was,"J.P" which lead to the understanding that severe bi-frontal damage caused serious social learning problems. Finally, the most famous patient, "H.M" who will be further discussed below, revealed the importance of the medial temporal lobes for memory (Benjamin et al., 2018). Careful research about these individuals and their unfortunate life circumstances has provided invaluable information about learning and memory in the brain.

Organ Level – Multiple Memory Systems

In the 20th century contributions from human amnesic patients, animal research and the development of imaging techniques have made it possible to study learning and memory at the organ level. From these studies the theory of Multiple Memory Systems (MMS) emerged, which is the idea that different kinds of memory (Hilgard & Bower, 1966; Moscovitch, 1992; Nadel, 1992) are mediated by different parts of the brain (Mishkin et al., 1984; O'Keefe & Nadel, 1978; Scoville & Milner, 1957; L. Squire et al., 1993; White & McDonald, 2002). The MMS theory continues to be upheld by evidence in the field and areas like the HPC, dorsal striatum, amygdala (White et al., 2013), cerebellum (Krupa et al., 1993), and cerebral cortex (McNaughton et al., 1989; Young et al., 1997), are thought to be central structures of complex networks supporting different types of learning and memory.

The Hippocampus

Fundamental research in support of the MMS theory came from the study of patient H. M. H. M had an intractable seizure disorder that led to the drastic decision of having a portion of his medial temporal lobe removed including a large portion of his HPC (Scoville & Milner, 1957). After the operation H. M showed deficits in episodic and biographical memory, however other types of memory like procedural memory remained intact (L. R. Squire, 2009). Episodic memory refers to the ability to recall information about personal experiences that are grounded in a time and place, whereas procedural memory refers to actions and skills (Mitchell et al., 1990). After over 60 years of research, it was concluded that the HPC and surrounding structures in the medial temporal lobe cause severe anterograde amnesia and temporally graded retrograde amnesia if disrupted (L. R. Squire, 2009). Anterograde amnesia is the clinical term used to describe the inability to form new memories, whereas retrograde amnesia refers to the loss of information that

was obtained before the symptoms began (Smith et al., 2013). This research provided strong evidence for the localization of different kinds of memory in different parts of the brain. More importantly the HPC became a locus for an intensive research effort to study the neural basis of learning and memory.

A debate ensued as to exactly what role does the HPC play in learning and memory. Some researchers believe its role is primarily learning and memory of spatial information (Ferbinteanu et al., 2003; Nadel, 1991; O'Keefe, 1991), others believe it is involved in memory processes that are more general and nonspecific like, working memory (Olton et al., 1979), declarative**,** (Cohen & Eichenbaum, 1991), configural learning (R. J. Sutherland et al., 1989), and episodic memory (Tulving, 1972; Tulving & Markowitsch, 1998) .Due to the internal circuit of the HPC and the important discovery of place cells, it is now accepted that a major role of the HPC is encoding information about space and context, creating a cognitive map of an environment (O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978). Evidence from animal lesions and pharmacological studies further supports this idea, concluding that the HPC is preferentially involved in spatial learning and memory and rapid acquisition of contextual information (Ferbinteanu et al., 2003; Jarrard, 1995; McDonald & White, 1994). Furthermore, following the structure equals function paradigm the HPC has extensive connections with cortical and limbic structures, which allow the HPC to form associative representations of stimuli in the environment to influence behaviour (Gruber & McDonald, 2012).

Cellular Level - LTP

The aforementioned studies paved the way to further our understanding of learning and memory processes in the brain. How might neurons communicate with each other to store and encode information, became a prominent question in the field. A huge advancement was made in

1973, with the important discovery of long-term potentiation (LTP) in the HPC (Bliss & Lømo, 1973). LTP is an increase in response due to tetanic stimulation. Specifically, LTP is the amplification of the excitatory post synaptic potential because of simultaneous high frequency inputs (Bliss & Lømo, 1973). Initially LTP was only thought to be induced artificially *in vitro*. However, experiments in intact animals allowed for the assessment of the longevity of LTP in the HPC. Using chronically implanted recording and stimulating electrodes LTP has been observed to last up to a year in rats (Abraham et al., 2002). LTP can also be induced following behavioral experiences like spatial learning and exposure to new contexts (Barnes, 1979, 1988). This discovery was crucial because LTP was proposed as a cellular mechanism by which long term learning and memory could be occurring in the brain.

LTP provided experimental evidence that satisfied criteria proposed by Donald Hebb for a synaptic memory mechanism in his book "The Organization of Behaviour" (Hebb, 1949). Hebb postulated that if a cell is close enough to excite another cell, some sort of growth process takes place and as a result the efficiency of one cell increases the firing in the other cell. Currently, in the field the growth process Hebb was most likely referring to is known as synaptic plasticity and is the strengthening (LTP) or weakening (Long-term depression (LTD)) of synaptic connections between neurons. LTP/LTD are thought to be the cellular process by which synaptic plasticity occurs (Bear & Malenka, 1994). Indeed, a large body of evidence *in vitro* and *in vivo* points to a strong correlation between LTP/LTD and synaptic plasticity (Bear & Malenka, 1994; E. P. Huang, 1998; Larkman & Jack, 1995) .

Molecular Level - Underpinnings of LTP

Once determined that LTP and consequent synaptic plasticity is a cellular mechanism by which long term memory storage could occur, next would be to ask how is learning and memory

organized in the brain at the molecular level? *In vitro* preparation of hippocampal slices using pharmacological manipulations, along with genetic knockout mice studies have uncovered a lot of the molecular underpinnings of LTP. An important component of LTP is that it requires simultaneous activity of pre and post synaptic neurons. This is achieved via a coincidence detection mechanism involving N-methyl-D-aspartate- receptors (NMDARs), which are a type of G-protein-coupled ionotropic glutamate receptors found in the post-synaptic neuron (Newcomer et al., 2000). Pre-synaptic neuronal activity causes glutamate release into the synapse which then binds to NMDARs. Additionally, post-synaptic depolarization needs to occur for the ejection of a magnesium plug. Then the receptors open and allow influx of calcium ions (Nowak et al., 1984). Evidence for the role of NMDARs in LTP, comes from experiments where selective antagonists, 2-amino-5-phosphonopentanoate (AP5) and 3-(2-Carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP) were administered and LTP did not occur (Collingridge et al., 1983). Calcium influx through NMDARs is crucial for LTP, and this was determined because intracellular application of calcium chelators, like 1, 2-bis (o-amino phenoxy) ethane-N, N, N', N'-tetra acetic acid (BAPTA) and ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetra acetic acid (EGTA) prevented its induction (Lynch et al., 1983; Mulkey & Malenka, 1992).

LTP independent of NMDARs has also been reported via voltage-gated calcium channels (VGCCs). Previous experiments used higher-frequency activity than needed to induce NMDARs-LTP, the LTP produced was only partially blocked by NMDARs antagonists, the remaining LTP was blocked by VGCC blockers (Grover, 1998; Grover & Teyler, 1990; Weisskopf et al., 1999). VGCCa are abundantly found in the post-synaptic neuron and are opened by membrane depolarization to allow the influx of calcium ions. Another type of LTP independent of NMDARs occurs via the activation of metabotropic glutamate receptors (mGluRs) (Huber et al.,

2000; Kemp & Bashir, 1999; H. Wang et al., 2016). When activated mGluRs can initiate a signalling cascade which leads to the release of intracellular calcium (Bashir et al., 1993; Bolshakov & Siegelbaum, 1994; O'Malley et al., 2003).

NMDARs, VGCCs, and mGluRs all have one thing in common and that is that their activation increases the calcium concentration in the post-synaptic neuron. Indeed, it has been shown that the uncaging of calcium itself can induce LTP and/or LTD (Malenka et al., 1988). Therefore, a crucial step in the mechanism of LTP is the intracellular increase of calcium ions. Beyond the induction of LTP, calcium detection mechanisms have been determined to play a crucial role in the expression of LTP. Kinases like, cyclic AMP-dependent protein kinase A (PKA) and calcium calmodulin-dependent kinase 2 (CaMKII), detect elevations in the calcium concentration. The kinases then phosphorylate proteins like α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), which are involved in the expression of LTP (Lee et al., 2003). Phosphorylation of AMPARs at specific protein sites can increase channel conductance. Phosphatases like protein phosphatase 1 and calcineurin also detect calcium but at lower concentrations and can then dephosphorylate AMPAR and reduce the receptors efficacy (Lee et al., 2000). In summary, these calcium detection mechanisms have been known to alter AMPARs function in ways that enhance or decrease synaptic efficacy, as well as playing a role in spatial memory (Lee et al., 2003).

For proper LTP expression there needs to be efficient communication from the synapse to the nucleus to initiate gene transcription. This occurs via signaling cascades involving the activation of cAMP, PKA, mitogen activated protein kinases (MAPK) and transcription factor cAMP-responsive element binding protein (CREB) (Frey et al., 1993; Y. Y. Huang et al., 2000). Once CREB has been activated in the nucleus, it sends out a signal for gene transcription and *de novo* protein synthesis. Activity-dependent gene expression via cAMP-CREB pathways is

important for learning and memory (Dash et al., 1990; reviewed in Kandel, 2001). Further downstream, the specific molecular mechanisms are complex and still widely unknown. However, it has been proposed that newly synthesized proteins can cause structural changes in the size and shape of synapses, leading to alterations in synaptic efficacy (K. M. Harris et al., 2003). From LTP induction via calcium influx into the post-synaptic neuron, to LTP expression via calcium mediated signaling pathways, it is evident that calcium plays a crucial role in synaptic plasticity.

MWT – From molecules to behaviour

At the time when many researchers in the field where devoted to the study of LTP and the molecular mechanisms of learning and memory, others were attempting to link LTP results to behavioral experiments in rodents. This is a daunting, but crucial task since learning and memory are ultimately cognitive functions in the brain and should also be studied at the organism level.

In the early 1980s Richard G. M. Morris developed a behaviour paradigm known as the Morris Water-Maze task (MWT) (R. Morris, 1984). The MWT quickly became the gold standard behavioural task to study spatial learning and memory because, hippocampal lesions produced reliable performance impairments (R. G. M. Morris et al., 1982; R. J. Sutherland et al., 1983; R. Sutherland & Rudy, 1988). In a classic MWT experiment, rodents are placed in a pool of opaque water, and must swim to a hidden escape platform using external/extra-maze cues. Animals are given several trials per day for few days to learn the platform location. Then the platform is removed from the pool, and they are allowed to freely swim, this is known as a probe trial. Control animals quickly learn the platform location and spend more time in that location during the probe trial. HPC lesion animals show impaired learning and do not prefer the platform

location during the probe trial (R. Morris, 1984; Othman & Hassan, 2015). Further supporting the role of the HPC in spatial learning.

The linking of LTP to behavioural learning and memory became possible via the study of NMDARs previously mentioned. Morris and others guided by the evidence that NMDARs antagonists blocked LTP *in vitro*, infused AP5 into the ventricles of rat's HPC and observed a blocking of LTP *in vivo* (R. G. M. Morris et al., 1986). When these rats where then trained in the MWT they failed to accurately find the escape platform, performing like rats with HPC lesions. These pharmacological experiments were foundational in developing a more comprehensive view from the molecular to the organism level of how learning and memory is organized in the brain.

NMDARs

Further NMDARs inactivation research showed that it was linked to impairments in other learning tasks, like operant condition (Tonkiss et al., 1988) and 8 arm radial maze (Ward et al., 1990). Experimental results also indicated that if rats acquire spatial information and are given NMDARs antagonists 24 hours later before being tested for retention there is no learning impairment (Shapiro & Caramanos, 1990). Therefore, NMDARs blockade was not found to impair the retrieval of already formed memories. Based of the aforementioned research the view that NMDARs were crucial for inducing synaptic plasticity, and therefore are needed for the formation of memories became widely accepted (Collingridge & Bliss, 1987).

On the other hand, contradictory results began to build up which suggested that the role of NMDARs in learning and memory was not as simple as previously stated. All the forementioned studies blocking NMDARs were done by administering the blockade intraperitoneally or intraventricular (R. G. M. Morris, 1989; R. G. M. Morris et al., 1986; Ward et al., 1990) . However, this method of injection may have multiple effects on behaviour because NMDARs are

blocked throughout the entire central nervous system (Monaghan & Cotman, 1985). Some brain regions which contain NMDARs provide support for learning and memory functions but are not specifically involved in these operations (sensory processing, motor control, motivation, attention, etc.). As such, blocking NMDARs in different brain regions could cause impairments outside of learning and memory. Indeed, research has shown that NMDARs blockade was linked to effects in motor coordination (Cain et al., 1996), sensory (Salt, 1986) and anxiolytic response (Stephens et al., 1986). Researchers opposing the popular view of the role of NMDARs at the time, accurately stated that the effects seen by NMDARs blockade were not due to memory impairment but were a result of motor impairments. In the MWT motor impairments like poor swimming, falling of the platform, and reduced swim speed, could hinder a rat's ability to learn (Cain et al., 1996). Admittedly, studies looking at the role of NMDARs in spatial learning, did report motor problems in their rats after drug blockade (R. G. M. Morris et al., 2013; Robinson et al., 1989; Shapiro & Caramanos, 1990). These results brought into question what the true role of NMDARs might be in learning and memory.

To clearly understand the role of NMDARs in the HPC a few researchers began to use variations of the MWT classic design. This shift was mainly based on results that pre-training rats on the MWT eliminated the learning deficits associated with NMDARs antagonists (Bannerman et al., 1995; Hoh et al., 1999; Saucier & Cain, 1995). The idea behind this, is that if a rat is pre-trained on the protocol of the task before drug infusion and standard training, the potential effects of NMDARs blockade due to motor impairments are diminished.

Interesting and important results for the current thesis were observed by an electrophysiological experiment looking at place fields in the HPC. They found that NMDARs blockade did not prevent the formation of new place field representations in a new environment, but the drug did prevent long term stability of the representation 24 hours later (Kentros et al.,

1998). As a results, a new idea emerged that NMDARs may not be needed for acquisition of learning but instead for consolidation of information. Consolidation is a process by which short term memory becomes long term memory over time (Urcelay & Miller, 2007). Support for the idea that new spatial information is possible without NMDARs was found in a conditioned fear experiment. Santini et al., (2001) peripherally injected rats with CPP (NMDARs antagonist) and found that the rats could acquire extinction memories, however they could not remember the extinction 24 hours later. The same results were found when the rats were injected immediately after acquiring extinction memory. This pointed to the idea that NMDARs are not needed to acquire information but were needed for consolidation.

Further support for the consolidation role of NMDARs came from McDonald et al., 2005. The researchers pre-trained rats to find an escape platform daily for four days. The next day they blocked NMDARs via intrahippocampal injections of CPP and then rapid acquisition training (mass-training) was done to a new escape location. All the rats successfully learned the mass-training location while under NMDARs blockade, demonstrating proper acquisition. However, when tested 24 hours later the rats did not recall the mass-training platform location. Rather the rats displayed a preference for the pre-trained platform location, acting as if the mass-trained location training did not occur. In summary, their ability to learn was intact while their ability to consolidate was impaired.

Given that there was still not a clear consensus on if NMDARs were crucial for acquisition and/or consolidation. Follow up experiments from the ones done in 2005, tested the role of NMDARs in familiar vs. novel contexts. The idea was that NMDARs were not needed for new learning in familiar contexts because the HPC had already made a representation of the environment. However, in new learning with novel context the HPC had not yet made a representation and therefore needed the activation of NMDARs. Bye & McDonald, 2019, pre-

trained rats to a platform location in room A andthen administered intrahippocampal CPP injections before mass-training to a new location, in either room A (the familiar room) or in room B (the novel context). In this set of experiments, they found: 1) blockade of HPC NMDARs did not impair the rapid acquisition of new spatial learning in a familiar context; 2) NMDARs blockade severely impaired rapid new learning in a novel context; 3) NMDARs activation seems critical for the long-term expression of new learning in both contexts. These results differ from the popular view that HPC NMDARs are always necessary for encoding spatial information (R. G. M. Morris, 2013). Perhaps more importantly these results follow the trend of previously mentioned studies that new learning can occur independently of NMDARs (Bannerman et al., 1995; Hoh et al., 1999; McDonald et al., 2005b; Saucier & Cain, 1995; Taylor et al., 2014), specifically when the animals have been familiarized with the context. The Bye & McDonald (2019) study will be the foundation for the research in this thesis.

VGCCs

Currently NMDARs are still postulated by many to be crucial for new learning in the HPC. However, based on the literature here for mentioned, there is data to suggest that there are at least two exceptions to this, one is when distributed pre-training prior to new learning occurs, and the other is when the new learning is done in a familiar environment. Studies have shown that NMDARs independent forms of LTP can be induced in the HPC (Bramham et al., 1991; Grover, 1998; E. W. Harris & Cotman, 1986). This means that other non-NMDA mediated synaptic mechanism could be responsible for spatial learning and memory in tasks like MWT. As a reminder, when NMDAR are activated, they allow calcium ions to flow from the synapse into the post-synaptic neuron, which increases the intracellular concentration of calcium, causing depolarization leading to LTP and synaptic plasticity.

Interesting work has shown that activation of VGCCs and associated calcium influx can induce LTP independent of NMDARs (Moosmang et al., 2005). VGCCs are present all throughout the brain with the L-type being the most abundant in the HPC. These channels are located on the postsynaptic membrane and can provide calcium into the intracellular space of neurons (Striessnig et al., 2015). Induction of LTP with 200 Hz of stimulation in CA1 pyramidal neurons was not impaired with NMDARs antagonist, but was impaired when using nipefidine, a VGCC antagonist (Grover & Teyler, 1990). Similar effects were seen in genetic knockout mice missing the L-type VGCC receptor. The mice can exhibit normal NMDARs dependent LTP in CA1 neurons, however when NMDARs antagonist is given LTP does not occur (Moosmang et al., 2005). Intracellular plasticity signalling cascades involving extracellular signal-regulated kinase; mitogen-activated protein kinase (ERK/MAPK) and CREB mentioned previously have been shown to be affected by knockout mice with genetic manipulations of VGCCs (Dolmetsch et al., 2001). These mice express impaired performance on many HPC dependent spatial behavioural tasks. As a results VGCCs may play a role in NMDARs independent LTP and could support spatial learning and synaptic plasticity in the HPC.

The Calcium theory of new learning

To explain and expand on the research to date, a theory has been developed by our lab, referred to as The Calcium theory of New Learning. This states that the amount of intracellular calcium influx needed to support new learning depends on the conditions of the new learning. Specifically, if pre-training has occurred and if the context of the new learning is familiar or novel. NMDARs in the HPC rapidly encode a complex representation of the context. Once this representation is formed new learning can occur within that representational framework needing less intracellular calcium. For example, in MWT, learning a new escape location in the same

room where pre-training occurred. This new learning in a familiar context requires less calcium for the relevant plasticity and can occur through VGCCs. Explaining the NMDARs independent new learning seen in these studies (Bye & McDonald, 2019; McDonald et al., 2005). On the other hand, in the new room, new learning, an entirely new representation must be rapidly formed and therefore requires all available calcium via NMDARs and VGCCs. This would explain the results that NMDARs are important when new learning occurs in a novel context (Bye & McDonald, 2019; McDonald et al., 2005).

Purpose of thesis

The overarching goal of this thesis is to provide a greater understanding of how learning and memory is organized in the brain. This is attempted by answering a series of more specific questions which focus on how different glutamate-based plasticity mechanisms contribute to spatial learning and memory in the HPC. Using neuropharmacology and behavioural animal learning procedures to test the role of NMDARs and VGCCs in the HPC. Based on previous research the hypothesis is that disruption of new spatial learning in a familiar context is dependent on a blockade of both NMDARs and VGCCs.

Experiment 1: Does the number of pre-training trials effect the 24-hour probe in a rapid acquisition variant of the Morris Water Task?

There were three pilot studies done prior to the main experiments described in this thesis. The purpose of these pilots was to find an appropriate dose for intrahippocampal VGCCs blockade. Behavioural testing was done following previous experiments using a variant of the MWT (Bye & McDonald, 2019; McDonald et al., 2005). This protocol involves four days of pre-training to a platform location. The next day a receptor antagonist is injected followed by mass-training to a new platform location. The following day a probe trial is given. During the mass-

training if the rats decrease their latency and path length its indicative of proper learning. The elegance of this task design is showcased in the probe trial. If the rats can learn the new platform location and can recall that information due to proper consolidation, then they should show a preference for the new location quadrant during the probe trial. If the rats can learn the new location but cannot consolidate the information, then they should show a preference for the pre-trained location during the probe. Unexpectedly, results from the pilot studies showed that the control rats did not prefer the mass-training quadrant. They also did not show a preference for the pre-trained quadrant. Having a proper control group on the probe is important to compare the drug groups and clearly answer the hypothesis of this thesis. These results were puzzling and required further investigation.

An idea to explain the lack of preference for the mass-trained location during the probe of the control rats, was the effect of pre-training trials. The rats were given twice as many trials to the pre-training location (32 trials over four days) than to the mass-training location (16 trials in one day). This means that because of the greater number of trials, rats could have a stronger representation for the pre-trained location compared to the mass-trained location. In Experiment 1 the number of pre-training and mass-training trials was equated to better understand the 24-hour probe preference. The hypothesis was that equating the number of pre-training and mass-training trials would cause the rats to prefer the mass-training location during the probe because it was the last escape location they were trained to.

Experiment 2: Does multi-targeted hippocampal calcium receptor blockade effect spatial learning and memory in a rapid acquisition variant of the Morris water task?

Studies looking at spatial learning and memory using pre-training and mass-training (Hoh et al., 1999; McDonald et al., 2005), have argued that most of the important learning for the task takes place during the pre-training phase and later training may not require the activation of

plasticity mechanism in the HPC. Following this idea, in the MWT when the platform is moved to a different location during mass-training, the rat can learn the new platform location without HPC plasticity mediated by NMDARs. This may be because they use previously acquired HPC representations or cortical plasticity (Inglis et al., 2013). However, maybe HPC plasticity does still exists but is mediated through other calcium sources like VGCCs. To examine this possibility, in Experiment 2 rats will be tested in a pre-training MWT protocol while blocking NMDARs & VGCCs independently and together in the HPC. The hypothesis is that NMDARs and VGCCs blockade alone will not impair new location learning in a familiar context. However, blocking both NMDARs & VGCCs will severely impair new learning in a familiar context.

Chapter 2

Does the number of pre-training trials effect the 24-hour probe in a rapid acquisition variant of the Morris Water Task?

Introduction

Why the MWT?

The Morris Water Task (MWT) is a behavioural paradigm that has been around for over four decades in the study of learning and memory (R. Morris, 1984; R. G. M. Morris et al., 1982). It is arguably the "Gold standard" task used to study spatial learning and hippocampal-dependent memory. The reason for the popular use of this task is that it has many advantages over other paradigms. For example, it is extremely effective at evaluating hippocampal-dependent learning and memory (Vorhees & Williams, 2014). This is in part because a key feature of the spatial version of the MWT is the use of pseudo-random start points, which make it difficult for subjects to navigate based on a simple turning response or guidance strategy like approaching a distal cue, therefore the subjects must rely on a hippocampal strategy (McDonald et al., 2004). The MWT is a water maze, and the water is an equal-opportunity motivator that balances motivation to escape over a wide range of body weight difference among groups of animals. An effect that does not apply to appetitive tasks that are inherently problematic when a treatment causes difference in body weight, due to reliance on appetite and the reward value of the reinforcer (Cravens, 1974). Another important advantage is that in rats and most strains of mice, 100% of the animals complete the task, this avoids the problem of selection bias due to dropout rates (Vorhees & Williams, 2014). In addition, rats are natural swimmers and only a few trials are needed for animals to learn that active searching leads to escape from the maze. Above all the MWT is often

employed because its effects on performance after treatment have been widely replicated by many researchers, as well as, its reliability in various cross-species studies like guinea pigs, rats, and mice (Klapdor & van der Staay, 1996; Lewejohann et al., 2010; R. Morris, 1984). All of these make the MWT ideal for answering questions of learning and memory in the HPC.

Different Versions of MWT

In the classical version of the MWT, spatial learning is assessed (R. Morris, 1984). Rats are trained to find a hidden platform at a fixed location for multiple trials over a few days. Twenty-four hours later the platform is removed from the pool and a probe trial is performed, which measures the spatial bias of the rats. Typical control rats decrease their latency and path length during the training phase, eventually swim directly to the platform from all start points and spend more time in the platform quadrant during the probe phase (Blokland et al., 2004).

Slight modification of MWT protocol allow the experimenter to test different kinds of memory. For example, to test discrimination learning two visible platforms are used that are distinct from one another like one being white and one being black. One platform is sturdy and the other one sinks when the rats stand on it. The rats must learn which platform can be used to escape from the water and which cannot, therefore testing their ability to differentiate the stimulus information (D'Hooge & de Deyn, 2001). To test spatial working memory a protocol called matching – to – sample can be used (Steele & Morris, 1999). Here the platform is relocated every day and the rat is given four trials per day. The first trial each day is known as a sample trial, and the rat must learn the new location by trial – and – error. During the following successive trials if the animal recalls the sample trial, it will swim a shorter path to the location. Since the platform location is moved daily no learning from previous days can be used to find the escape platform and therefore learning is a result of only temporary or working memory.

In 2005 McDonald et al, designed a three-phase rapid acquisition version of the MWT to test the effect of NMDARs blockade. This version was used to better understand the role of NMDARs, because previous research had shown that pre-training ameliorated the blockade impairments reported in other experiments (Cain et al., 1996; Saucier & Cain, 1995). The procedure consisted of a prolonged pre-training phase, a brief, intense phase of mass-training to a new location after NMDAs blocker infusion, and a final probe test between pre-trained and mass-trained location (McDonald et al., 2005). This version proved to be a good test of the rats' ability to quickly learn new spatial information in a familiar context and was used in subsequent experiments (Bye & McDonald, 2019).

Importance of Probe trial

The three-phase version of the MWT is optimal because it has the potential to test both the rat's learning and memory. The learning is tested during the second phase of the task, by measuring the decrease in path length over the mass-training session. During phase three when the probe trial is given, measuring the time spent in the pre-trained vs. the mass-trained quadrant allows memory to be tested. If the rat properly learned the mass-training location, then it should in theory spend more time there during the probe. On the other hand, if the rat did not consolidate the mass-training location during learning, it should show a quadrant preference for the pre-trained location. The foundation for this thesis was based on the findings that intra-hippocampal NMDARs blockade did not impair new learning in a familiar context using this version of the MWT (Bye & McDonald, 2019). The overarching goal of this thesis was to test the hypothesis that disruption of new learning in a familiar context required blocking calcium flow via both NMDARs and VGCCs.

Problem with pilot studies

While conducting pilot studies to choose a concentration for the VGCC blocker needed to inject into the HPC, the probe phase results become puzzling. The protocol used was the same as in the forementioned 2005 and 2019 studies, 4 days of 8 trials each day (total = 32 trials) of pre-training to a platform location in the SW quadrant. Then one day of 16 trials of rapid mass-training to a new platform location in the NE quadrant. Followed 24 hours later by a 30 second probe with no platform. Contrary to what was expected the control rats did not show a quadrant preference during the probe trial. This could be interpreted as an impairment in remembering or consolidation of the mass-trained location 24 hours after training. However, the control rats displayed proper learning during the mass-training day because there was a decrease in path length throughout the training session. Another possibility is that the total number of pre-training trials affects the quadrant preference. Given that the rats are given 32 trials to the pre-trained location over 4 days, and 16 trials in one day to the mass-trained location, both representations compete for behavioural control resulting in an absence of preference for the mass-trained location. Thinking that this was likely what was happening and believing the probe preference to be crucial to answering one of the main questions of this thesis, the following experiment was conducted.

Pre-training effect

To test if the number of pre-training trials affects the quadrant preference of the 24-hour probe, rats were divided into two groups. The rats were pre-trained for 8 trials a day over 2 or 4-days to amount to either 16 or 32 trials of pre-training. The following day, all the rats were mass-trained to a new platform location for 16 trials. Then 24 hours later they were given a 30 second probe, where the platform was removed from the pool and the rats were allowed to freely explore. The probe was done to test the quadrant preference of the rats depending on the amount of pre-training trials they were given.

Hypothesis 1 – Both the 2-day and 4-day pre-trained rats will learn the mass-training platform location.

Hypothesis 2 - The 4-day rats will not show a preference for neither the pre- nor mass-trained location, whereas the 2-day rats will prefer the mass-trained location.

Methods

Subjects

Eighteen male Long-Evans rats were used in the experiments described in this chapter. The rats were divided into two conditions, a 2-day pre-training condition (n=10) and a 4-day pre-training condition (n=8). Their age at the start of the experiment was approximately 3 months and their weight range was between 350g – 450g. The rats had *ad labitum* access to water and food. They were housed in pairs and kept on a 12h light /12h dark cycle with lights on at 7:30 and off at 19:30. After arrival in the housing facility the rats were given 7 days to acclimate to their home cages. All the animals were handled for 5 minutes a day for 5 days before starting the behavioural experiment. This was done to familiarize the rats with the experimenter.

Training apparatus

Training occurred in a white circular fibreglass pool which was 46 cm in height and 155 cm in diameter. The pool was located in the center of a room that contained simple geometric shaped posters on all four walls to serve as extra maze cues. In addition to the posters there was a computer, two black shelfs, a sink, and the experimenter, whom remained in the same location during training and testing. The pool was filled with water to a level where the animals cannot escape by using the pool walls but can also see the extra maze cues on the walls. The water was made opaque with non-toxic white paint and the pool was emptied, cleaned, and refilled daily

with fresh water. The escape platform was placed 2.5 cm below the surface of the water, in the center of one of the four quadrants (NW, NE, SW, SE). The escape platform was a clear plastic square (12 x 12 cm) that covered approximately 1% of the total surface area of the pool. The surface of the platform had small holes which provided grip for the rats to mount the platform and balance on the platform. A weight was used to hold down the platform and ensure that it did not move out of place during the task.

Statistical Analysis

Behavioural data was collected with a ceiling mounted camera and movement tracking software Noldus Ethovision 3.1. Statistical analysis was performed using IBM SPSS Statistic Version 28. Learning acquisition and probe test data was analyzed with a repeated measures ANOVA. When a significant interaction occurred, Bonferroni post hoc pairwise comparison was done between the conditions as differences were expected to occur. The following measures were analyzed for the acquisition of the task. Escape latency: time needed to find and climb onto the platform. The maximum value was 60 s. Distance moved: the total distance swam before reaching the platform (cm).

Behavioural protocol

The training protocol consisted of a three-phase version of the Morris water task. All the training and testing took place in the same room and at the same time of day. Two groups of rats were run in this experiment, differing only in the amount of pre-training they had in phase I of the task.

Phase I – pre-training

During phase I the rats were brought into the experimental room in individual cages on a cart and placed in the west side of the room. The animals were run in squads of 4 or 5 rats, one right after the other. During this phase, all rats were trained to find a hidden platform located in

the middle of the SW quadrant of the pool. The rats were given 8 trials a day for 2 days or 4 days, for a total of 16 or 32 trials, respectively. The starting position of each trial was randomly assigned to equidistant locations labelled NW, NE, SE, and SW. Each day the animals experienced a novel sequence of starting points for each trial. Each individual rat was placed in the pool at one of the starting positions facing the pool wall. The experimenter would then walk back to the same position every time and remain there until the trial ended. Each trial was terminated when the rats located and mounted the hidden platform or until 60 seconds had elapsed. After 60 seconds if the rat had not found the hidden platform, it was led to the platform by hand. Each rat was then left on the platform for 10 seconds prior to being placed back in its assigned cage, where it would remain while the other rats were being trained. The training session took 40-50 minutes with an average inter-trial interval of 5 minutes. The purpose of phase I was to teach the animals a pre-trained platform location and to familiarize the rats with the behavioural task.

Phase II – mass-training

Phase II began 24 hours following phase I . The platform location was moved to the middle of the NE quadrant of the pool, directly across from where phase I pre-training occurred. As in phase I, the rats were placed in the pool at one of the equidistant locations in a randomized order. The rats were allowed 60 seconds to find the new hidden platform location and were left on the platform for 10 seconds prior to being removed. All the rats were given 16 trials to learn the new platform location, within a two-hour period. The purpose of phase II was to rapidly train the rats to a new platform location.

Phase III - probe

A probe test occurred 24 hours after the phase II mass-training session. Each rat was placed in the pool as previously described, however there was no platform in the pool. The

starting position was the SE quadrant, and this was determined because it was equidistant from each of the previous platform locations. The rats were allowed to swim for 60 seconds and the first 30 seconds of the probe test was analyzed. The purpose of phase III (the probe test) was to determine if the number of pre-training trials 16 (2-day training) vs. 32 (4-day training) trials would affect the quadrant preference of the rats.

Euthanasia

A few days following the completion of phase III the rats were euthanized with an intraperitoneal injected of Sodium Pentobarbital (300mg/kg).

Results

Pre-training

To assess learning the latency and path length to the platform was analyzed. Latency results were identical to the path length results therefore they will not be discussed here, see Appendix 1. Figure 1 represents the average of the 2 trials across 2 or 4 training days depending on the experimental group. The average path length of the first trial block was 789.3 cm for 2-day rats and 1079.1 cm for 4-day rats. At the end of the pre-training phase, the path length for the last trial block was 296.1 cm for 2-day rats and 126.1 cm for 4-day rats (Fig. 1.1). Since sphericity is violated ($\varepsilon = 0.571$), Greenhouse-Geisser corrected results are reported. A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(4, 64.003) = 18.868$, $p = 0.004$) with no effect of group and no interaction. Over the 2-day or 4-day pre-training period, all the rats learnt the hidden platform location.

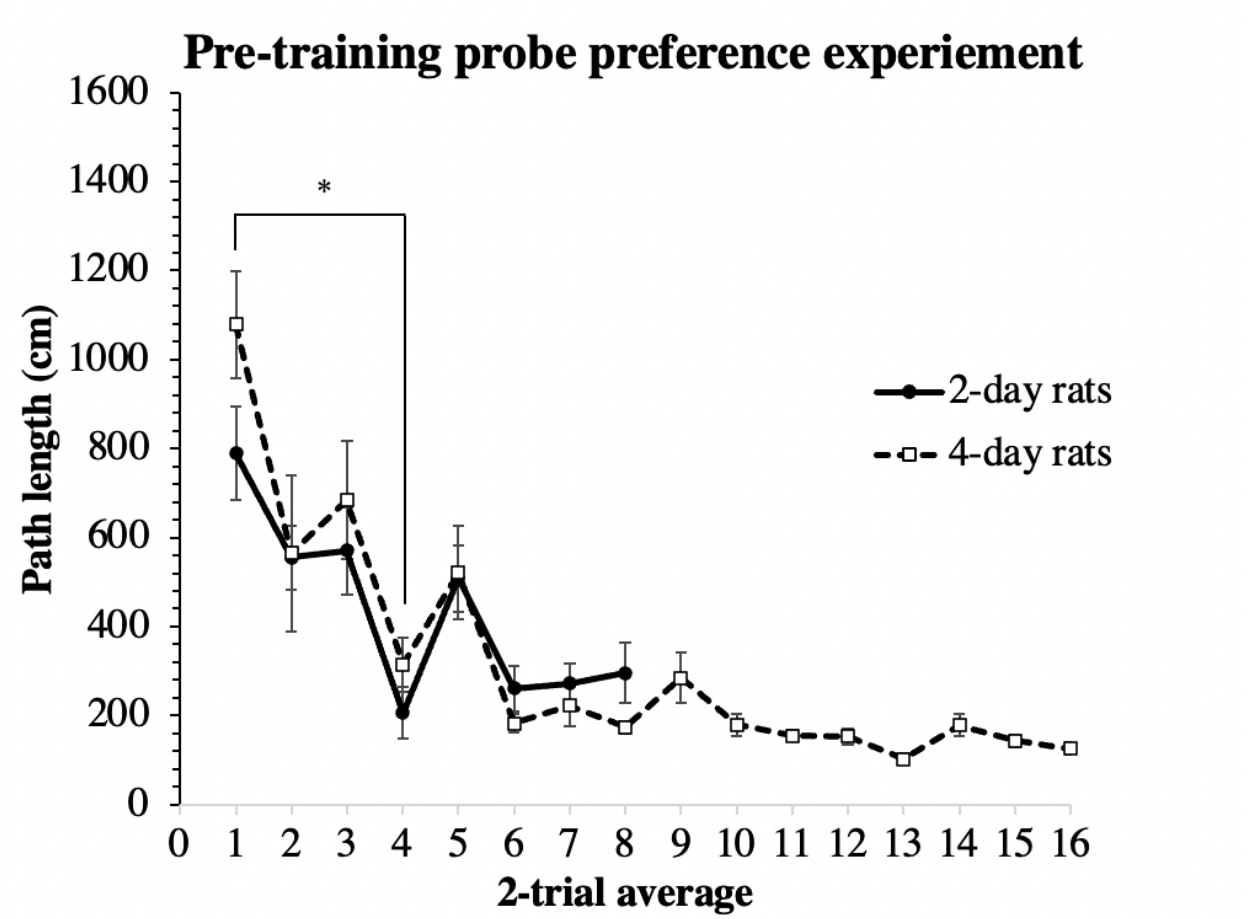

Figure 1.1: *Path length to platform as a 2-trial average during pre-training of all rats in experiment 1 on the Morris Water Task. Rats were given either 2 or 4 days of pre-training, 8 trials per day. Both groups learned. P = 0.004, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training

The mass-training phase was done 24 hours after the pre-training phase. The average path length of the first trial block during mass-training was 444.2 cm for 2-day rats and 699.6 cm for 4-day rats. At the end of mass-training, the path length was 180.4 cm for 2-days rats and 174.9 cm for 4-day rats (Fig 1.2). Since sphericity is violated ($\varepsilon = 0.355$), Greenhouse-Geisser corrected results are reported. A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(2.486, 39.770) = 14.613$, $p < 0.001$). There was no group effect, however there was an interaction of trail 1 and 2 on rat group ($F(2.486, 39.779) = 3.832$, $p = 0.022$). Post

hoc pairwise comparisons revealed a significant difference within 2-day rats between trial block 3 (292 cm) and 8 (180.4 cm) ($p = 0.019$). As well as a significant difference within 4-day rats between trial block 1 (699.6 cm) and 2 (199.96 cm) ($p = 0.008$). Over the 2-hour mass-training period both groups learned the new platform location.

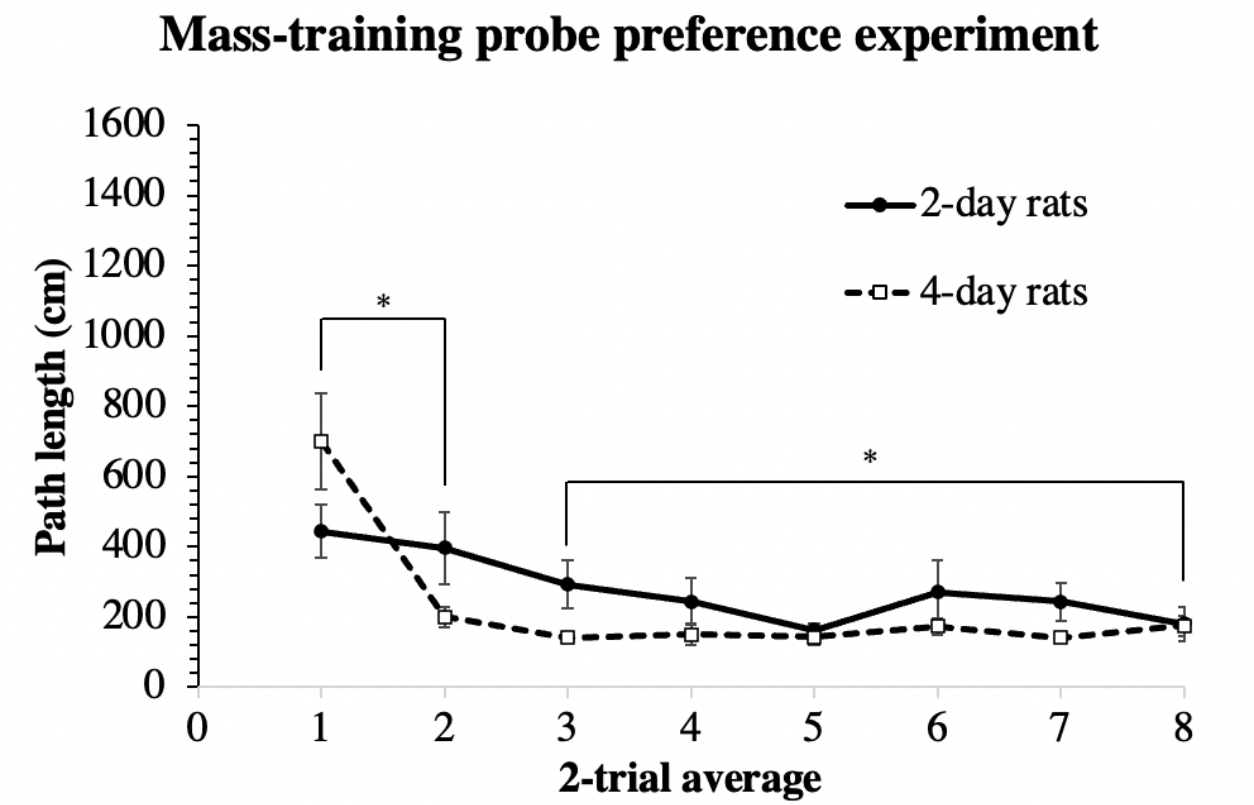

Figure 1.2: *Path length to platform as a 2-trial average during mass-training of all rats in experiment 1 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The 4-day rats show significant learning early in training whereas the 2-day rats learn late in training. 4-day rats p =0.008, 2-day rats p = 0.022. There was a significant interaction p = 0.022. Error bars: +/- 1 SE. Significance is denoted by "*".*

Probe

The probe test for learning and memory was done 24 hours after the completion of mass-training to assess the rat's quadrant preference for either the pre-trained or mass-trained location. The percentage of time spent in the two target quadrants was compared within and between the groups. A repeated measures ANOVA revealed a significant effect of quadrant ($F(1,16) = 6.948$, $p = 0.018$), no significant effect of group and no interaction. Post hoc pairwise comparison revealed a significant difference within the 2-day condition, with rats spending more time in the mass-trained quadrant (38 %) then in the pre-trained quadrant (16 %) ($p = 0.006$). There was no significant difference found within the 4-day rats. The 4-day rats spent 32.2 % in the mass-trained quadrant and 26.7 % in the pre-trained quadrant (Fig. 1.3). These results indicate that the 2-day rats preferred the mass-trained platform location 24 hours after training, while the 4-day rats did not. This means that the number of pre-training trials effected the quadrant preference of the 24-hour probe.

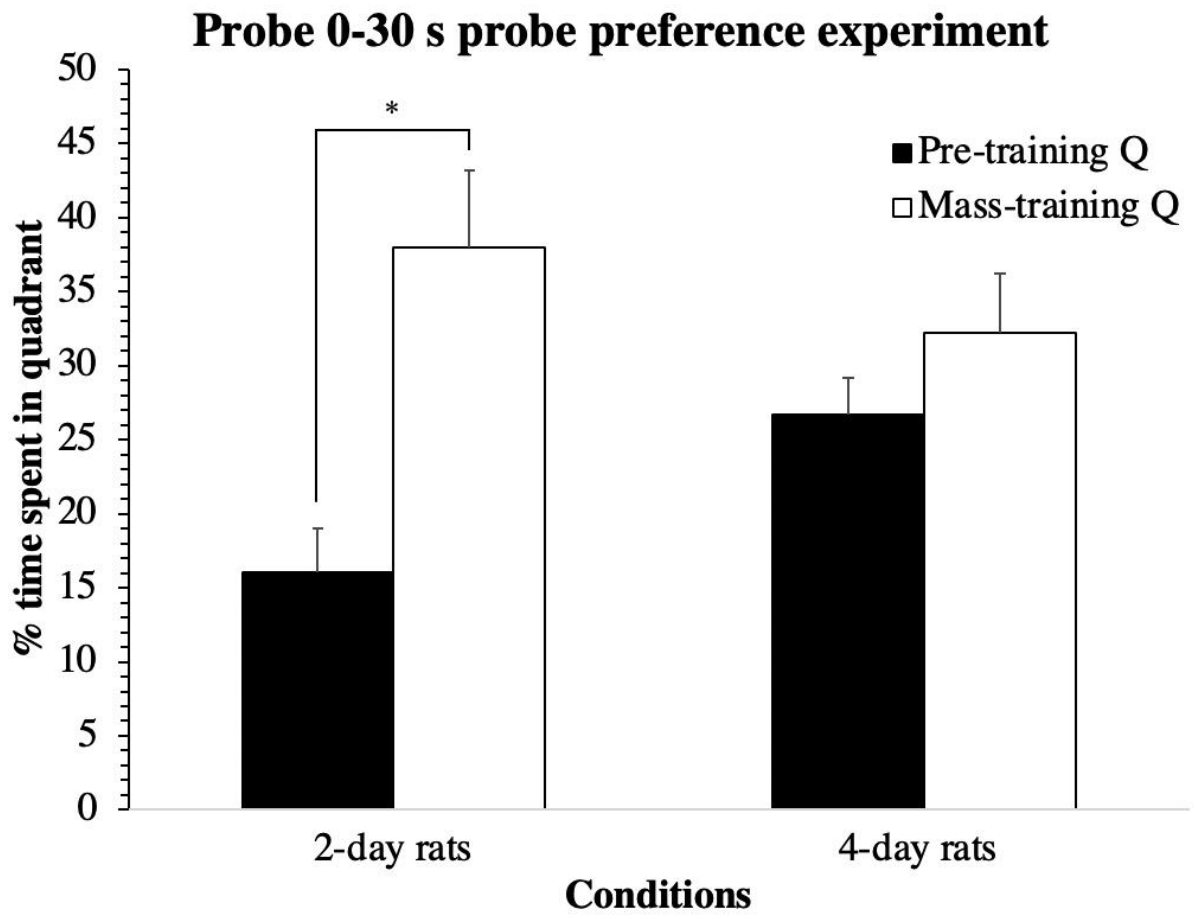

Figure 1.3: *Percentage of time spent in pre-trained quadrant and mass-trained quadrant of rats in experiment 1, during 30 second probe test done 24 hours after mass-training. The results revealed a significant within-subjects effects (F (1, 16) = 6.948, P = 0.018). 2-day rats display a preference for mass-trained quadrant, 4-day rats do not. P = 0.006 Error bars: +/- SE. Significance is denoted by "*".*

Discussion

In this experiment, rats where trained in a three-phase version of the MWT. One group of rats was given 16 trials of pre-training over 2 days to learn to locate a hidden platform. The alternate group was given 32 trials over 4 days of pre-training to learn the location of a hidden platform. Following pre-training, both groups did a mass-training session of 16 trials to locate a hidden platform in a novel location. Both groups were then given a probe test, 24 hours later, to assess their quadrant preference depending on if they received 16 or 32 trials of pre-training.

There were no significant differences between the two groups throughout the pre-training phase. All the rats had an increased path length to locate the hidden platform at the start of pre-training. The path length of the rats significantly decreased by the end of training, suggesting that independent of the number of pre-training trials (16 or 32) all the rats were able to learn to locate the hidden platform. Both groups significantly decreased their path length by trial block 4 (the last trial on day 1). By the end of the pre-training phase, the 2-day rats and the 4-day rats had a similar path length to locate the hidden platform. The largest decrease in path length for both groups of rats was seen throughout the first 2 days of pre-training, suggesting that by day 2 all the rats were able to locate the hidden platform. The path length of the 4-day rats did not significantly decrease following day 2 of pre-training, further suggesting that the rats were able to learn the location of the hidden platform by day 2.

During the mass-training session the rats were trained to a new hidden platform location in the opposite position of the pool. During the first trial of mass-training the path length of both groups was increased from their final pre-training measure. The increased path length during trial one was because the platform location was moved to a novel quadrant. Interestingly, the 4-day rats showed a significant decrease in path length sooner (by trial block 2) than the 2-day rats. Nonetheless, over the course of the mass-training session the path length decreased for both groups. Suggesting that the 2-day and 4-day rats were still able to learn the location of the hidden platform during the 16 mass-training trials.

The probe test performed 24 hours after mass-training revealed that the 2-day rats had a significant preference for the mass-training quadrant. The 4-day rats did not have a significant preference for the mass-training quadrant and spent a similar amount of time in both the pre-training and mass-training quadrant.

Pre-training learning

Ultimately, the results provide insights into the effects of pre-training trials on the 24-hour probe quadrant preference. Given the pre-training results most of the platform learning occured during the first 2 days of training (16 trials). Both groups had the greatest decrease in path length during this period and further training did not significantly decrease their path length. These findings are supported by previous research, which has shown that rats are able to quickly learn a new platform location in this task (Bye & McDonald, 2019; McDonald et al., 2005; Rossato et al., 2018). The 4-day rats also got to asymptote level of performance by the second day of pre-training and the last 2 days of pre-training did not significantly decrease their path length. This experiment pushes the limits on what is the minimum amount of pre-training that can be given to a rat to learn a platform location in the MWT. One could argue from the pre-training results, which show a significant decrease from block trial 1 to block trial 4, that by the end of the first day the rats learn the location and one day might be sufficient training. However, when looking at the mass-training results the following day, this number of trials might not be enough to properly familiarize the rats with the task. Therefore, a minimum of 2-days of pre-training would be preferred.

Mass-training 2-trial average learning

During the mass-training the 4-day rats learnt the new platform location quicker than the 2-day rats. The 4-day rats had significantly decreased their path length by the 2nd trial block, whereas the 2-day rats significantly decreased their path length by the 8th trial block. This shows that a greater number of pre-training trials enhances new learning in the same context. The 2-day rats were able to learn the new platform location, but it was by the end of the mass-training

session. Because it took them the entire session to show a significant decrease in path length, this might mean that 2 days of pre-training is the minimum number of trials needed for the rats to learn during the mass-training phase. On the other hand, there could be an alternative explanation for the group differences in learning time. The 4-day condition in the first trial block had an average of 699.6 cm, whereas the 2-day condition had an average of 447.3 cm. As a result, the 2-day condition needed to decrease their path length to a shorter amount than the 4-day condition to show a significant decrease. Nonetheless, statistically the starting path length between the groups was not statistically different. Overall, the mass-training day results show that all rats learnt the new platform location, but the 4-day rats learnt quicker compared to the 2-day rats.

Probe memory

The 24-hour probe analysis indicated that the 2-day rats preferred the mass-training location whereas the 4-day rats did not. These findings were in line with the original hypothesis for this experiment, which predicted that changing the number of pre-training trials would affect the quadrant preference for the 24-hour probe. Indeed, it would make sense that if the trials to one location equalled the trials to the other location, the rats would show a preference for the last trained location to escape the water. This effect had been shown before, though to a lesser degree in a previous experiment (McDonald et al., 2005). In that experiment they used the 4-day condition and analyzed the probe in 10 seconds time bins. Interestingly, the rats would display a searching strategy, where during the first 10 seconds they spent most of their time in the mass-trained location, then the following 10 seconds would search in the pre-trained location, and during the final 10 seconds would search both. The data from this experiment indicates that if the same total number of trials is given during the pre-training and mass-training sessions, rats will have a stronger recall and preference for the latest trained location.

Answering the hypotheses

Hypothesis 1 predicted that both the 2-day, and the 4-day rats would be able to learn the mass-trained platform location. The evidence supports this finding by showing a decrease in path length of both rat groups throughout the mass-training session. Hypothesis 2 predicted that the 4-day rats would not prefer the mass-trained location during the probe test, while the 2-day rats would. The data supported this claim, by showing a significant quadrant preference of the 2-day rats to the mass-trained location, whereas the 4-day rats did not show a significant quadrant preference. Rather the 4-day rats split their time between the pre- and mass-trained locations.

Summary

The findings from this experiment were the following: 2-day pre-training is sufficient for rapid mass-training to a new location learning to occur; if the rats properly learnt the mass-trained location during the probe they would display a preference for this location. As a result, the hypothesis that the number of pre-training trials affects the 24-hour quadrant preference can be accepted. This shorter 2-day pre-training version of the MWT then provided a great framework to test the role of multiple sources of calcium in spatial learning and memory in the hippocampus. Using this protocol, it could be tested if rats with multiple sources of calcium receptor blocked in the hippocampus could learn, by assessing their performance during the mass-training phase. In addition to testing if they could recall and display a memory for the learning the following day, analyzing the probe phase. The function of calcium flow via VGCCs and NMDARs in the HPC in learning and memory will be explored in the next experiment.

Chapter 3

Does multi-targeted hippocampal calcium receptor blockade effect spatial learning and memory in a rapid acquisition variant of the Morris water task?

Introduction

Learning and memory is believed to have a physical basis and understanding the mechanism responsible for it in the mammalian brain is an important question neuroscientist strive to answer. Arguably the main theory of what this mechanism might be is known as synaptic plasticity. A central tenant of this theory is that neurons that fire together will strengthen their synaptic connections to be able to effectively communicate with each other (Hebb, 1949). The plasticity, which can also be thought of as the change in activated neurons when a stimulus is presented, allows a representation of the information to be encoded and retrieved later.

Support for the theory of synaptic plasticity in learning and memory came from work done by Bliss and Lomo (1973). While studying the patterns of electrical activity in neurons in the rabbit HPC the researchers demonstrated an increase in synaptic efficacy of post-synaptic neurons after tetanic stimulation of pre-synaptic neurons. This was an important finding because it provided a model to study how synaptic strength can be modified by experience. Soon after these results were published the mechanism termed LTP became the standard for investigating the physical basis of learning and memory in the mammalian brain. LTP was described as the mechanism through which synapses are strengthened, by causing a greater post-synaptic potential due to a tetanic stimulus (Bliss & Lømo, 1973).

Consensus view of NMDARs

Mechanistically it is believed that this type of LTP causes an influx of calcium ions via NMDARs, which triggers biochemical and structural changes in the synapse (Dunwiddie &

Lynch, 1979). NMDARs are commonly referred to as coincidence detectors because they can only be opened when pre-synaptic glutamate is released. Glutamate then binds to NMDARs and post-synaptic depolarization occurs to remove its magnesium plug from the receptor's ion channel. Key experiments were done during the 1980s which discovered that glutamate was a critical neurotransmitter for the activation of post-synaptic receptors such as, NMDARs and AMPARs which were important for LTP. Pharmacological agents were then designed and manufactured to block these glutamate receptors (Bear & Malenka, 1994). Important experiments were done by Collingridge et al. (1983), indicating that blocking NMDARs terminated LTP induction, but did not affect LTP expression, whereas blocking AMPARs in HPC stopped LTP induction and expression. These studies provided the foundation connecting glutamate receptors to LTP in the HPC of mammalian brains.

Learning and memory are important brain functions that are best measured at the behaviour level of an animal. Richard Morris set out to link the molecular evidence of LTP to a freely behaving animal. In these experiments, rats were trained on the spatial version of the MWT while intraventricular infusions of AP5, an NMDA-antagonist was administered. AP5 has been shown to block LTP induction in the HPC. Morris et al. (1986) provided support for previous findings of Collingridge et al. (1983) by showing that blocking NMDARs during acquisition of the MWT impaired spatial learning . Ultimately, the combined evidence made a strong case for the canonical idea that NMDARs in the HPC were crucial for the initial encoding of spatial information, and linked NMDARs-dependent LTP in HPC with spatial learning and memory (Morris et al., 1990; Morris, 1989; Morris et al., 2013).

NMDARs

Contradictory view of NMDARs

While the view that NMDARs-dependent LTP in the HPC is crucial for learning and memory was popular, opposing evidence began to emerge. Some researchers expressed concern with the techniques used to administer NMDARs blockers, primarily peripheral and intraventricular injections. The idea was that the drug administration was too general and could be affecting other regions of the brain which provided support for learning and memory functions like motor control, motivation, and sensory processing. As a result, the effects that NMDARs blockers displayed would be because other processes were affected like motor control and not because there were true learning impairments (Cain, 1997; Keith & Rudy, 2013). Saucier and Cain (1995) decided to investigate if pre-training could resolve some of the suspected secondary effects due to the way in which the drug was administered. During the pre-training phase the rats were allowed to freely swim in the pool with no escape platform. The following day, NMDARs blockers were administered peripherally, 1 hour before standard MWT training. Interestingly, the researchers found that if pre-trained, rats with NMDARs blocked had normal acquisition of spatial learning in the MWT, a task which is dependent on HPC function (R. G. M. Morris et al., 1982; R. J. Sutherland et al., 1982, 1983). This led to the conclusion that pre-training ameliorated possible pharmacological effects of supporting neuronal circuits and that NMDARs function may not be critical for learning and memory.

Further research findings continued to build up, opposing the idea that NMDARs in the HPC were critical for spatial learning and memory. After the discovery of place cells, which are neurons in the HPC that play a role providing information to an animal about its location in its environments, interesting results emerged showing that the formation and short-term maintenance of place cells in HPC was not disrupted by blocking NMDARs, however long-term

place cell representations were compromised (Kentros et al., 1998). At the behavioural level researchers have also found that rats with intrahippocampal NMDARs blockade show normal rapid acquisition of a new platform location in a context where they had been previously pre-trained (McDonald et al., 2005). Interestingly, supporting the place cell experiment, they found that long-term consolidation of the new platform location was impaired with NMDARs blockade. McDonald et al. (2005) hypothesized that NMDARs in the HPC may be crucial for new learning to occur in a novel environment but may not be crucial for new learning in a familiar environment (where pre-training had occurred). To investigate this hypothesis, a series of experiments were done which involved a three-phase version of the MWT (Bye & McDonald, 2019). A similar protocol to what McDonald et al. (2005) used was implemented, rats were pre-trained (4 days, 8 trials per day) to a platform location and then given a mass-training session (one day, 16 trials) to a new platform location in either the same room (familiar context) or a new room (novel context). A NMDARs blocker was administered before the mass-training rapid acquisition session at a dosage that had been shown to block LTP in HPC. There were three key findings; 1) blockade of HPC NMDARs did not impair the rapid acquisition of new learning in a familiar context; 2) NMDARs blockade severely impaired rapid new learning in a novel context; 3) NMDARs activation seems critical for the long-term expression of new learning in both the familiar and novel context (Bye & McDonald, 2019).

Calcium theory of new learning

Based on these findings an interesting theory began to emerge to make sense of the behaviour and electrophysiological results. The calcium theory of new learning is the idea that learning new information in a novel environment requires more calcium influx into post-synaptic neurons. On the other hand, learning new information in a familiar environment requires less calcium. Furthermore, the calcium influx can occur via multiple sources like NMDARs which

would be capable of inducing LTP independent of each other. To fully test out the above hypothesis a variety of experimental techniques like behavioural pharmacology, immunohistochemistry, electrophysiology, and imaging techniques would have to be used. In this chapter a behavioural pharmacological approach will be used to test a portion of what is predicted by the above hypothesis. As stated, new learning with familiar information does not require NMDARs activity and this is because it requires less calcium influx which could occur via other receptors. However, new learning with novel information does need NMDARs activity and this is because a larger amount of calcium influx needs to occur via NMDARs and other receptors for proper LTP and synaptic plasticity to support the learning. The main goal of the experiments described in this chapter was to uncover which neurobiological mechanism could be responsible for new learning with familiar information.

NMDARs independent LTP

LTP in the HPC can be induced independent of NMDARs if the frequency of stimulation is higher than what is normally used for NMDARs induction (Grover & Teyler, 1990). Indeed, NMDARs independent form of LTP have been induced in many synapses of the HPC *in vivo* (Bramham et al., 1991; Grover, 1998; E. W. Harris & Cotman, 1986). Furthermore, these forms of LTP initiate similar plasticity cascades and downstream gene transcription activation than NMDARs dependent LTP. VGCCs are a good candidate to mediate NMDARs independent LTP. VGCCs are postsynaptic channels located all throughout the brain with the L-type being the most abundant in the HPC. Once opened VGCCs allow the influx of calcium ions. LTP can be induced in the HPC when NMDARs are blocked with AP5, however this LTP was blocked with nifedipine, a VGCCs antagonist (Grover & Teyler, 1990). Mice missing L-type VGCCs due to genetic knockout, display normal NMDARs LTP in the HPC, but when NMDARs blockers are administered all LTP is blocked (Moosmang et al., 2005). These mice also showed impaired

performance on a variety of HPC dependant spatial learning and memory tasks. Another study which blocked either NMDARs or VGCCs in rats, tested them on spatial memory and concluded that each receptor had a different role in memory, VGCCs in memory retention whereas NMDARs played a role in rapid acquisition (Woodside et al., 2004). NMDARs and VGCCs have in common that when activated they increase intracellular calcium concentration, which is crucial for LTP expression. Additionally, the intracellular signaling cascades following LTP induction via ERK/MAPK and CREB phosphorylation linked to NMDARs activation have also been found to be affected by disruption of postsynaptic VGCCs function (Dolmetsch et al., 2001). In summary VGCCs are a good mechanism for NMDARs independent LTP and may play a role new learning with familiar information because; 1) they have been shown to block NMDARs independent LTP; 2) VGCCs blockade has led to spatial learning and memory impairments; 3) responsible for calcium influx which is crucial for LTP induction; 4) VGCCs are linked to the phosphorylation of proteins involved with LTP expression.

Effective drug administration

To mitigate concerns raised previously about mode of administration of drug blockers causing unintended effects not involved with learning and memory, direct intrahippocampal drug infusion in freely behaving animals is critical. Fortunately, previous experiments have done this and have found an effective dosage using AP5, an effective NMDARs antagonist, which will be used in this experiment (Day et al., 2003; R. G. M. Morris, 1989; R. G. M. Morris et al., 1986, 2013). AP5 disrupts the entire NMDAR complex, including the NR1 subunits, which are critical for channel function, and the NR2 subunits that appear to regulate channel function (Monyer et al., 1992). To date there is significantly less research on appropriate intrahippocampal dosages for L-type VGCCs antagonists, however, verapamil seems to be a good candidate. Verapamil is a phenylalkylamine which binds to a drug binding region near the pore and to the proposed

activation gate of the channel's alpha-1 subunit (Striessnig et al., 2015). Verapamil was chosen for three key reasons as the VGCCs blocker for this experiment. Firstly, just like AP5, verapamil reversibly interacts with the channel, inhibiting its activity only for a few hours (Striessnig et al., 1998). Secondly, it can be dissolved in 0.9 NaCl % saline like AP5 (Woodside et al., 2004). Lastly, verapamil has been shown to impact VGCC LTP in vivo (Lashgari et al., 2006; Morgan & Teyler, 1999; Niikura et al., 2004), as well as impair some aspects of learning and memory in freely behaving rats (Bauer et al., 2002; S. H. Wang et al., 2012; Woodside et al., 2004). Based on the forementioned studies three pilot experiments were conducted to determine an appropriate dosage for intrahippocampal verapamil infusion.

Proposed research

To determine which neurobiological mechanism might be responsible for new learning with familiar information described in Bye and McDonald (2019) two different LTP inducing mechanisms were blocked in the rat HPC during the three-phase version of the MWT. Rats were given 2 days of spatial pre-training, followed by either NMDARs blockade, VGCCs blockage, or NMDARs and VGCCs blockade prior to mass spatial training to a new platform location. A probe test was then performed to test the role of these receptors in memory consolidation. Path length and latency to locate the hidden platform, as well as heading angle error were used to determining learning performance. Based on previous studies, it was hypothesized that disruption of new learning with familiar information is dependent on a blockade of both NMDARs and VGCCs and that consolidation would be compromised in all receptor blocker conditions.

Hypothesis 1 – Blocking VGCCs WILL NOT impair new spatial learning with familiar information but will impair long-term memory of this location 24 hours later.

Hypothesis 2 – Blocking NMDARs WILL NOT impair new spatial learning with familiar information but will impair long-term memory of this location 24 hours later.

Hypothesis 3 – Blocking VGCCs and NMDARs WILL impair new spatial learning with familiar information and long-term memory of this location 24 hours later.

Methods

Subjects

Male Long-Evans rats were the subjects of the experiments described in this chapter. They were divided into three cohorts, cohort 1 (n=16), cohort 2 (n=20), and cohort 3 (n=18). The subjects age and weight were the same as in experiment 1. The experiment in this chapter was very similar to the 2-day pre-training condition of experiment 1. Phase I and phase III were identical, as well as all the data and statistical analysis. An additional measure was analyzed in this chapter, which was heading angle error. This quantifies the deviation of the rat from swimming straight to the platform and is therefore an additional measure of learning performance. For this experiment all the rats underwent intrahippocampal canulation surgery to block VGCCs and NMDARs prior to phase II (mass-training).

Surgery

Four permanent guide canulae were implanted bilaterally into the dorsal and ventral hippocampi of all rats. Rats were subcutaneously injected with Baytril at 10 mg/ kg one day prior to surgery and three days post-op to prevent infection. Rats were subcutaneously injected with buprenorphine at 0.03 mg/kg 30 minutes prior to surgery for pain management. Rats were then anesthetized using 4% isoflurane gas in oxygen with a flow of 2 L/min. Surgical anesthetic plane was maintained using 1.5 – 2% isoflurane throughout the surgery. An area above the skull approximately 3 by 6 cm was shaven using an electric shaver, to ensure minimal hair was on the

surgical field. Rats were positioned in a standard stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). A 4 cm incision was made in the scalp, the skin retracted, and four 0.7 mm and two 1 mm holes were drilled into the skull. The two 1 mm holes served as anchoring screws, and the four 0.7 mm holes were guide cannulae. Two 23-guage stainless steel guide cannula were inserted bilaterally into the dorsal (A/P -3.5mm, M/L: +(-) 2mm, D/V: - 3.15 mm) and ventral (A/P -5.8mm, M/L: +(-) 5.2mm, D/V: - 6.0 mm) hippocampus and were held in position using dental acrylic. The guide cannulae were plugged using 30-guage wire obturators, which stayed inside until infusion. At the end of surgery, and every 24 h until returned to home cages, rats were subcutaneously injected with Metacam 1mg/ kg for pain management. Rats were held in ventilated cabinets under observation for 48 h. Then they were returned to their home cages and allowed between 7 to 10 days of recovery before beginning behavioural testing.

Behavioural protocol – phase II

All the training and testing took place in the same room and at the same time of day. Three cohorts of rats were run in this experiment, differing only in the drug they were injected during phase II infusion day. The assignment to each condition was counterbalanced to ensure there was no difference in the pre-training acquisition between pre-treatment conditions. For cohort 1 the verapamil rats (n=8) received intrahippocampal infusion of verapamil (7ug/ul) dissolved in NaCl 0.9% saline. The control rats (n=8) received NaCl 0.9% saline. Obturators were removed and infusion was done at a rate of 0.25 ul/minute for 4 minutes, for a total of 1 ul per infusion site. Dosage was determined based on previous pilot experiments. For cohort 2 and 3 the procedure was done in the same manner, the only difference was a different drug infused. In cohort 2 the AP5 rats (n=10) were administered AP5 (5.9ug/ul) dissolved in NaCl 0.9% saline, and the control rats (n=10) received NaCl 0.9% saline. This dose has been shown to impair spatial water maze performance (Steele & Morris, 1999) . In cohort 3 the combo rats (n=9) were

infused with a combo solution containing verapamil (7ug/ul) and AP5 (5.9ug/ul) in NaCl 0.9% saline, and the control rats (n=9) received NaCl 0.9% saline.

After the 4 minutes infusion was completed, the infusion cannulae were left inside the permanent guide cannula for an extra minute to allow proper diffusion of the drug. Once this 5-minute procedure was completed, new obturators were placed into the permanent guide cannula. Approximately 45 minutes later phase II training began. Like phase I training, rats were placed in the pool at one of the equidistant locations in random order, were allowed 60 seconds to find the new platform location and were left on the platform for 10 seconds. All training occurred within a two-hour period, a time frame for which verapamil and AP5 has been shown to block LTP in the hippocampus (Day et al., 2003; Morgan & Teyler, 1999; S. H. Wang et al., 2012). The purpose of phase II was to determine whether rats can learn a new platform location after being pre-trained in the same environment while glutamate receptors are blocked in the hippocampus.

Perfusion and Histology

The day after phase III the rats were perfused with an intraperitoneal injected of Sodium Pentobarbital (300mg/kg). Approximately 10 -15 minutes later they were transcardially perfused with 4% paraformaldehyde solution and phosphate-buffered saline. For cryoprotection the brain was left in a 4% paraformaldehyde solution for 24 hours, then placed in a 30% sucrose + 0.2% sodium azide solution for 5 days. The brain was sliced using a freezing microtome at 40 microns per section. Hippocampal sections were stained using cresyl violet Nissl. Proper cannulation placement was analyzed using a light microscope and all subjects with cannulations outside the hippocampus were excluded from statistical analysis.

Results

Cohort 1- VGCC blocker

Pre-training

To assess learning, latency and path length to the new platform location was analyzed. The latency data was identical to the path length data, therefore will not be discussed here, see Appendix 2. There was a total of 16 rats in cohort 1, 8 rats were in the control group and 8 rats were in the verapamil (VGCC blocker) group. Figure 2.1 represents the average of 2 trials across 2 days of training. The average path length of the first trial block was 1244.6 cm for control rats and 1079.7 cm for pre-verapamil rats. At the end of pre-training phase, the path length for the last trial block was 320.3 cm for control rats and 240.1 cm for pre-verapamil rats (Fig 2.1). A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(7, 98) = 19.252$, $p < 0.001$) with no effect of group and no interaction. Both the control and pre-verapamil rats had a significant difference in path length from trial block 1 to 4 ($p = 0.003$). Both groups successfully learned the pre-training platform location.

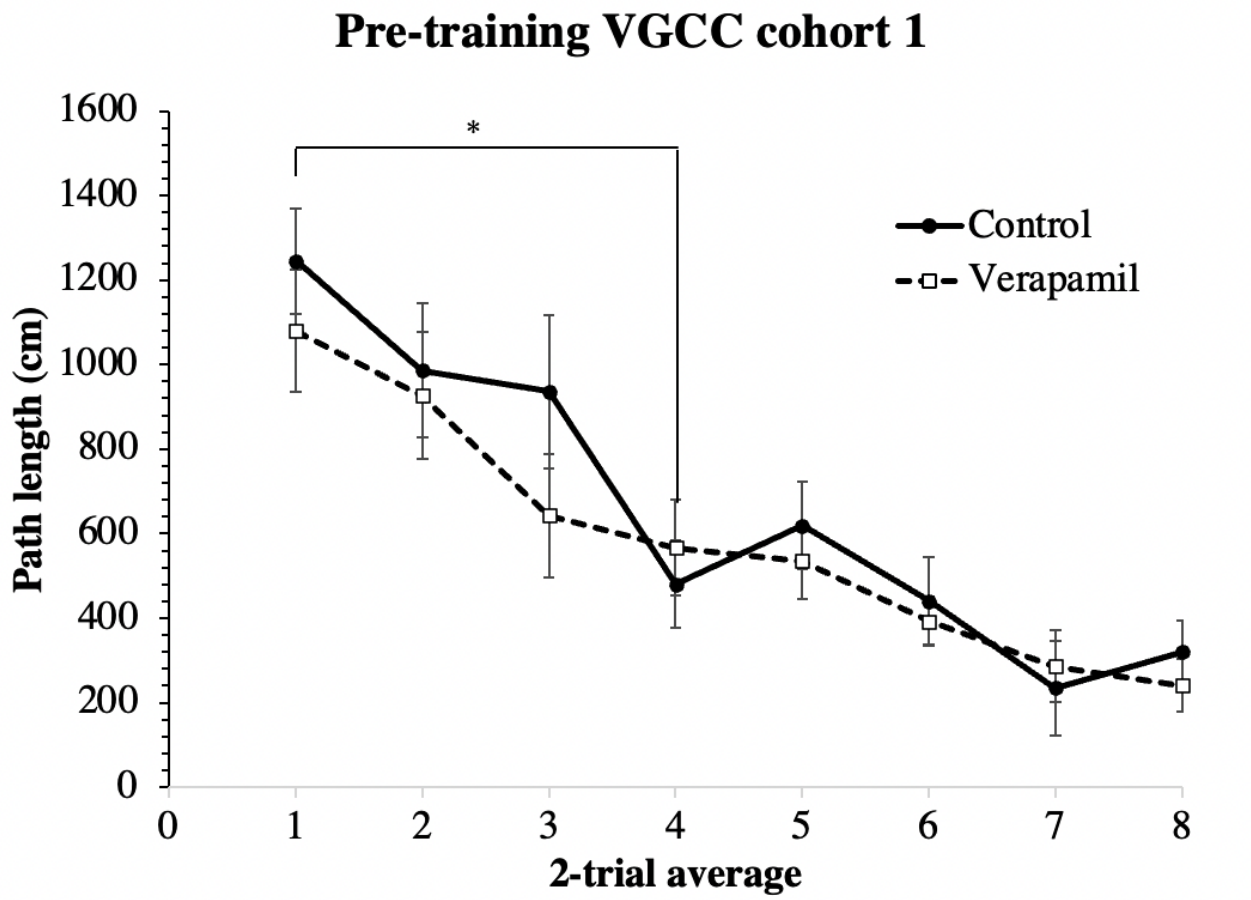

Figure 2.1: *Path length to platform as a 2-trial average during pre-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 days, 8 trials per day. Both groups learned. P = 0.003, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training

The mass-training phase to a new platform location was completed 24 hours after the pre-training phase. The average path length of the first trial block during mass-training was 1027.3 cm for control rats and 728.7 cm for verapamil rats. At the end of mass-training, the path length was 379.6 cm for control rats and 277.8 cm for verapamil rats (Fig 2.2). A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(7, 98) = 7.094$, $p < 0.001$). There is an interaction ($F(7, 98) = 2.164$, $p = 0.044$). Post hoc pairwise comparisons revealed a significant effect of trial block 1 vs. 6 ($p = 0.052$) for the control rats. However no

significant effect of trial for the verapamil rats. Over the 2-hour mass-training period the control rats show significant learning whereas the verapamil rats do not.

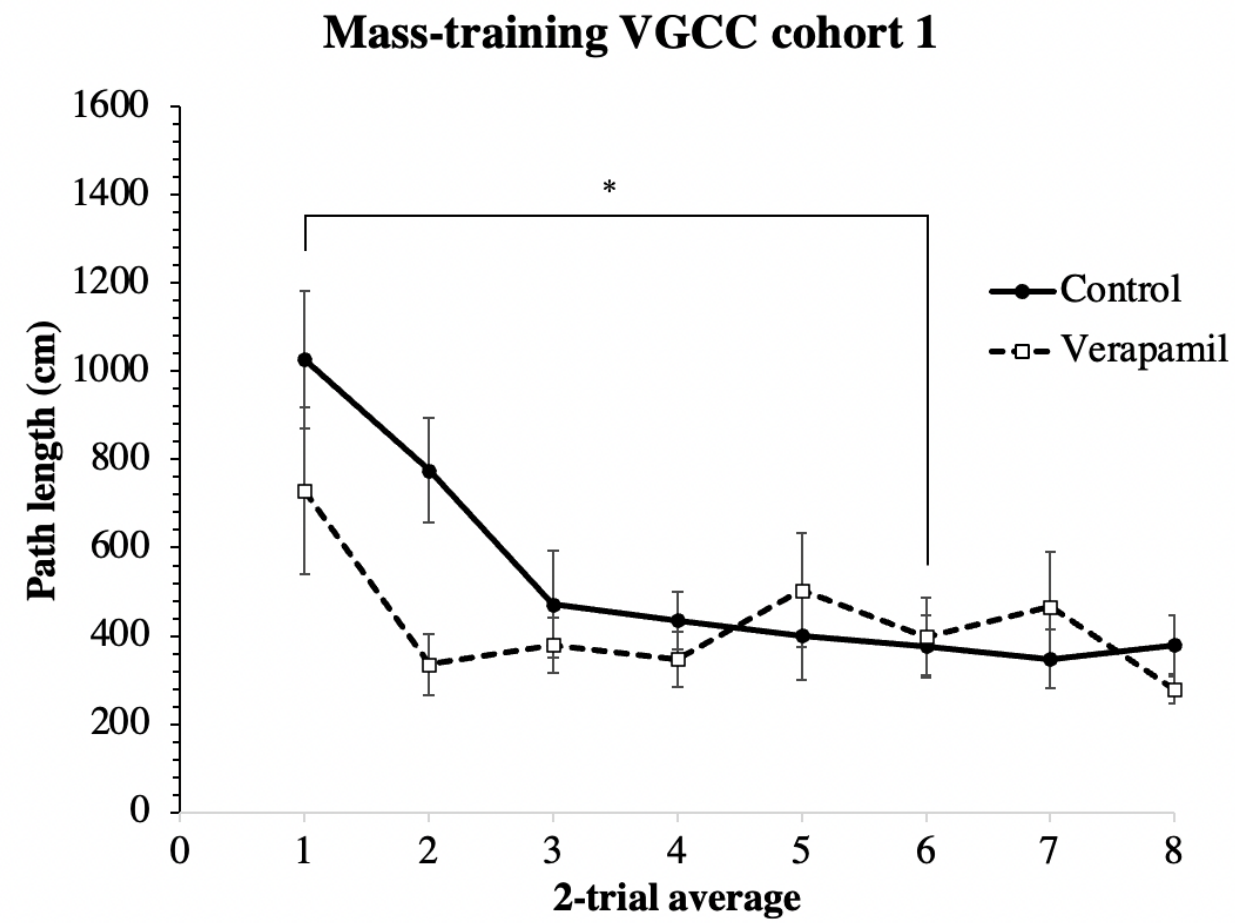

Figure 2.2: *Path length to platform as a 2-trial average during mass-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The control rats show significant learning whereas the verapamil rats do not. P = 0.052, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training was also analyzed as the first 8 trials average (early in training) and last 8 trials average (late in training). The average of the first 8 trials was 677.5 cm for the control rats and 447.5 cm for the verapamil rats. The average of the last 8 trials was 376.1 cm for the control rats and 411.5 for the verapamil rats (Fig 2.3). A repeated measures ANOVA indicated that there was a significant effect within subjects $F(1, 14) = 5.363$, $p < 0.036$). Post hoc pairwise

comparisons revealed a significant difference between the control rats first 8 vs. last 8 trial averages ($p = 0.002$), however no significant difference of the verapamil rats. Over the 2-hour mass-training period the control rats showed significant learning whereas the verapamil condition did not.

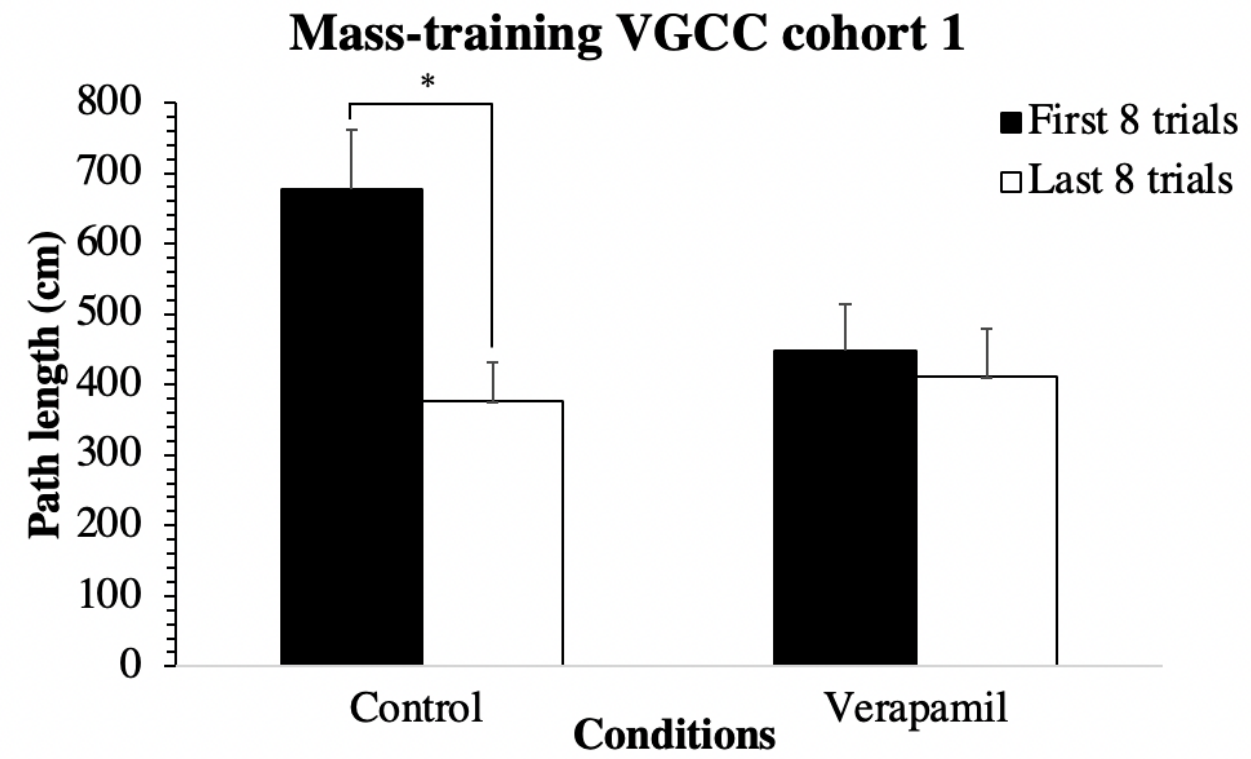

Figure 2.3: *Path length to platform early vs. late in mass-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The control rats show significant learning whereas the verapamil rats do not. P = 0.002, Error bars: +/- 1 SE. Significance is denoted by "*".*

In a similar manner heading error was analyzed for the first 8 trials and the last 8 trial average. The initial heading error was determined as the average deviation from the line between the start point of the rat and the platform location over the time from 1s to 2s (Bye et al., 2019). The average of the first 8 trials was 66.5 degrees for the control rats and 66.74 degrees for the verapamil rats. The average of the last 8 trials was 63.38 degrees for the control rats and 57.83

degrees for the verapamil rats (Fig 2.4). A repeated measures ANOVA indicated that there was not a significant effect within subjects F (1, 14) = 3.496, $p = 0.083$). Over the 2-hour mass-training period the control and verapamil rats had similar heading angle error.

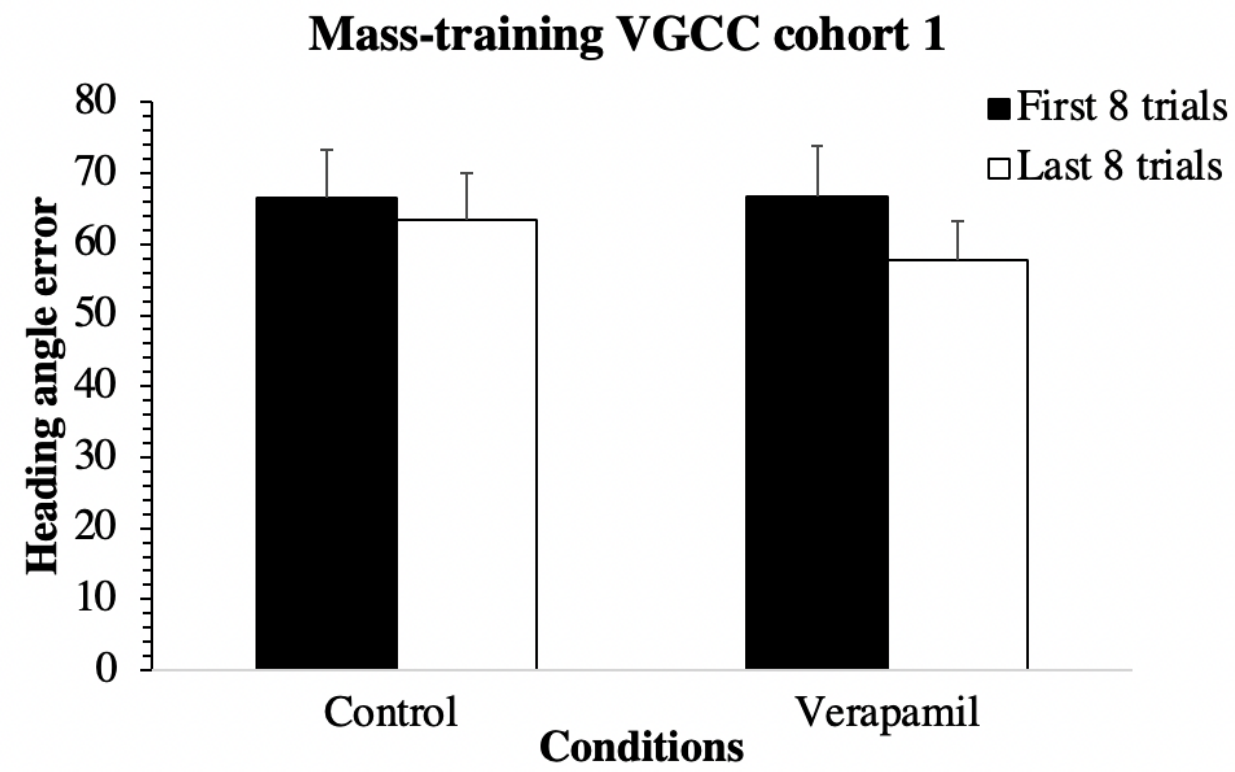

Figure 2.4: *Heading angle error was measured as the average deviation from the line between the rat starting point and the platform over the interval from second 1 to 2 during early vs. late in mass-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. All the rats show similar heading error throughout the mass-training session. Error bars: +/- 1 SE. Significance is denoted by "*".*

Probe

The probe test was done 24 hours after the completion of mass-training to assess the rat's quadrant preference for either the pre-trained or mass-trained location. The percentage of time spent in the two target quadrants was compared within and between the groups. The average time the control rats spent in the pre-trained quadrant was 26.7 % and 19.3 % in the mass-trained quadrant. The average time the verapamil rats spent in the pre-trained quadrant was 35.8 % and 12.6 % in the mass-trained quadrant (Fig 2.5). A repeated measures ANOVA revealed a

significant within subjects' effect of quadrant (F (1,14) = 10.194, $p < 0.007$). Post hoc pairwise comparison revealed a significant between the verapamil rats average time spent in pre-trained quadrant vs. mass-trained quadrant ($p = 0.04$), however no significant difference for the control rats. These results indicate that the control rats did not prefer either quadrant, however the verapamil rats preferred the pre-trained quadrant.

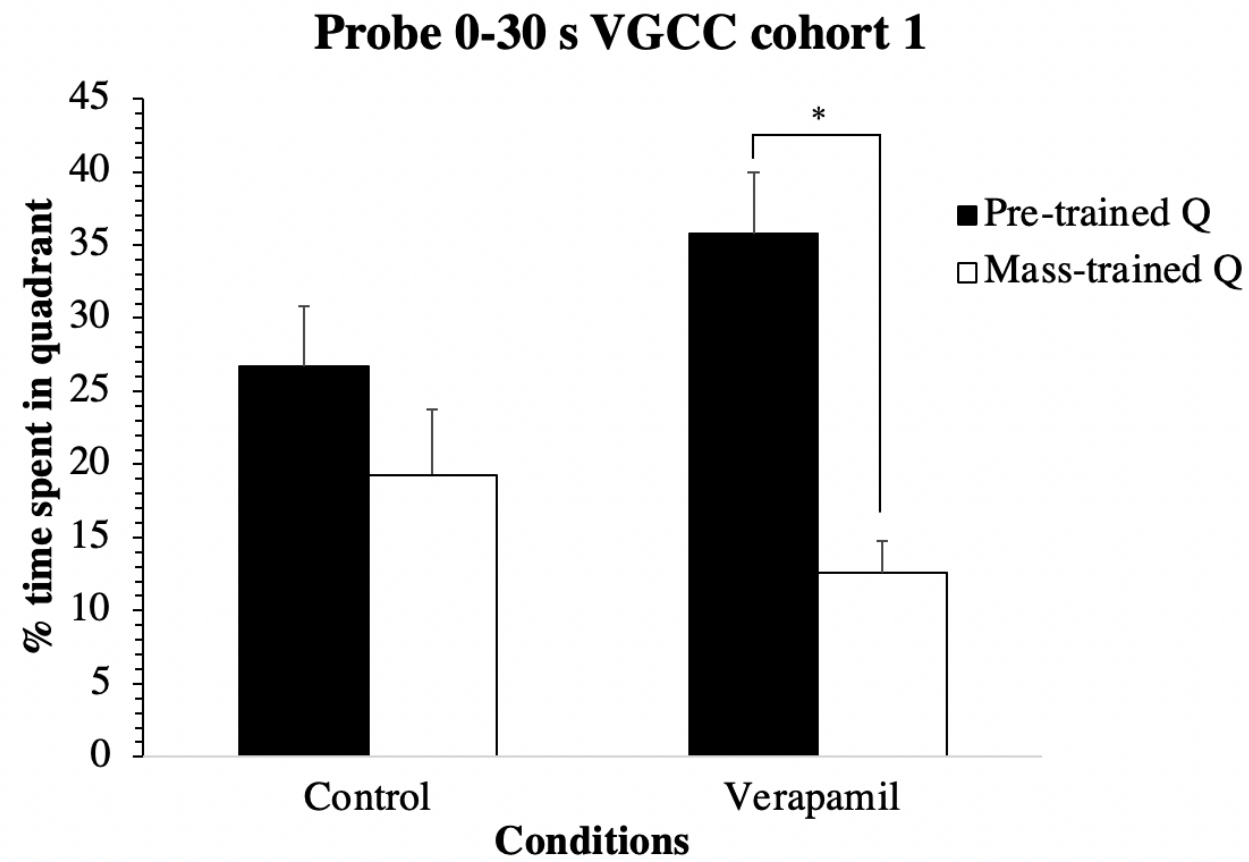

***Figure 2.5**: Percentage of time spent in pre-trained quadrant and mass-trained quadrant of rats in cohort 1, experiment 2, during 30 second probe test done 24 hours after mass-training. Verapamil rats display a preference for pre-trained quadrant, control rats do not. P < 0.05, Error bars: +/- SE. Significance is denoted by "*".*

Cohort 2 – NMDA blocker

Pre-training

There was a total of 20 rats in cohort 2, 10 rats were in the control group and 10 rats were in the AP5 (NMDA blocker) group. Figure 3.1 represents the average of 2 trials across 2 days of pre-training. The average path length of the first trial block was 1407.4 cm for control rats and 1269.6 cm for pre-AP5 rats. At the end of pre-training phase, the path length for the last trial block was 371.7 cm for control rats and 273.8 cm for pre-AP5 rats. Sphericity test was violated ($\varepsilon = 0.623$), Greenhouse-Geisser corrected results are reported. A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(4.36, 78.477) = 27.105$, $p < 0.001$) with no effect of group and no interaction. Both the control and pre-AP5 rats had a significant difference in path length from trial block 1 to 3 ($p < 0.001$). Both groups successfully learned the pre-training platform location.

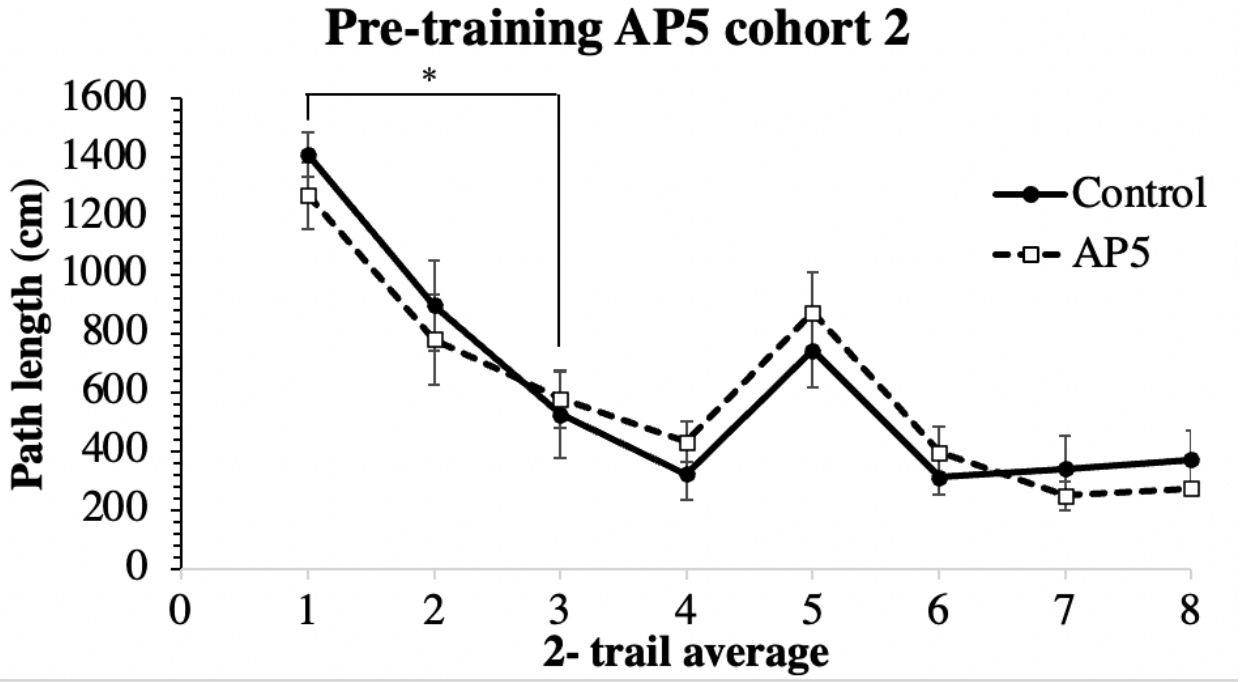

Figure 3.1: *Path length to platform as a 2-trial average during pre-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 days, 8 trials per day. Both groups learned. P < 0.001, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training

The mass-training phase was done 24 hours after the pre-training phase was done. The average path length of the first trial block during mass-training was 655.5 cm for control rats and 916.6 cm for AP5 rats. At the end of mass-training, the path length was 121.2 cm for control rats and 426.2 cm for verapamil rats (Fig 3.2). Sphericity test was violated ($\varepsilon = 0.623$), Greenhouse-Geisser corrected results are reported. A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(4.794, 86.291) = 8.162$, $p < 0.001$). There was no effect of group and no interaction. Post hoc pairwise comparisons revealed a significant effect of trial block 1 vs. 8 ($p = 0.036$) for the control rats. As well as a significant effect of trial block 1 vs. 5 ($p < 0.028$) for the AP5 rats. Over the 2-hour mass-training period both groups had a significant decrease in their path length.

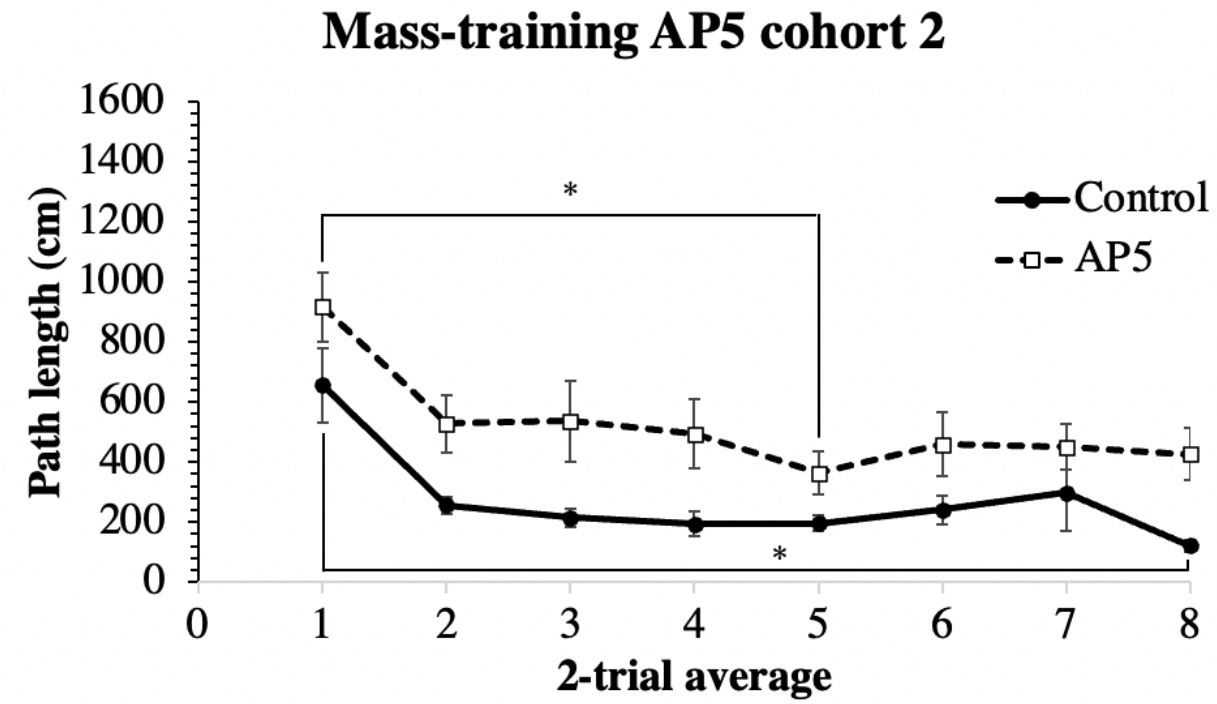

Figure 3.2: *Path length to platform as a 2-trial average during mass-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. All rats displayed learning. Control rats p = 0.036, AP5 rats p = 0.028, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training was also analyzed as the first 8 trials average (early in training) and last 8 trials average (late in training). The average of the first 8 trials was 330.2 cm for the control rats and 618.5 cm for the AP5 rats. The average of the last 8 trials was 214 cm for the control rats and 424.6 for the AP5 rats (Fig 3.3). A repeated measures ANOVA indicated that there was a significant effect within subjects F (1, 18) = 9.890, $p < 0.006$). Post hoc pairwise comparisons revealed a significant difference between the AP5 rats first 8 vs. last 8 trial averages ($p = 0.012$), however no significant difference of the control rats. From early to late in the mass-training period the AP5 rats had a significant decrease in their path length, whereas the control rats did not.

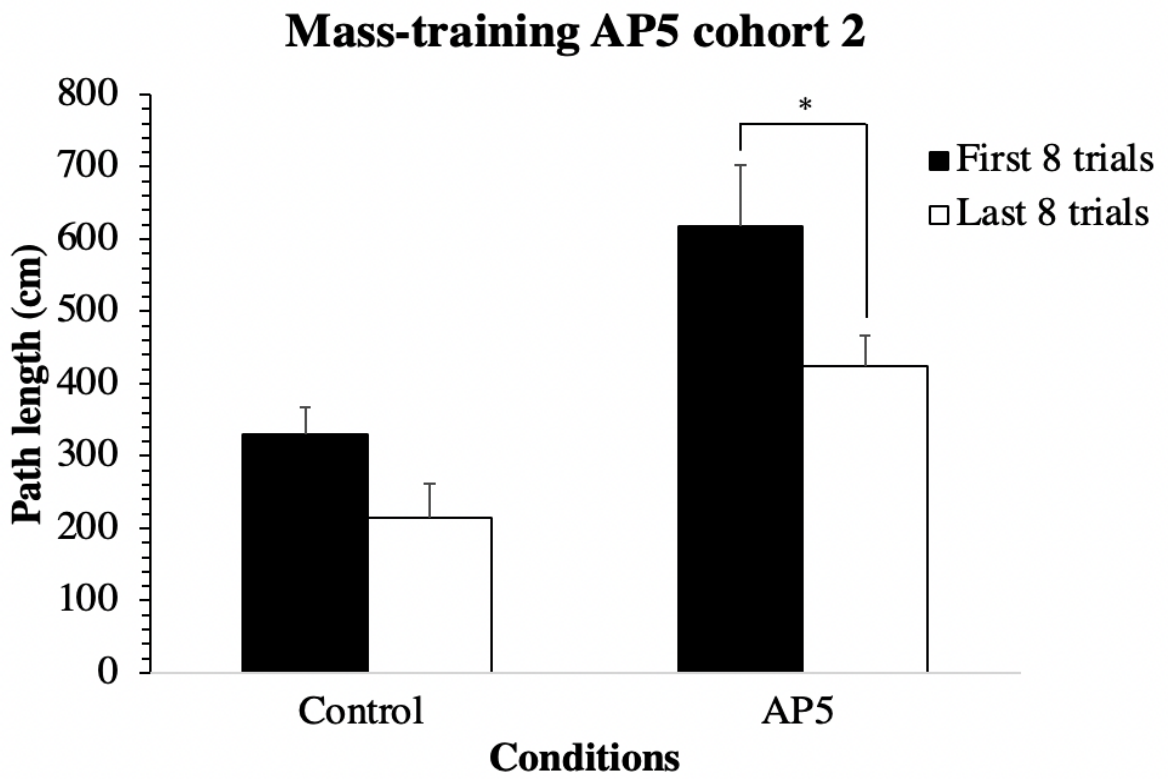

Figure 3.3: *Path length to platform early vs. late in mass-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The AP5 rats show significant learning whereas the control rats do not. P = 0.012, Error bars: +/- 1 SE. Significance is denoted by "*".*

Heading error was analyzed for the first 8 trials and the last 8 trial average. The average of the first 8 trials was 50.42 degrees for the control rats and 59.31 degrees for the AP5 rats. The average of the last 8 trials was 40.22 degrees for the control rats and 55.35 degrees for the verapamil rats (Fig 3.4). A repeated measures ANOVA indicated that there was a significant effect within subjects F (1, 18) = 6.978, $p = 0.017$). Post hoc pairwise comparisons revealed a significant difference between the control rats first 8 vs. last 8 trial averages ($p = 0.015$), however no significant difference of the AP5 rats. Over the 2-hour mass-training period the control rats had a decrease in their heading angle error, whereas the verapamil rats did not.

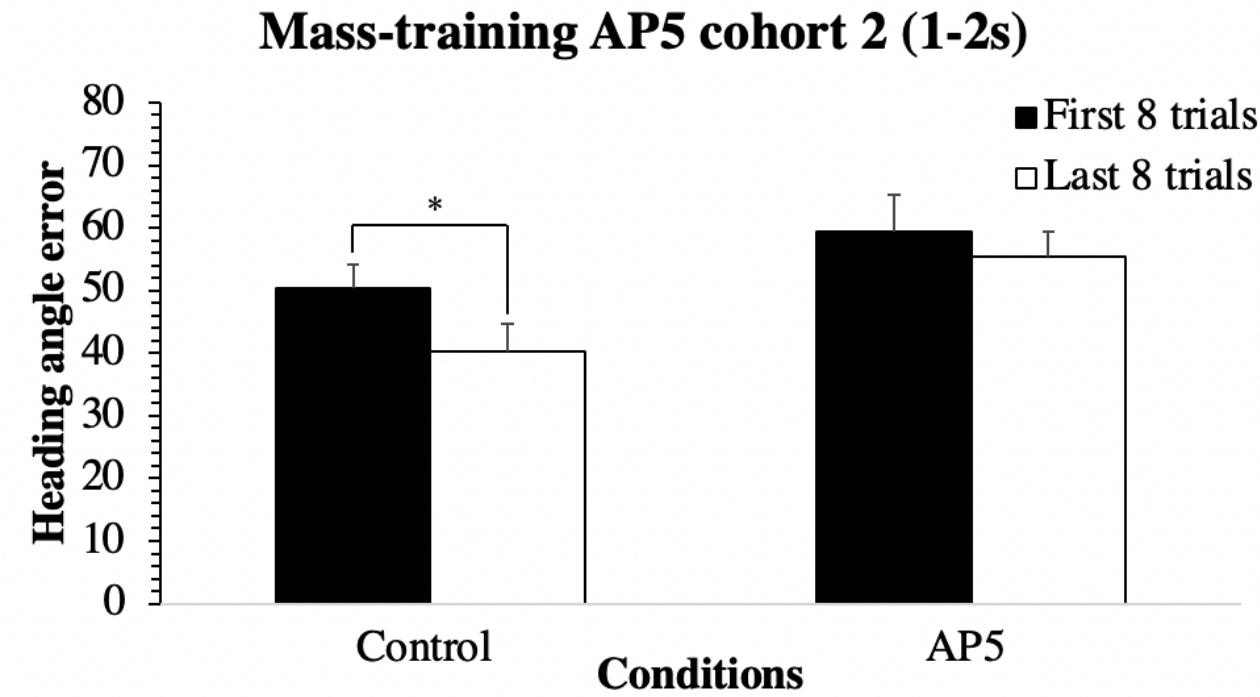

Figure 3.4: *Heading angle error was measured as the average deviation from the line between the rat starting point and the platform over the interval from second 1 to 2 during early vs. late in mass-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The control rats significantly decreased their heading angle error while the AP5 rats did not. Error bars: +/- 1 SE. Significance is denoted by "*".*

Probe

The probe test was done 24 hours after the completion of mass-training to assess the rat's quadrant preference for either the pre-trained or mass-trained location. The percentage of time spent in the two target quadrants was compared within and between the groups. The average time the control rats spent in the pre-trained quadrant was 21.6 % and 25.13 % in the mass-trained quadrant. The average time the AP5 rats spent in the pre-trained quadrant was 28.8 % and 15.6 % in the mass-trained quadrant (Fig 3.5). A repeated measures ANOVA revealed a significant within subjects' effect of quadrant ($F(1,18) = 6.565$, $p < 0.020$). Post hoc pairwise comparison revealed a significant difference between the AP5 rats average time spent in pre-trained quadrant vs. mass-trained quadrant ($p = 0.014$), however no significant difference for the control rats. These results indicate that the control rats did not display a significant preference for either quadrant, however the AP5 rats preferred the pre-trained quadrant.

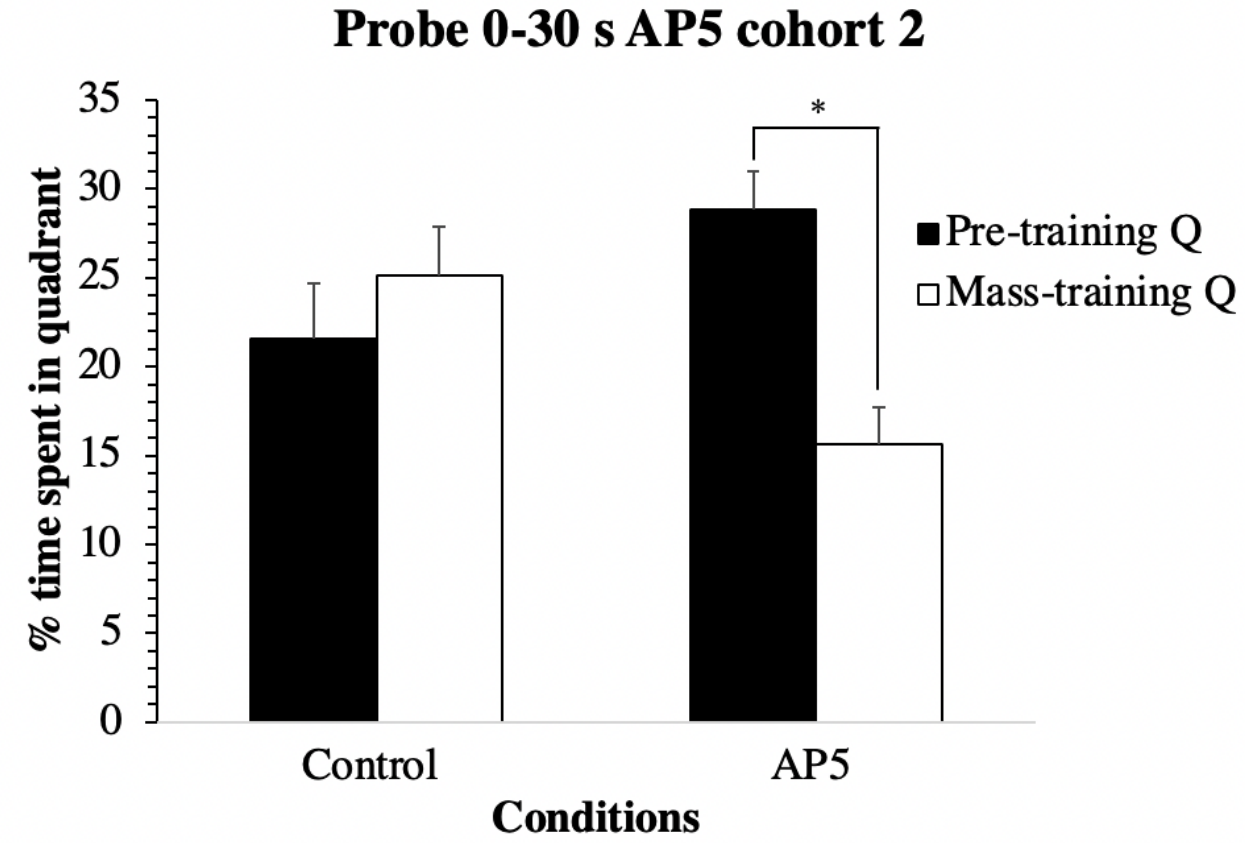

Figure 3.5: *Percentage of time spent in pre-trained quadrant and mass-trained quadrant of rats in cohort 2, experiment 2, during 30 second probe test done 24 hours after mass-training. AP5 rats display a preference for pre-trained quadrant, control rats do not. P = 0.014, Error bars: +/- SE. Significance is denoted by "*".*

Cohort 3 – Combo Blocker

Pre-training

There was a total of 18 rats in cohort 1, 9 rats were in the control group and 9 rats were in the combo (VGCC and NMDA blocker) group. Figure 4.1 represents the average of 2 trials across 2 days of pre-training. The average path length of the first trial block was 1223.1 cm for control rats and 1039.8 cm for pre-combo rats. At the end of pre-training phase, the path length for the last trial block was 393.3 cm for control rats and 311.8 cm for pre-combo rats (Fig 4.1). A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(7, 112) = 10.561$, $p < 0.001$) with no effect of group and no interaction. Both the control and

pre-AP5 rats had a significant difference in path length from trial block 1 to 4 ($p = 0.008$). Both groups successfully learned the pre-training platform location.

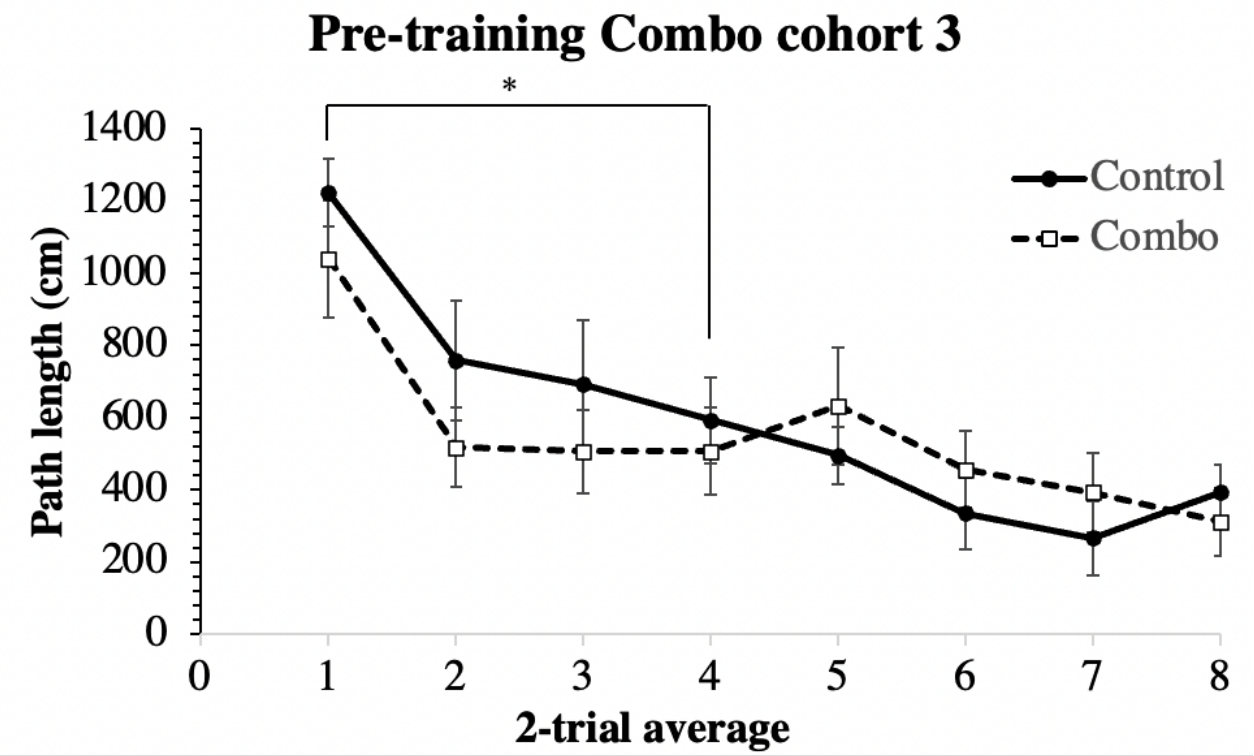

Figure 4.1: *Path length to platform as a 2-trial average during pre-training of all the rats in cohort 3, experiment 2 on the Morris Water Task. Rats were trained over 2 days, 8 trials per day. Both groups learned. P = 0.008, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training

The mass-training phase was done 24 hours after the pre-training phase was done. The average path length of the first trial block during mass-training was 1008.1 cm for control rats and 1027.5 cm for combo rats. At the end of mass-training, the path length was 319.8 cm for control rats and 437.1 cm for verapamil rats (Fig 4.2). Sphericity test was violated ($\varepsilon = 0.515$), Greenhouse-Geisser corrected results are reported. A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(3.603, 57.648) = 9.081$, $p < 0.001$). There was no between subject effect or interaction detected. Post hoc pairwise comparisons revealed a

significant effect of trial block 1 vs. 4 ($p = 0.015$) for both the control and combo rats. Over the 2-hour mass-training period both groups had a significant decrease in their path length.

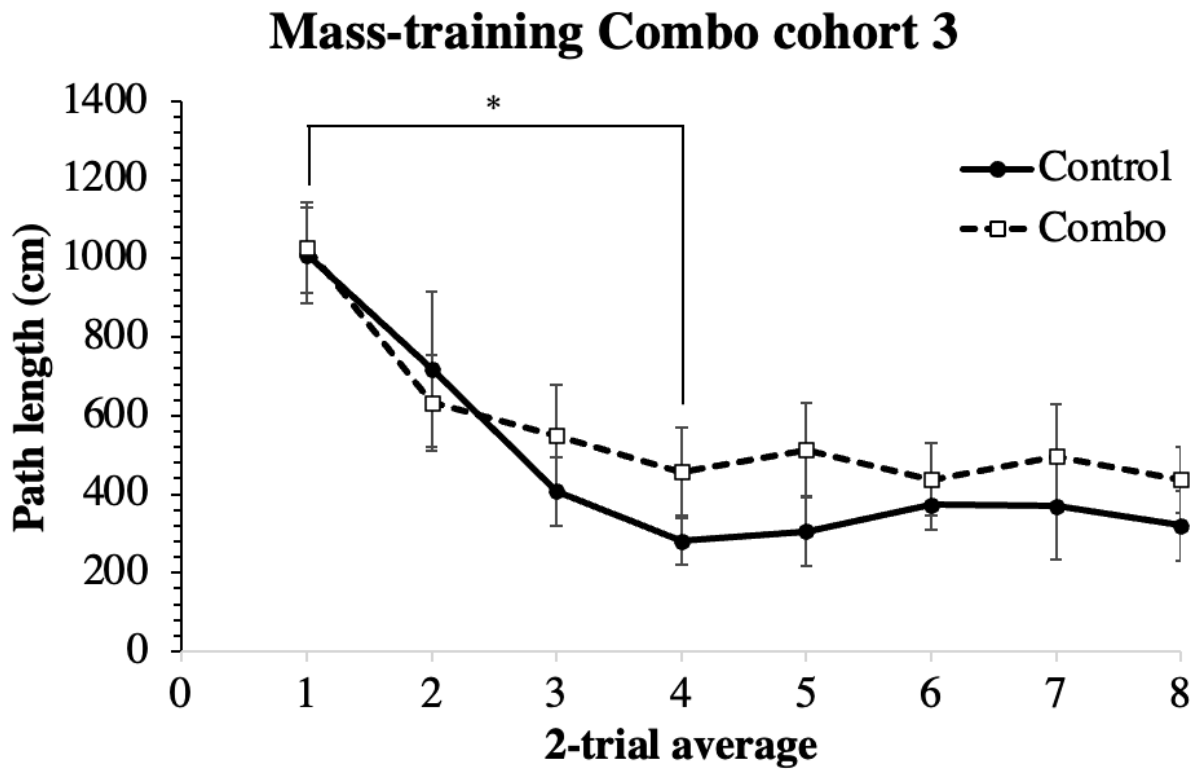

Figure 4.2: *Path length to platform as a 2-trial average during mass-training of all the rats in cohort 3, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. All rats learnt. P = 0.015, Error bars: +/- 1 SE. Significance is denoted by "*".*

Mass-training was also analyzed as the first 8 trials average (early in training) and last 8 trials average (late in training). The average of the first 8 trials was 603.6 cm for the control rats and 666.8 cm for the combo rats. The average of the last 8 trials was 342.1 cm for the control rats and 471.2 cm for the combo rats (Fig 4.3). A repeated measures ANOVA indicated that there was a significant effect within subjects $F(1, 16) = 18.151$, $p = 0.001$). Post hoc pairwise comparisons revealed a significant difference between the control rats first 8 vs. last 8 trial averages ($p = 0.003$), as well as a significant difference of the combo rats ($p = 0.02$). From early to late in the

mass-training period both the control and combo rats had a significant decrease in their path length.

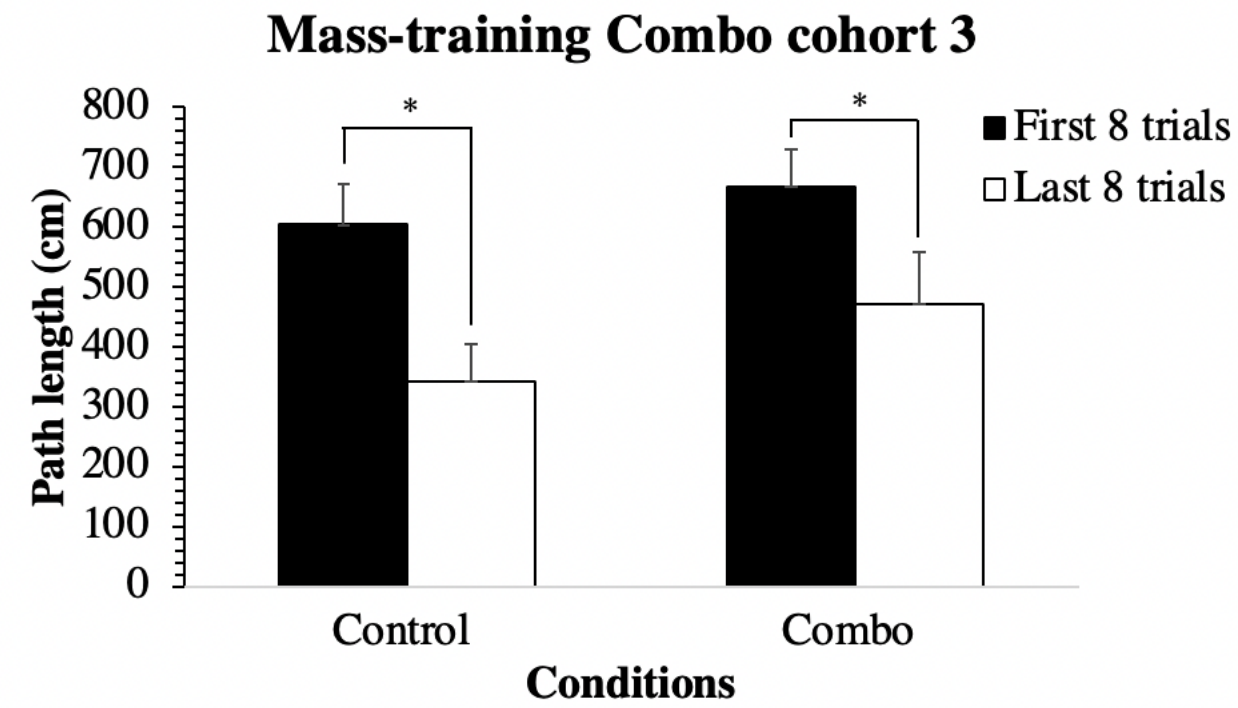

Figure 4.3: *Path length to platform early vs. late in mass-training of all the rats in cohort 3, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. Both the control and combo rats show significant learning. P = 0.003, p = 0.02, Error bars: +/- 1 SE. Significance is denoted by "*".*

Heading error was analyzed for the first 8 trials and the last 8 trial average. The average of the first 8 trials was 58.66 degrees for the control rats and 54.84 degrees for the combo rats. The average of the last 8 trials was 52.18 degrees for the control rats and 50.77 degrees for the verapamil rats (Fig 4.4). A repeated measures ANOVA indicated that there was not a significant effect within subjects F (1, 16) = 2.581, *p = 0.128*). Over the 2-hour mass-training period the control and combo rats had similar heading angle error.

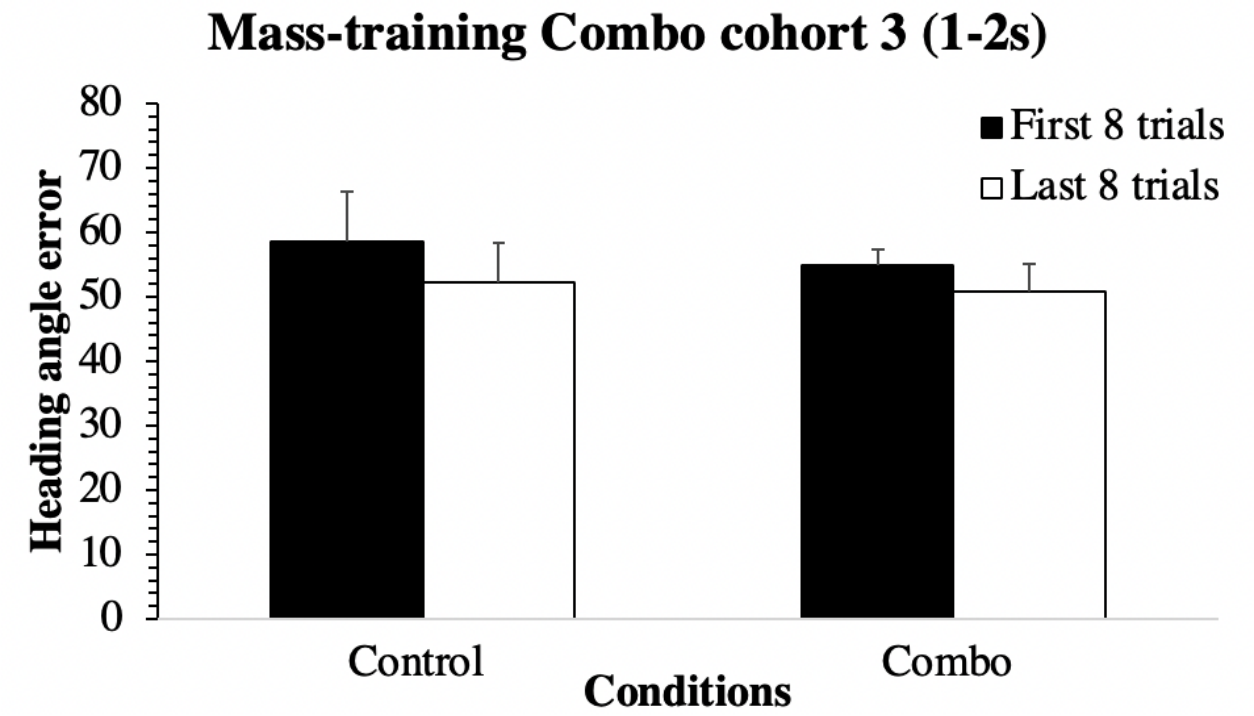

Figure 4.4: *Heading angle error was measured as the average deviation from the line between the rat starting point and the platform over the interval from second 1 to 2 during early vs. late in mass-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. All the rats show similar heading error throughout the mass-training session. Error bars: +/- 1 SE. Significance is denoted by "*".*

Probe

The probe test was done 24 hours after the completion of mass-training to assess the rat's quadrant preference for either the pre-trained or mass-trained location. The percentage of time spent in the two target quadrants was compared within and between the groups. The average time the control rats spent in the pre-trained quadrant was 22.2 % and 23.3 % in the mass-trained quadrant. The average time the combo rats spent in the pre-trained quadrant was 23.6 % and 22.8 % in the mass-trained quadrant (Fig 4.5). A repeated measures ANOVA revealed no significant within subjects' effect of quadrant, as well as no between subject's effect. These results indicate that that both the control and the combo rats did not prefer either quadrant location.

The probe results in *Figure 4.5* showed that both the control and combo rats had split their time between the pre- and mass trained location. Further analysis was done to assess the time each condition spent in the other quadrants as well. The average time the control rats spent in the SE quadrant was 40.1 % and 14.3 % in the NW quadrant. The average time the combo rats spent in the SE quadrant was 33.6 % and 19.9 % in the NW quadrant. The results indicate that the control rats preferred the pre- and mass-trained over the SE quadrant, whereas the combo rats split their time equally between the three quadrants.

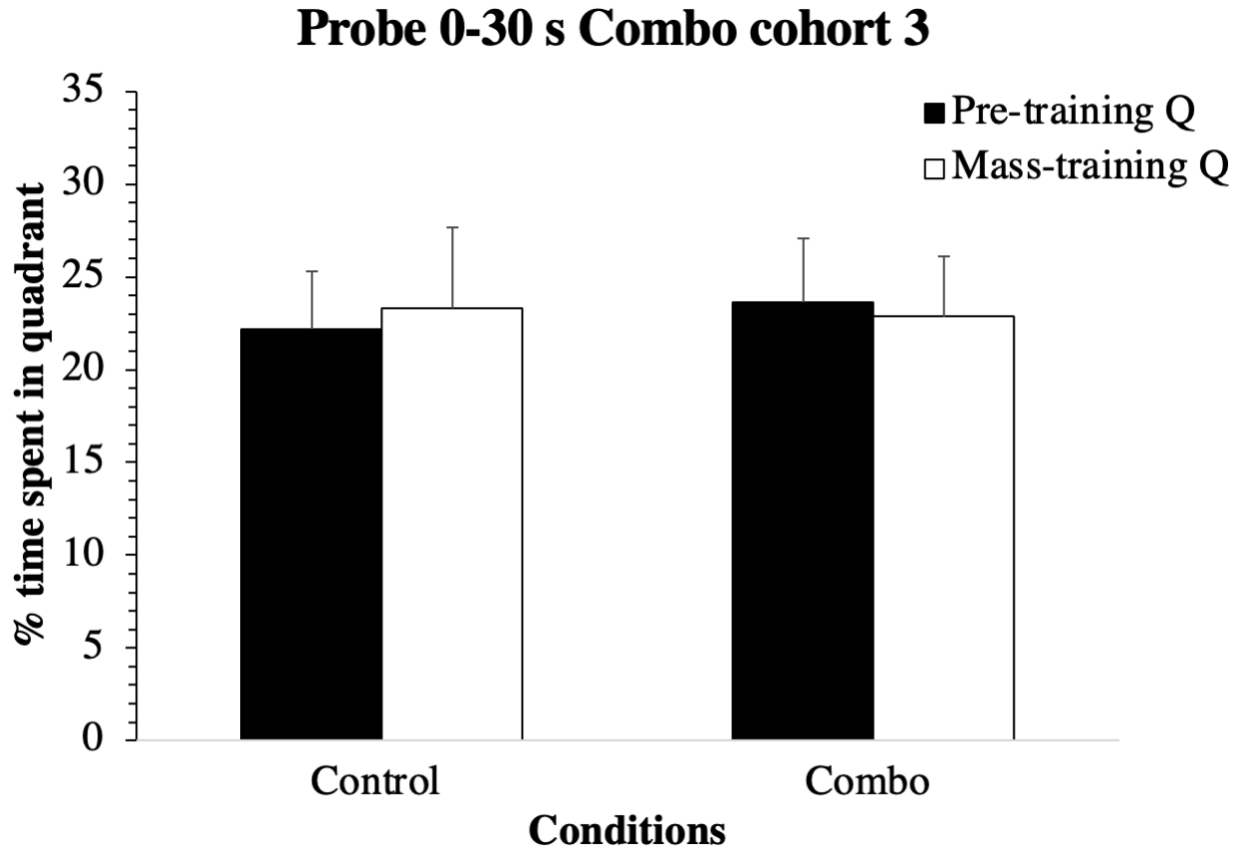

Figure 4.5: *Percentage of time spent in pre-trained quadrant and mass-trained quadrant of rats in cohort 3, experiment 2, during 30 second probe test done 24 hours after mass-training. Control and combo rats did not have a quadrant preference. Error bars: +/- SE. Significance is denoted by "*".*

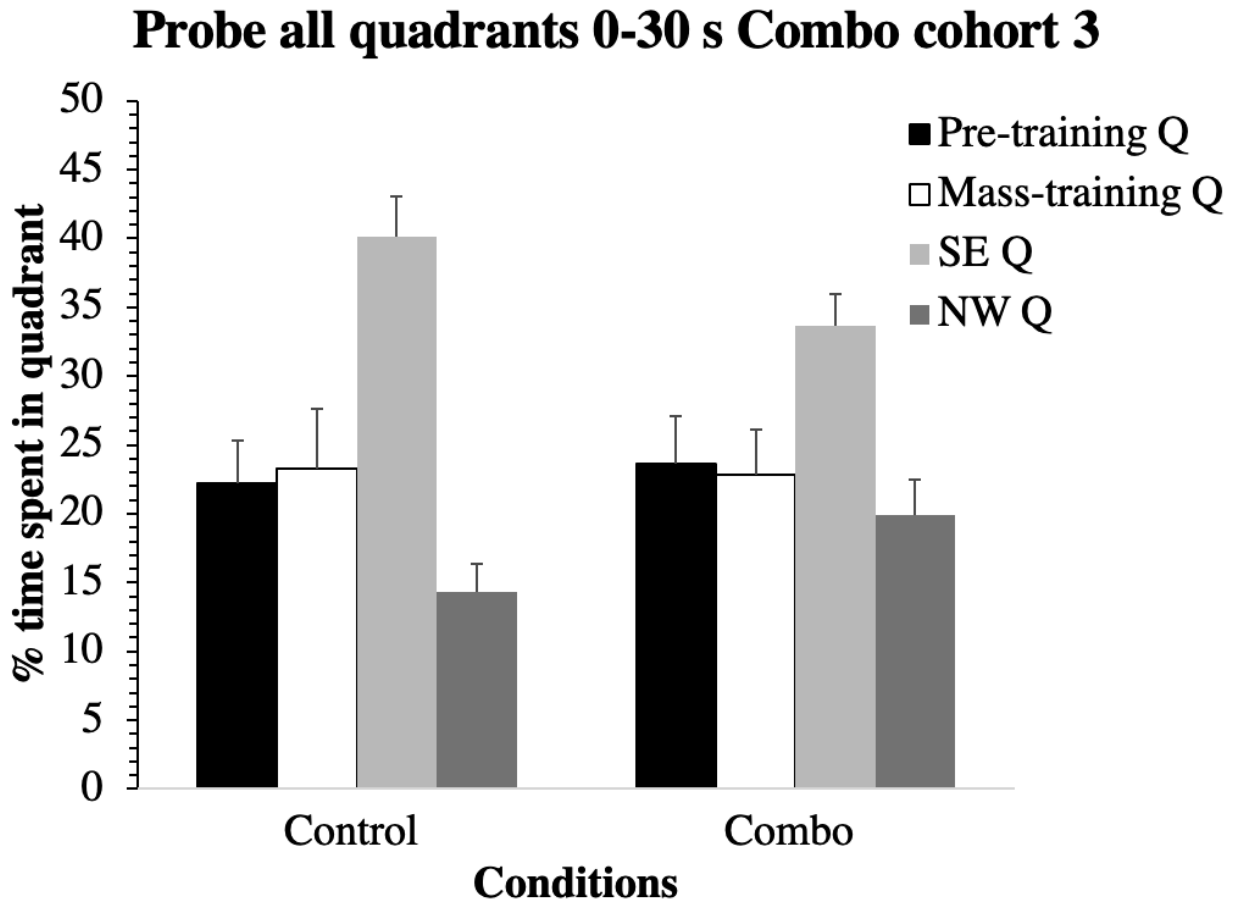

Figure 4.6: *Percentage of time spent all quadrant of rats in cohort 3, experiment 2, during 30 second probe test done 24 hours after mass-training. Control and combo rats both spent increased time in the SE Q (starting location). Control rats then split their time between pre- and mass-trained quadrant whereas the combo rats split their time between the pre, mass, and NW quadrants. Error bars: +/- SE. Significance is denoted by "*".*

Additional Analysis

At first glance some of the results discussed above lead to puzzling conclusions. In cohort 1, the verapamil (VGCC blocker) rats do not significantly decrease their pathlength throughout the mass-training session (Fig 2.2 and 2.3). This could lead to the conclusion that blocking VGCC impaired learning of the new platform location during mass-training. However, by the end of the mass-training session the path length of the verapamil rats was almost identical to the path length of the control rats. To further analyze if this conclusion was correct, all the experimental groups path length during the mass-training were graphed together (Fig 5.1). This

allows for direct comparison of all the drug groups, to identify if there were any differences in their performance.

The average path length of the first trial block during mass-training was 728.7 cm for verapamil rats, 916.6 cm for AP5 rats and 1027.5 cm for combo rats. At the end of mass-training, the path length 277.8 cm for verapamil rats, 426.2 cm for AP5 rats and 437.1 cm for combo rats (Fig 5.1). A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(7, 168) = 7.601$, $p < 0.001$). There was no between subject effect or interaction detected. Post hoc pairwise comparisons revealed a significant effect of trial block 1 vs. 4 ($p = 0.049$) for the combo rats. There was no significant difference in the verapamil or AP5 rats. Over the 2-hour mass-training period all drug groups path length decreased and did not significantly differ from each other, however the within effect of trial was only significant in the combo rats. These results suggest that all the drug group rats learned the new platform location as shown by a similar learning curve, despite only the combo rats showing significance throughout the session.

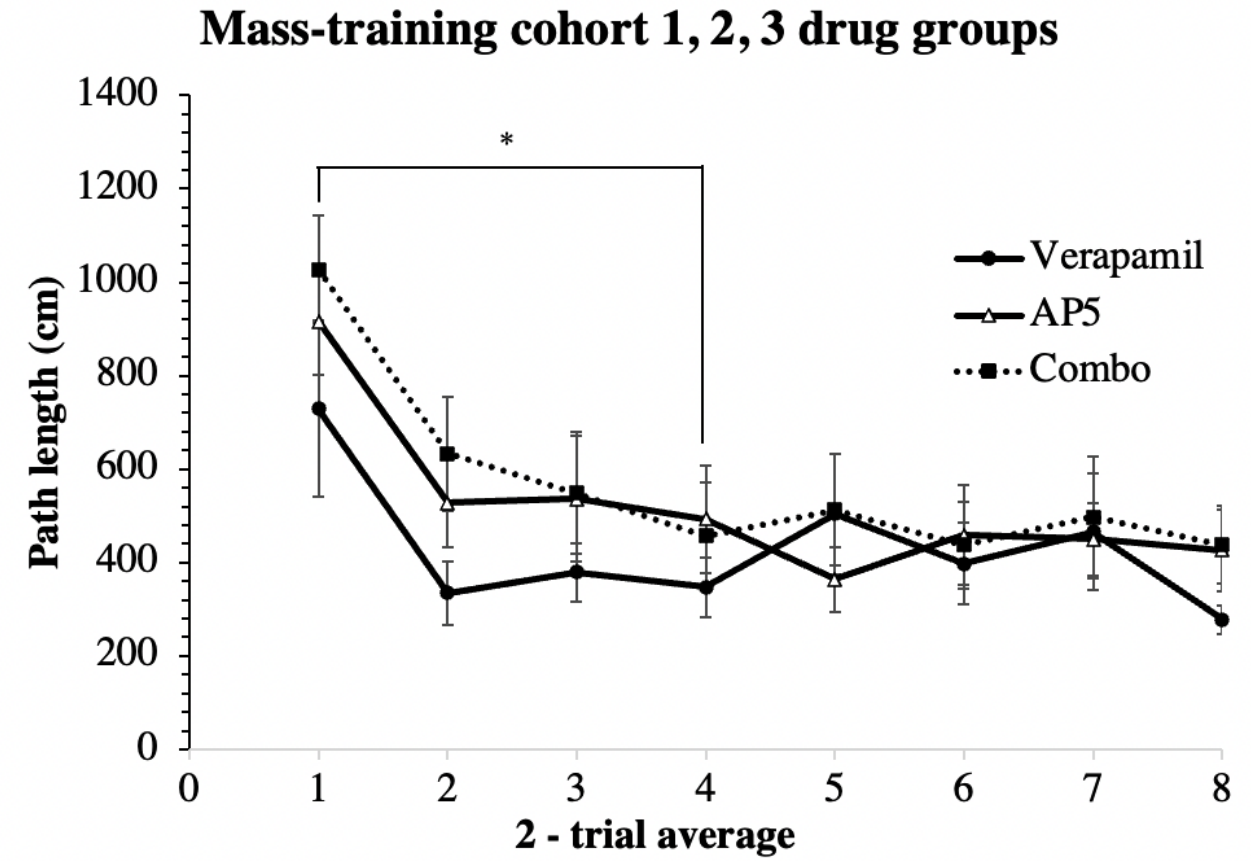

Figure 5.1: *Path length to platform as a 2-trial average during mass-training of all drug group rats in all 3 cohorts of experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. Combo rats has a significant decrease in path length p = 0.049, Error bars: +/- 1 SE. Significance is denoted by "*".*

Another unexpected result from the original analysis was in cohort 2. In figure 3.3 the control rats of this cohort did not display a significant difference early vs. late in mass-training session. However, figure 3.2 shows that by the last trial block they did have a significant decrease in their path length. To better understand the performance of the control rats, all the control rats from the different cohorts during mass-training was graphed together (Fig 5.2). This allows for direct comparison of all the control groups to see if there were any difference in their performance.

The average path length of the first trial block during mass-training was 999.7 cm for control verapamil rats, 493.6 cm for control AP5 rats and 1061.6 cm for control combo rats. At the end of mass-training, the path length 357.7 cm for control verapamil rats, 102.4 cm for control AP5 rats and 351.1 cm for control combo rats (Fig 5.2). Since sphericity is violated ($\varepsilon = 0.553$), Greenhouse-Geisser corrected results are reported. A repeated measures ANOVA indicated that there was a significant effect of trial on path length ($F(3.874, 77.485) = 15.726$, $p < 0.001$). There was no interaction detected, however there was between subjects' effect ($F(1, 20) = 11.135$, $p < 0.001$). Post-hoc pairwise comparisons revealed significant difference in the verapamil control condition between trial 1 and 4 ($p = 0.014$). Post-hoc pairwise comparisons revealed a significant difference in the combo control condition between trial 1 and 3 ($p = 0.028$). Post-hoc pairwise comparisons did not reveal a significant difference within the AP5 control condition. Post-hoc pairwise comparisons revealed a significant difference in the first trial block between groups, verapamil rats vs. AP5 rats ($p = 0.036$), combo rats vs. AP5 rats ($p = 0.013$). Over the 2-hour mass-training period the VGCC controls and Combo controls had a significant decrease in path length, whereas the AP5 controls did not. The starting path length of the AP5 controls significantly differed from the starting path length of the verapamil and combo control rats. These results indicate that control AP5 rats had an unusually short path length at the beginning of the mass-training session and their performance was different than the other control rats due to having a shorter path length.

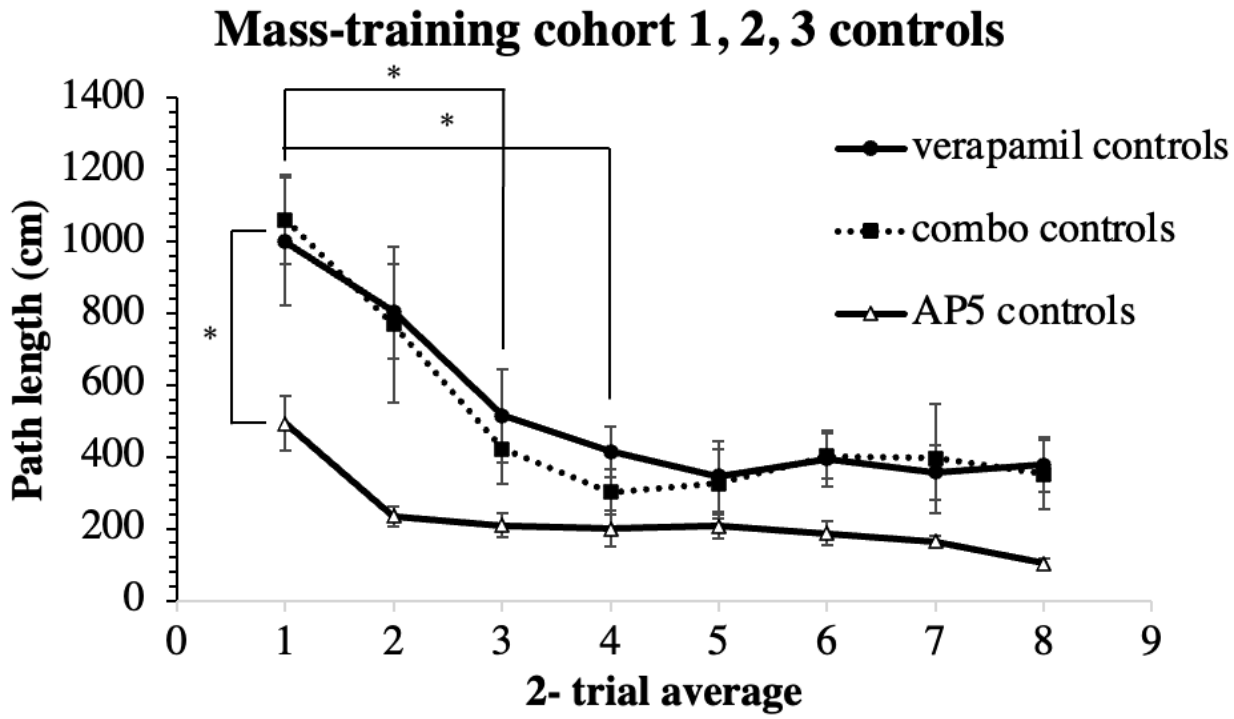

Figure 5.2: *Path length to platform as a 2-trial average during mass-training of all control group rats in all 3 cohorts of experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. Verapamil and combo controls had a significant decrease in path length (p = 0.014) and (p = 0.028) respectively. Verapamil and combo control were significantly different than AP5 control in the first trial block (p = 0.036) and (p = 0.013) respectively. Error bars: +/- 1 SE. Significance is denoted by "*".*

The swim path of a rat chosen at random from each experimental condition at the end of the mass-training session was examined (Fig. 5.3). As seen by previous measures there is not much difference in the swim path of the rats from each group. The swim path shows that all the rats regardless of the experimental condition learned the new platform location during the mass-training session.

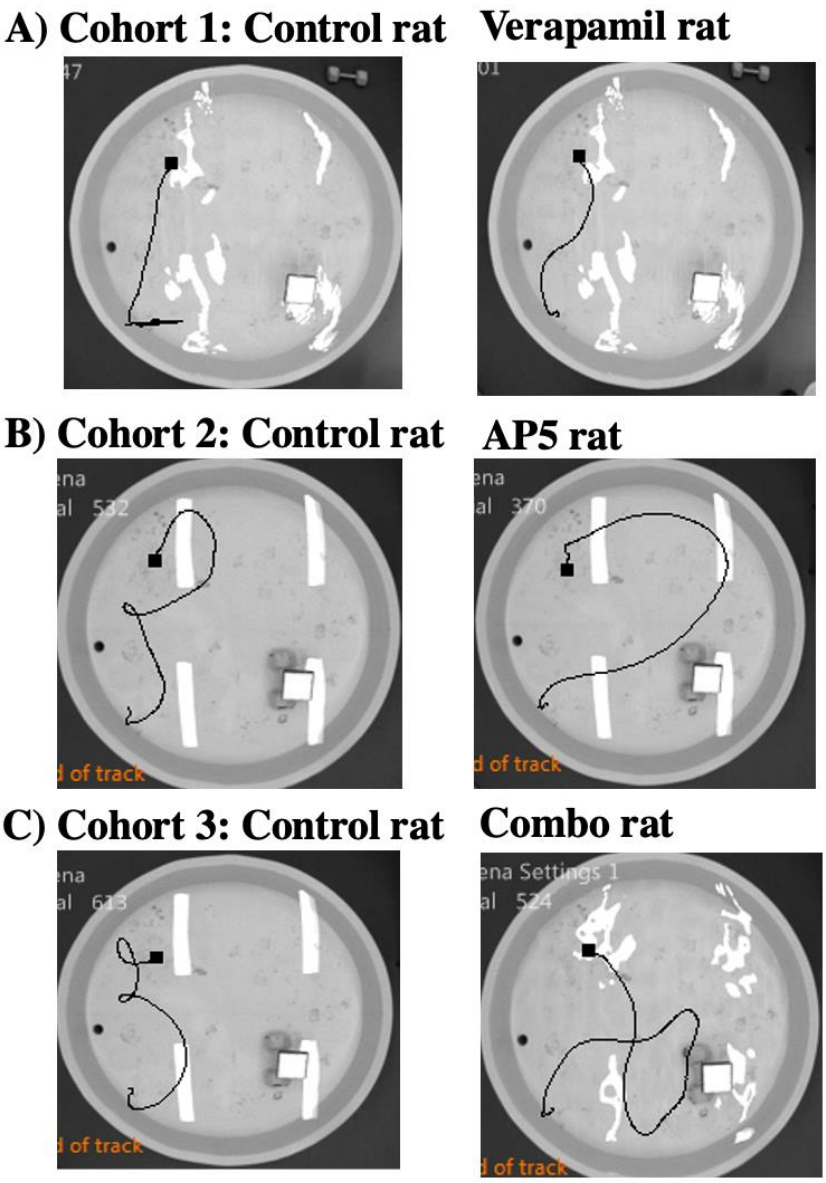

Figure 5.3*: The swim path to platform for a typical rat in each condition at the end of the mass-training session (trial 15).*

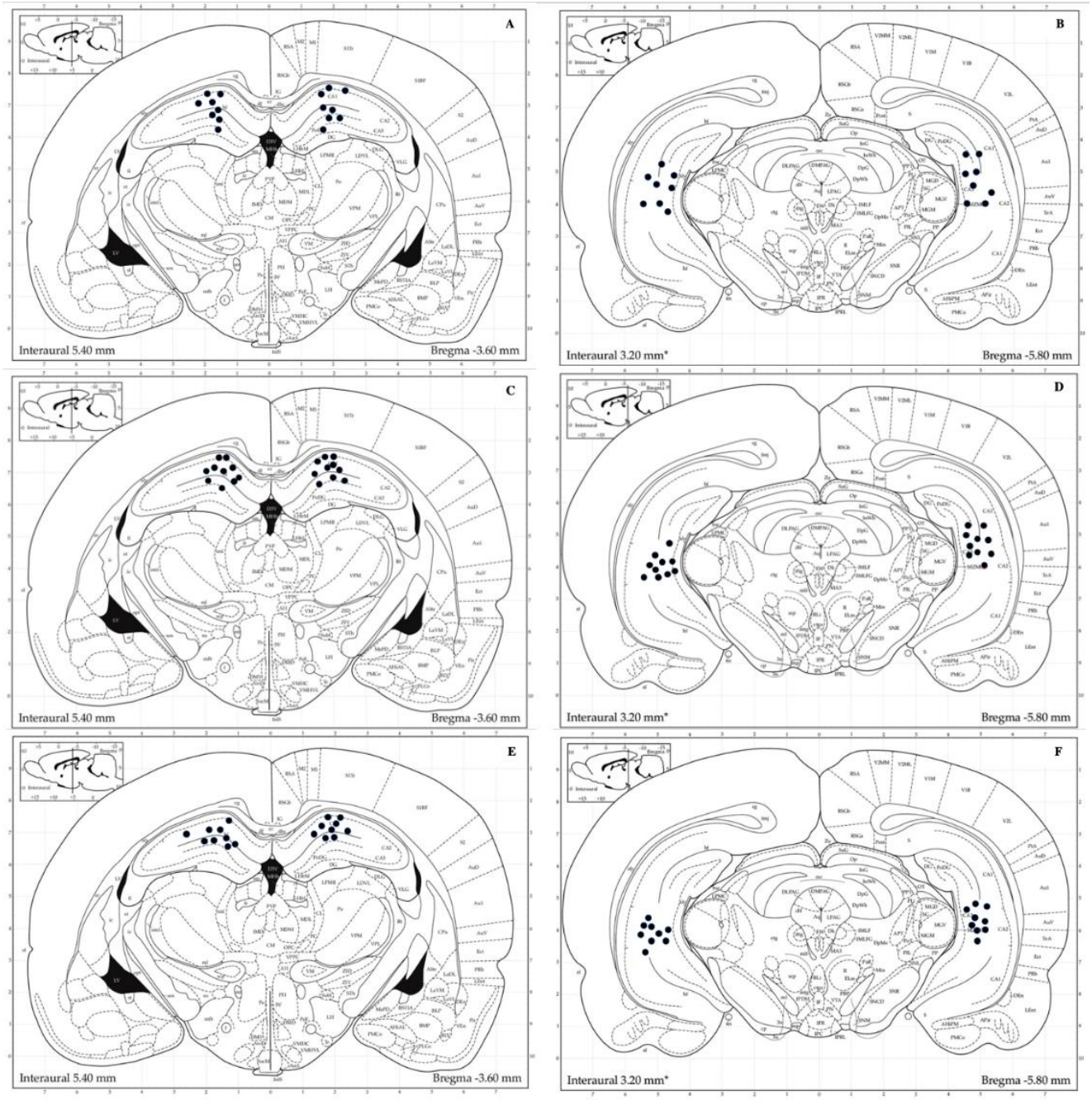

Figure 5.4*: Dorsal and ventral hippocampal cannulation placements for VGCCs and NMDARs blocker rats in experiment 2. Cannula end points were taken from hippocampal slices and imposed onto images of the dorsal and ventral hippocampi.* ***A****. dorsal cohort 1 (verapamil rats),* ***B****. ventral cohort 1 (verapamil rats),* ***C****. dorsal cohort 2 (AP5 rats),* ***D****. ventral cohort 2 (AP5 rats),* ***E****. dorsal cohort 3 (combo rats),* ***F****. ventral cohort 3 (combo rats).*

Discussion

In this experiment, rats with blocked NMDARs and/or VGCCs where trained in a three-phase version of the MWT. All the rats were given 2 days (16 trials) of pre-training to a hidden platform. Then receptor blockade was administered, and they were mass-trained (16 trials) to a new platform location in the same room to evaluate their learning. The rats were given a probe trial to assess their memory of the mass-trained platform.

Pre-training learning

There were no significant differences between all the rats during the pre-training phase. All the rats had an increased path length to locate the platform at the start of pre-training and their path length significantly decreased by the end of training. From this it can be concluded that all the rats learned the pre-training platform location.

Mass-training 2-trial average learning

During the mass-training the rats were trained to a new platform location, in the opposite quadrant (NE) to the pre-trained location in the pool. Cohort 1 (control and verapamil rats) both had an increased path length during the first trial block. This was because the platform location was moved to a new location than that during pre-training. The control rats had a significantly decreased path length throughout the mass-training, whereas the verapamil rats did not. At first glance it seems like the blockade of VGCCs may have caused a learning impairment, however the graph trend demonstrated a decreased in path length. This result will be further explored later in the discussion. Cohort 2 (control and AP5 rats) also had an increased path length during the first trial block. Again, this is understandable because the platform was moved to a new location. The control rats took all training session to show a significant decrease in path length, whereas the AP5 rats had a significantly decreased path length approximately half-way through the session. The results indicate that the AP5 rats were able to properly learn the new platform

location. The control ratsalso learnt the new platform location, however from the data it seems like the control rats acquired the new platform location slower relative to the AP5 rats. These results will also be further explored. Cohort 3 (control and combo rats) had an increased path length during the first trial block. As seen before this is a reliable trend that occurs because the rats first look in the pre-trained location before realizing that it is no longer there and begin to search all around the pool. The control and combo rats displayed a very similar learning curve, both demonstrated a significantly decreased path length half-way through the training session. The significantly decreased path length is suggestive of learning. As such the control and combo rats were able to learn a new platform location.

Mass-training early vs. late learning

Another way to analyze if learning occurred during the mass-training session is to look at the average path length of each rat early (1-8 trials) vs. late (9-16 trials) in training. Cohort 1 control rats show a significant decrease early in the training vs. late in training, clearly indicating learning. The verapamil rats on the other hand do don't show a significant decrease in early vs. late. Interestingly, the verapamil rats early in training average is very similar to the control rats late in training average. This suggests that the reason why there was not a significant decrease in path length in because the verapamil rats path length is already short and close to asymptote levels of performance. Meaning that just because there was not a significant decrease in path length this does not mean that they did not learn the platform location. If compared the late in training path lengths of the control and verapamil rats, there is no significant difference, arguing that the verapamil rats did learn the new platform location. Cohort 2 control rats show a similar trend to the verapamil rats in cohort 1. Control rats do not show a significant decrease in early vs. late in training path length. However, the average early path length is very short and therefore, even though the late in training path length did decrease it was not enough to indicate a

significant difference. Nonetheless, given the path length it would be okay to assume that the control rats in cohort 2 did learn the new platform location. The AP5 rats show a significant decrease of early vs. late in training path length, which indicates that they learned the new platform location. Cohort 3 control and Combo rats show a significant decrease in early vs. late in training path length, which indicates that they learned the new platform location.

Heading angle error during mass-training

The heading angle error was analyzed early vs. late in mass-training to see if there was any difference between the conditions. The initial heading angle during second 1 to 2 can provide information about the rats learning of the new platform location during mass-training. Rats that knew the location and went straight to it, had a lower number whereas rats that were heading away from the platform location had a bigger number. Cohort 1 control and verapamil rats both have similar heading angle error and don't significantly differ from each other. Meaning that when VGCCs are blocked rats were able to learn a new platform location. In cohort 2, the control rats have lower heading angle error compared to the AP5 rats. At first instance this might be seen as an impairment of the AP5 rats, however this is not likely. The control AP5 rats have a lower number even compared to the control rats in the other cohorts. Furthermore, the AP5 rats heading angle error is like the other cohorts control rats. What most likely occurred is that the control rats of cohort 2 are an anomaly and not typical controls. This was also the case when path length was analyzed previously (Fig. 5.2) when the cohort 2 controls were significantly faster than the other cohorts' controls. Therefore, it can be concluded that blocking NMDARs does not impair rapid new learning to occur. Cohort 3 controls and combo rats both have similar heading angle error and don't significantly differ from each other. Meaning that when VGCCs and NMDARs are blocked rats were able to learn a new platform location in the same context as pre-training.

Probe memory

A 30 second probe test was done 24 hours after the mass-training to investigate VGCCs or NMDARs blockers impact on the rat's long-term memory of the mass-trained location. Given chapter II results it was expected that the control rats would show a reliable preference for the mass-trained quadrant location in the probe. Unfortunately, this was not the case with any of the control rats in all 3 cohorts. The control rats did not have a significant preference for either the pre-trained quadrant or the mass-trained quadrant, splitting their time between both locations. As a result, the probe data is hard to interpret and compare with the drug groups. Nonetheless, one can compare the behaviour of the controls (splitting time equally between both pre- and mass-trained quadrants) with the behaviour of the drug groups. Cohort 1 verapamil rats showed a strong preference for the pre-trained location during the probe. This may indicate that the mass-trained location learning did not consolidate as well as the pre-trained location. Similarly, Cohort 2 AP5 rats showed preference for the pre-trained location during the probe. Again, this may mean that AP5 rats did not consolidate the mass-trained location. Surprisingly, Cohort 3 combo rats showed the same behaviour as the control rats, not having a preference and splitting their time equally between the pre- and mass-trained locations. However, when looked further and assessed the time the control and combo rats in cohort 3 spent in each of the four quadrants. The results show that the control rats preferred the pre- and mass trained location over the other quadrants, whereas the combo rats split their time between all the quadrants. This indicates that the controls did have a strong preference for both pre- and mass trained locations, but the combos where randomly going to all the quadrants. This could imply that there was a consolidation effect and memory might have been affected due to the NMDA and VGCC blockade.

Learning across cohorts

These experimental cohorts were conducted successively, and statistical comparison between them should be treated with caution. However, to look further into the unexpected

results from Cohort 1 verapamil rats and the Cohort 2 control rats on mass-training day, additional analysis was done. The 3 experimental groups from the different cohorts were plotted all in one graph and the 3 control groups in another. The combo rats showed a significant decrease in path length throughout the mass-training day when plotted against the other two experimental groups. The verapamil and AP5 rats did not show a significant decrease in path length throughout the mass-training day. However, both verapamil and AP5 rats show a similar learning curve to the combo rats, differing only slightly on the first trial block path length (the combo rats have a slightly longer path length). Taking this into account it could be argued that the reason the verapamil and AP5 rats don't show a significant decrease is because their initial path length is shorter and not because of a learning impairment. This most likely means that all the experimental groups learned the new platform location.

All the control rats from the 3 different cohorts were plotted together for the mass-training day to investigate the AP5 control rat's inconsistent results previously mentioned. Both the verapamil and combo control rats had a significant decrease in path length and show a standard learning curve. The AP5 controls on the other hand, begin the mass-training at almost an asymptote level of performance and even though not significant, decrease their path length throughout the mass- training session. Because of this the AP5 control rats performed significantly different when compared to the verapamil and combo controls. Overall, it can be concluded that the AP5 control rats learned the new platform location because the trend line and learning curve is very similar to the other control group rats.

The swim paths from a rat chosen at random from each of the experimental groups and their respective control was analyzed as well. The swim paths for all the rats regardless of which groups they were part of have a direct swim path to the platform location. Within the first 5-10 seconds all the rats found their way to the platform location by the end of the mass-training

session. This provides further evidence to support the claim that all the rats learned the new platform location.

Answering the hypotheses

Hypothesis 1 stated that rats would be able to acquire a new spatial location with familiar information with VGCCs blocked and long-term memory would be impaired. It can be argued that the verapamil rats did learn a new location even though there was not a significant decrease in path length because; 1) the learning curve is similar to the control rats; 2) late in mass-training the verapamil rats had very similar path length and heading angle error as the controls; 3) when compared to the combo rats they are not significantly different from each other and the combo rats also had the same dosage of verapamil; 4) swim paths is almost identical to the control rats. The reason VGCCs may not be crucial for this new learning is because rats have already constructed a representation of the environment (during pre-training) and learning a new location with familiar information requires less calcium influx which can occur via other receptors like NMDARs (Crestani et al., 2019; Kroker et al., 2011). When comparing the control rats and verapamil rats probe data one could make the case that the verapamil had a weaker representation of the mass-trained location than the control rats. It is hard to conclude that they had an impairment in their consolidation or long-term memory because the control rat's behaviour was not as expected but working off the available evidence this could be the case. Prior research has suggested that VGCCs and NMDARs activity play a different role in learning and memory, specifically, that VGCCs are more involved in memory consolidation and long-term expression (Bauer et al., 2002; T. C. Foster, 2012; McKinney et al., 2008; Woodside et al., 2004). This might be the reason the verapamil rats showed the preference for the pre-trained location instead of the mass-trained (new) location. In summary hypothesis 1 can be partially accepted, because

blocking VGCCs did not impair learning of a new spatial location with familiar information but may have impaired long-term memory.

Hypothesis 2 stated that rats would be able to acquire a new spatial location with familiar information with NMDARs blocked and long-term memory would be impaired. AP5 rats were able to learn a new platform location while having blocked NMDARs. This result is supported by previous studies (Bye & McDonald, 2019; McDonald et al., 2005). As could be the case with VGCCs, if NMDARs are inactive when new learning with familiar information is occurring there might be less amount of calcium influx needed, which could be occurring via other receptors. AP5 rats during the probe behaved the same as the verapamil rats and showed a preference for the pre-trained location. If compared to the control rats which spent an equal amount of time in the pre- and mass-trained locations, it may indicate an impairment of consolidation and long-term memory. Indeed, Bye & McDonald (2019) reported similar results, finding that CPP (NMDAR antagonist) rats also preferred the pre-trained location and not the mass-trained location. In summary hypothesis 2 can be partially accepted, because blocking NMDARs did not impair learning of a new spatial location with familiar information and may have impaired long-term memory.

Hypothesis 3 stated that rats would not be able to acquire a new spatial location with familiar information and long-term memory would be impaired with VGCCs and NMDARs blocked. The results indicated that the combo rats which had both VGCCs, and NMDARs blocked were able to learn the new platform location. This is contradictory to what was hypothesized, however an explanation to this might be because there are still alternative plasticity mechanisms by which calcium influx can occur independent of VGCCS and NMDARs. For example, metabotropic glutamate receptors (mGluRs) have been shown to elicit NMDARs independent LTP in vitro (Kroker et al., 2011; Oliet et al., 1997; H. Wang et al., 2016) and in

vivo (Crestani et al., 2019). These receptors are metabotropic which means that when activated cause an internal conformational change in the receptor (Reiner & Levitz, 2018). This initiates a signaling cascade involving the inositol pathway leading to the endoplasmic reticulum activation and eventually causing intra-neuronal calcium release (Reiner & Levitz, 2018). This is a plausible mechanism by which combo rats which have already been pre-trained in an environment and are only required to learn a new platform location, therefore have a lessened demand on the amount of calcium entering the neuron could still learn. Another possibility is that the verapamil dosage used in this study was not effective at blocking all VGCCs channels. This is possible considering that no other studies to date have injected this drug into the hippocampus. On the other hand, these results might be evidence to disprove the overarching hypothesis that the amount of calcium influx is crucial when new learning is occurring with familiar information. A caveat to mention is that because the MWT version used in this study was shorten from 4 days of pre-training to 2-days due to the probe results in chapter II, this increased the behavioural variability in the rats. Meaning it would be harder to observe learning and memory impairments because the rats learning was more sporadic and less controlled. An example of this increased variability is the cohort 1 verapamil rats and cohort 2 control rats. The behavioural variability can be more clearly seen during the probe test, where not even the control rats which showed great learning of the new platform location, showed a clear preference for that location. Interestingly, the combo rats showed the same behaviour as the control rats, not having a preference and spending equal time in both quadrants' locations. From this it can be concluded that combo rats may have been able to consolidate and remember the new location 24 hours later. In summary hypothesis 3 must be rejected because blocking VGCCs and NMDARs did not impair learning of a new spatial location with familiar information, nor did it impaired long-term memory.

Summary

The experiments in this chapter provide insight into the effect of blocking NMDARs and VGCCs on rapid acquisition of a new spatial location and long-term memory. The overarching conclusion of this research is that rapid new learning in the MWT with familiar information is not impaired by NMDARs & VGCCs blockade. The learning curves for all the experimental groups were very similar to the control groups. Cautious interpretation of the probe data could argue that there was an effect in the long-term memory of the new location learning 24 hours later. However further researcher would have to be done to confidently state this.

Chapter 4

General Discussion

The goal of this thesis was to conduct experiments to further our understanding of the neurobiological mechanism involved in spatial learning and memory in the mammalian brain. Specifically looking at the effects of pre-training on the calcium influx demands needed to properly learn and remember novel spatial information. Experiments in Chapter II were designed to understand the effects of pre-training on rapid learning and long-term memory. Experiments reported in Chapter III were completed to investigate the effects of calcium influx blockade on rapid learning and long-term memory.

Hypotheses and summary of results

For Chapter II it was hypothesized that both the 2-day and 4-day pre-training rats would be able to rapidly learn new spatial information. Additionally, it was hypothesized that as previously seen in pilot experiments, the 4-day rats would not have a preference during the 24-hour probe, whereas the 2-day rats would show a preference for the mass-trained location. These predictions were supported by the results. All rats regardless of the number of pre-training trials were able to learn a new spatial location. As predicted the 4-day rats did not prefer either the pre-training or the mass-training location. On the other hand, the 2-day rats preferred the mass-trained location, matching the initial hypotheses.

For Chapter III it was hypothesized that learning and memory would be impaired when VGCCs and NMDARs were both blocked. However, blocking them individually would not impair the rat's ability to rapidly learn a new platform location, but would impair their long-term memory. The results of these experiments demonstrated that the rats could learn when VGCCs and NMDARs are blocked individually and together. The long-term memory effects where

harder to interpret, however tentatively show some long-term memory impairments with individual blockade of VGCCs or NMDARs.

Novel contributions

The results of experiments described in this thesis contribute several novel findings and were mainly discussed in each chapter's discussion, however the implications to the field in general will be discussed below.

Pre-training effects

The goal of the experiment in chapter II was to investigate the pre-training effects on the probe preference in the MWT. That is to ensure that the control rats would show a strong preference (memory) for the mass-training location, to later test calcium channel blockade effect on learning and memory (Chapter III). To investigate this all rats where trained in the three-phase version of the MWT. The rats were either given 2 days (16 trials) or 4 days (32 trials) of pre-training, then mass-trained (16 trials) to a new platform location, followed by a 30 second probe. As hypothesized, regardless of the amount of pre-training trials, all rats were able to learn the pre-trained platform location. All rats showed significant learning by the 8^{th} pre-training trial. Asymptote levels of performance was achieved by both pre-training rat groups by the 16^{th} pre-training trial, and additional training did not significantly decrease the path length. Previous studies have shown that 4 days of 4 or 8 trials per day is sufficient pre-training for rats to learn new spatial information (Blokland et al., 2004; McDonald et al., 2005b). Novel findings in this experiment reveal that half of that training, 2 days of 8 trials per day is sufficient for rats to learn new platform location on the MWT.

In the mass-training session both the 2-day and 4-day rats learned the new platform location. However, there was a significant difference in their learning. Interestingly, the 4-day

rats showed very quick learning, whereas the 2-day rats took the entire session. Additionally, there was an interaction, showing that learning performance for trial 2 was dependent on which pre-training group the rat had been a part of. This provides new insight into the effects of pre-training on the rat's ability to learn a new spatial location and suggest that a greater amount of pre-training allows for quicker learning. An interesting question might be whether the 4-day rats display quicker learning because of the total amount of pre-training trials (32 vs 16) or because of an increased days and therefore sleep nights. The latter seems to be more likely, since studies have demonstrated that sleep is crucial for memory consolidation (Diekelmann & Born, 2010; Stickgold, 2005; Tatsuno et al., 2016), and as a result a greater number of sleeps during learning might be beneficial. If this is the case, it means that the 4-day rats learned the new platform location quicker than the 2-day rats because they had more sleep bouts to better consolidate the information. However, to conclude this further research is needed. Nonetheless, this experiment suggests that differences in pre-training trials causes differences during new spatial learning.

The probe results described in chapter II, I believe, are important. This is because one key feature in testing the rats on the three-phase version of the MWT is that both learning (mass-training – phase II) and memory (probe test – phase III) can be assessed. In theory if the rats were able to learn during mass-training, then they should display a preference for the mass-trained location during the probe test. Unfortunately, initial pilot studies results were unreliable, and the control rats did not show a preference. Therefore, my chapter II hypothesis emerged, which was that equating the amount of pre-training and mass-training would produce the expected preference for the mass-trained location. Indeed, the results of experiment 1 in chapter II confirmed this. The 2-day rats but not the 4-day rats had a significant preference for the mass-trained location on the probe test. It is important to mention that the preference for the mass-trained location was found during the analysis of the first 30 seconds of the 24-hour probe.

Previous studies had not found this preference over the 30 seconds probe, instead they had analyzed the probe in 10 seconds bins to detect a preference for the mass-trained location (Bye & McDonald, 2019; McDonald et al., 2005). Though similar findings have been described in the forementioned studies, this experiment provides a stronger probe preference result to compare against future experimental conditions.

VGCCs and NMDA receptors and spatial learning

After determining that the 2-day pre-trained rats were able to learn a new platform location and display a strong preference for the new location, the next step was to use this version of the MWT to test the role of different calcium channel blockers on spatial learning and memory. The hypothesis was that only if all the major calcium channels were blocked, VGCCs (verapamil) and NDMARs (AP5) would new spatial learning with familiar information be impaired. Overall, the results showed that learning was possible when VGCCs and NMDARs were blocked individually and together. Providing evidence to reject the original hypothesis of the thesis. However, there were some puzzling results which were mitigated and interpreted by additional analysis. During the mass-training new platform learning the verapamil rats did not show a significant decrease in path length, whereas the control rats did. However, the early vs. late in training average path length analysis showed that the verapamil rats had a lower early in training average compared to the controls. Furthermore, their late in mass-training average was very similar to that of the control rats. It is probable that the verapamil group on average found the new platform earlier in training, which would explain their early average path length results. Heading angle error analysis indicated that both the control and verapamil rats had similar performance. Therefore, the data supports the claim that VGCCs blockade did not impair new spatial learning. Previous research on the role of VGCCs in the HPC is limited because there have not been many studies conducted in which verapamil was injected directly in the HPC.

Nonetheless, a study using a chronic oral administration of verapamil found that there was not impairment in the passive avoidance task, where rats learn to stay in a lighted area of a box to avoid an electric shock (Lashgari et al., 2006). Because verapamil was given orally it would most likely affect VGCCs all over the brain and not specifically just the HPC, nonetheless the findings are similar in that the rats were able to learn. Another study that injected verapamil into the lateral amygdala at a similar dosage which was used in this thesis, tested the rats learning and memory, concluded that VGCCs are not critical for the learning but may play a role in long-term memory formation (Bauer et al., 2002). This would align with the results of this thesis, where blocking VGCCs did not impair learning but might have had an effect in the 24-hour probe (will be discussed below).

During the mass-training session the control rats ran with the AP5 rats showed a similar pattern to the verapamil rats. The control rats early average path length was very short, and no significant difference was found between early vs. late in the mass-training session. Again, this is believed to have occurred because this control rat group on average might have found the new platform location quicker. However, even more puzzling is that these control rats had an astonishingly short path length by the end of the mass-training session. Individual animal behavioural is variable and could partly explain this effect, however one of the hallmarks of the MWT is that it produces reliable animal behaviour data (D'Hooge & de Deyn, 2001). A caveat to this, however, is that the three-phase-version of the MWT used in the present experiments had a shorten pre-training-phase I compared to previous studies (Bye & McDonald, 2019; McDonald et al., 2005). Therefore, the mass-training-phase II learning could be more behaviourally variable in each rat, explaining the performance variability in the different cohorts of control rats. Nonetheless, the AP5 rats even compared to the unique performance of their cohorts control rats, displayed significant learning throughout the mass-training session. These results are in line with

many studies in the literature which state that NMDARs role in spatial learning is more limited than originally proposed (Hoh et al., 1999; McDonald et al., 2005; Saucier & Cain, 1995; Taylor et al., 2014). Even though the role of NMDARs in new spatial learning on this three-phase version of the MWT had previously been investigated by (Bye & McDonald, 2019), they had used a different NMDAR antagonist (CPP) than the more standard NMDAR antagonist, AP5. A significant amount of work has been done showing that AP5 blocks LTP induction at the dosages used in these experiments. Therefore, a novel finding is that intra-hippocampal infusion of AP5, the most used NMDAR antagonist, does not impair rapid acquisition of new spatial learning following pre-training.

The main hypothesis of this thesis was that to impair new learning with familiar spatial information both VGCCs and NMDARs would need to be blocked. However, the experiments results disprove this by showing that the combo rats were able to learn a new spatial location following pre-training. The combo rats had a significant decrease in path length throughout the mass-training session, and their heading angle error was very similar to that of the control rats. The main reason for the original hypothesis of this thesis was based on my calcium theory of new learning. Based on previous findings that NMDARs blockade did not impair this new learning and other studies that stated that VGCCs might be responsible for NMDARs independent learning (Bauer et al., 2002), the idea emerged that the amount of calcium influx into the post-synaptic neuron could be the determining factor. This made logical sense because both NMDARs and VGCCs mediate calcium influx during LTP and activate similar downstream mechanism linked to LTP expression. Pending the field's consensus that LTP is needed for new learning is true, then the reason that blocking NMDARs and VGCCs individually did not impair new learning could be, because one source of calcium could be compensated by the other. Therefore,

the prediction that blocking both sources of calcium together would impair learning made sense, however the experimental results refuted this prediction.

Firstly, even though NMDARs and especially VGCCs are most likely the biggest sources of calcium influx during LTP induction, there are others like mGluRs. These receptors when activated can cause calcium influx into the post synaptic neuron, by releasing intracellular calcium from the endoplasmic reticulum (Reiner & Levitz, 2018). This makes use of stored intracellular calcium that could initiate the signalling cascade responsible for downstream activation needed for LTP expression. Therefore, it can be argued that the reason the combo rats were able to learn was because mGluRs were sufficient to provide enough calcium influx at the time of learning. Indeed, studies have argued for an independent role of these receptors in LTP and learning and memory (Bliss et al., 2018; W. J. Foster et al., 2018).

An alternative explanation could be that the dosage of verapamil used in this study is not enough to block all the VGCCs in the hippocampus. As mentioned previously VGCCs are proposed to be one of the biggest sources of calcium influx into the post-synaptic neuron (Berridge, 1998)**.** As a result, if not all VGCCs were blocked then the remainder VGCCs could allow enough calcium influx to encode the learning. Even though pilot studies were conducted to determine the best dosage for a full blockade, there is a limited amount of research using verapamil and no previous research on intra-hippocampal infusions of verapamil has been done. In addition, the problem of increased animal behaviour variability due to shortening the MWT pre-training phase could have impacted the results. Specifically, the shorter pre-training period could have caused the mass-training day learning to be more variable, which might have washed out minor impairments that could have otherwise been seen. Control rats for each cohort were ran to mitigate this, nonetheless, given the unusual short path length and learning performance of one

of the control cohorts, this leaves room for further investigation to fully understand what may have occurred.

Lastly, there is the possibility that the original prediction of this thesis is incorrect and new learning with familiar information is not dependent on the amount of calcium influx. Therefore, blocking a big source of calcium influx via NMDARs and VGCCs would not impair the learning as seen in these experiments. Studies have shown that the HPC is still needed for new spatial learning even when animals are pre-trained (Bannerman et al., 1995). Interestingly a recent study argues that "silent learning" might be occurring, in which new memory encoding happens in the absence of cell-firing. The idea is that the hippocampal network still mediates new memory encoding because LTP induction is intact, but this can occur even under conditions in which somatic cell-firing is blocked in the HPC (Rossato et al., 2018). Silent learning is an interesting hypothesis and given the current research in the field and the results shown here, suggests that a more intricate molecular mechanism might be responsible for new learning with familiar information. A potential molecular mechanism could be the role of calcium as a second messenger to activate a signalling cascade which are responsible for initiating gene transcription in the nucleus. This is crucial for *de novo* protein synthesis and proper LTP expression (Nakazawa et al., 2004). Perhaps intermediate signalling molecules are needed and are not properly activated if the calcium entering the post-synaptic neuron is not from the right sources.

VGCCs and NMDA receptors and spatial memory

The decision to use the 2-day pre-training condition based on the probe preference results seen in chapter II, turned out, to not be the best one for assessing memory impairments of VGCCs and NMDARs blockade. It was expected that the control rats during the 24-hour probe would display a strong preference for the mass-trained location. This could be interpreted as an indication of proper memory formation of the mass-training platform. Unfortunately, the strong

preference displayed by the rats in chapter II was not replicable in any of the control groups ran in chapter III. Instead, the control rats split their time between both the pre- and mass-trained locations but did not display a preference for either. Though at first these results seemed frustrating, an upside was that they were consistent across the 3 cohorts control rats. The only difference between the control rats in experiment 1 (2-day rats) and experiment 2 is that rats in the latter underwent hippocampal canulation surgery. According to previous research the cannulation procedure should not cause these effects (Hilliard et al., 1968). Nonetheless, some cell death does occur where the canulae are placed, though this is minimal and is unlikely to be responsible for this change in behaviour. More probable is that the probe preference results seen in experiment 1 might be more behaviourally variable than expected and repetition of this experiment would be best to confirm that the results are reliable. As a result, the probe data should be interpreted with caution.

All the cohorts control rats behaved in the same way during the first 30 seconds of the 24-hour probe. Splitting their time equally between the pre- and mass-trained location. Because this search strategy was consistent it could be compared against the verapamil and AP5 rats. Interestingly verapamil and AP5 rats during the probe showed a strong preference for the pre-trained location. This could indicate that their memory of the mass-trained location was not as strong as the control rats. Indeed, previous studies using VGCCs antagonist obtained similar results and argued that these receptors might be preferentially involved in memory consolidation (Bauer et al., 2002; Woodside et al., 2004). Additionally, the consolidation effects also seen in the AP5 rats have been similarly reported by other researchers (Bye & McDonald, 2019). Though seemingly in line with past research, these probe results should just be a starting point and not a concise answer to what the role of these receptors might be in spatial memory. Especially considering that the combo rats did not show the same preference for the pre-trained location and

instead behaved in the same manner as the control rats, splitting their time equally between both pre- and mass trained locations. Further analysis looking at all the quadrants revealed that the controls preferred the pre- and mass trained quadrants, whereas the combo rats split their time evenly amongst all quadrants. The control rats had a strong representation for both the pre- mass trained platform, however the combo rats swim randomly throughout the pool. This indicates that NMDA and VGCC blockade may have impair spatial memory on this version of the MWT.

Limitations

Throughout the experiments of this thesis there were some challenges and limitations some of which have been forementioned. These include increased behavioral variability due to shortening the pre-training phase of the MWT. The fact that this experiment is the first to inject verapamil a VGCCs antagonist directly into the hippocampus, therefore less is known about the ideal drug dosage. Additionally, as mentioned in the introduction answering complex questions about higher cognitive functions like learning and memory are best answered from multiple levels of analysis. The approach taken in this thesis was at the behavioural level and though it is a good place to start, an important limitation of this research is that there was no assessment at the molecular level. For example, it would have been beneficial to have done an immediate early gene (IEG) quantitative assessment in the hippocampus to see if indeed blocking these receptors was inhibiting IEG expression. IEGs like Arc protein are believed to be good correlates of LTP and therefore evidence for proper learning and memory function (Guzowski et al., 2001). Furthermore, proteins like CamKII and CREB phosphorylation are known to be downstream effects of calcium signalling leading to LTP expression (Nakazawa et al., 2004; Zamponi et al., 2015). Testing these would provide a clearer understanding of what is occurring molecularly and either support or disprove the working hypothesis. This is especially important given that as the

hypothesis in this thesis that blocking both VGCCs and NMDARs would impair new spatial learning with familiar information was founded on the theory that the amount of calcium influx during the learning is important. Since the theory makes molecular predictions, it would be ideal to test this at the molecular level in addition to the behavioural level. Lastly, all the rats used in this thesis were male and studies have reported behavioral differences between male and female rats based on differences in temperament. Male rats are sometimes more anxious, less impulsive and have lower activity levels than their female counterparts (Bonuti & Morato, 2022). This could translate to differing performance in the MWT because male rats on average might display more stressed and higher anxiety behaviours like "peripheral looping" which means persistent swimming around the pool wall (Brody & Holtzman, 2006). To account for this an experiment with equal number of female and male rats would be best.

Future directions

The results of this thesis state that VGCCs and NMDARs blockade in the hippocampus does not impair new learning with familiar spatial information. However, due to some of the conflicting results it good be optimal to further investigate these results. Specifically, a good future experiment would be to replicate the combo blocker group in the 4-day pre-training version of the MWT. This version of the task has been used before and has yielded replicable results. If this experiment yielded similar results than that would further support the conclusion of this thesis. Additionally, if the rats could still learn, an intracellular calcium chelator experiment would be ideal to fully answering the theory behind the hypothesis of this thesis that proper learning is dependent on the amount of calcium influx into the post-synaptic neuron. If this idea is correct, then the calcium chelator should completely block new spatial learning and therefore affirm the theory. If the calcium chelator blocks new spatial learning, then that might mean that

there are additional calcium sources other than VGCCs and NMDARs that might be playing a role. In this case, mGluRs as previously mentioned would be a good candidate and an experimental condition in which VGCCs, NMDARs, and mGluRs were blocked in the hippocampus might yield promising results.

Additionally, to mitigate some of the behavioural variability results observed throughout the experiments in this thesis, a within-subjects design could be used. A protocol known as delayed matching to place can be used on the MWT (Rossato et al., 2018). The rats would be given four trials per day, however the first trial each day is to a different platform location. Therefore, the rat learns a new platform location every day. Given that the calcium channel blockers are reversible after a few hours, a rat can be given the blocker one day and the vehicle the next day. In this manner each animal would act as its own control. This would decrease the behavior variability and allow for clearer results.

Conclusion

This thesis examined the effects of pre-training trials and calcium channel blockade on spatial learning and memory in a three-phase-version of the MWT. Several novel contributions were made throughout these experiments. It was observed that rats can learn a new platform location with as little as 16 pre-training trials over 2 days. Also, longer pre-training periods may speed up and facilitate new spatial learning. Intra-hippocampal blockade of VGCCs does not impair new spatial learning and neither does blockade of VGCCs and NMDARs, however long-term spatial memory may be impaired. These findings are crucial because they add to the field of knowledge regarding the neurobiological mechanisms involved in spatial learning and memory in the mammalian brain. Understanding the molecular underpinnings of learning and memory is of

the outmost importance because these cognitive abilities are the foundations which makes us humans.

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Appendix 1: Supplemental Figures from Chapter 2

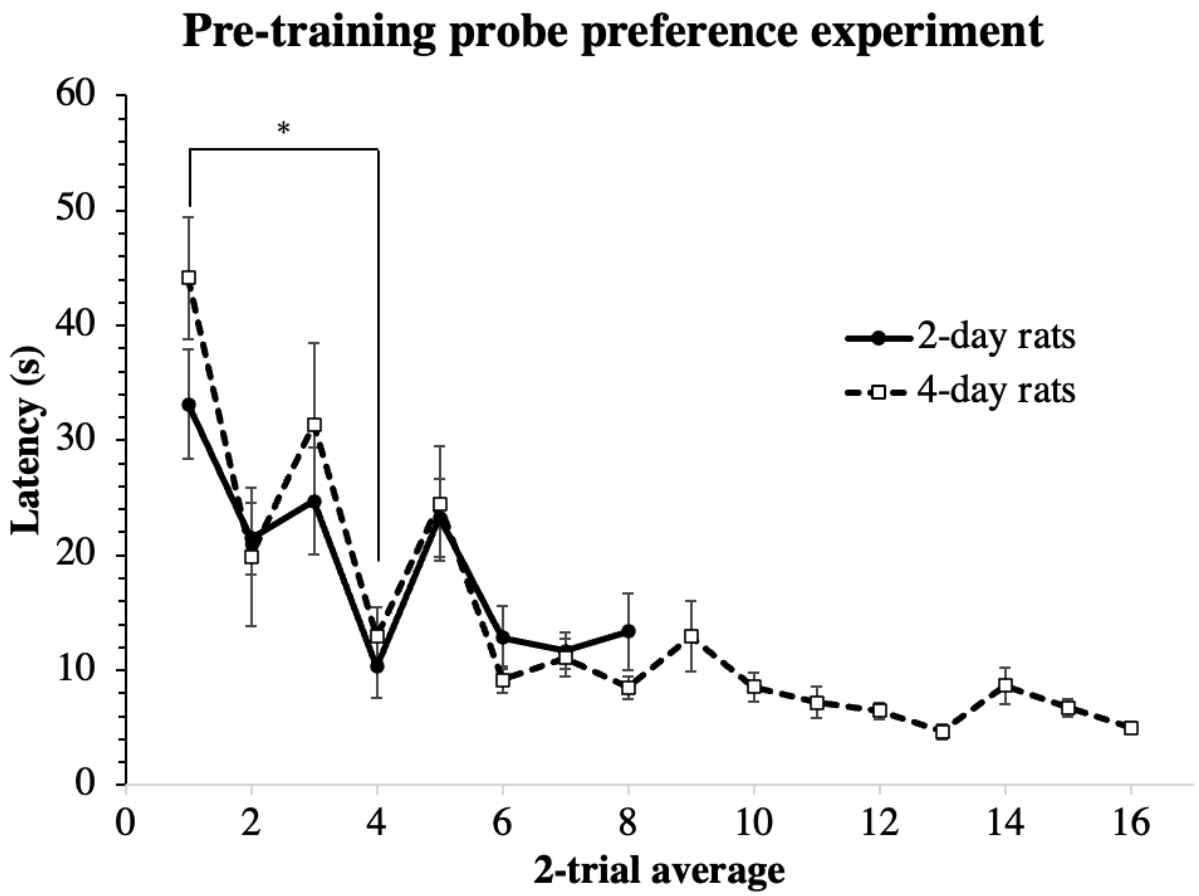

Figure S1: *Latency to platform as a 2-trial average during pre-training of all rats in experiment 1 on the Morris Water Task. Rats were given either 2 or 4 days of pre-training, 8 trials per day. Both groups learned. P < 0.001, Error bars: +/- 1 SE. Significance is denoted by "*".*

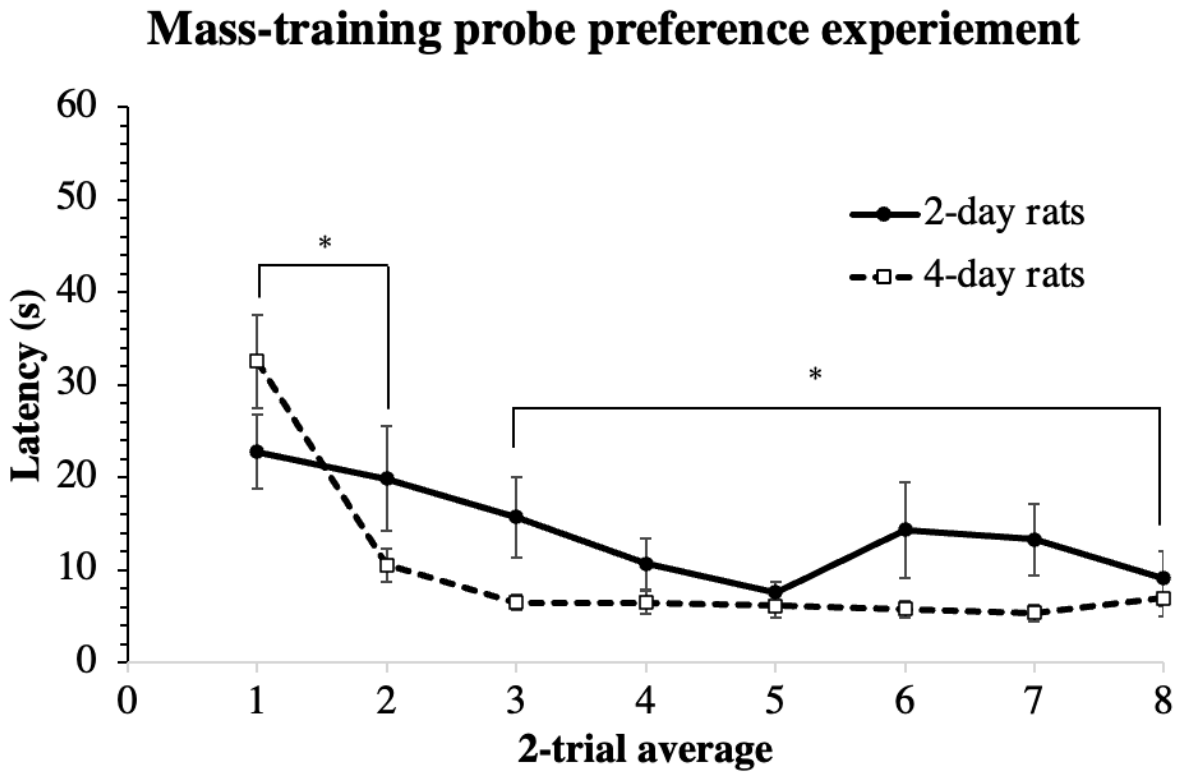

Figure S2: *Latency to platform as a 2-trial average during mass-training of all rats in experiment 1 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The 4-day rats show significant learning early in training whereas the 2-day rats learn late in training. 4-day rats $p < 0.005$, 2-day rats $p = 0.043$. There was a significant interaction $p = 0.014$. Error bars: +/- 1 SE. Significance is denoted by "*".*

Appendix 2: Supplemental Figures from Chapter 3

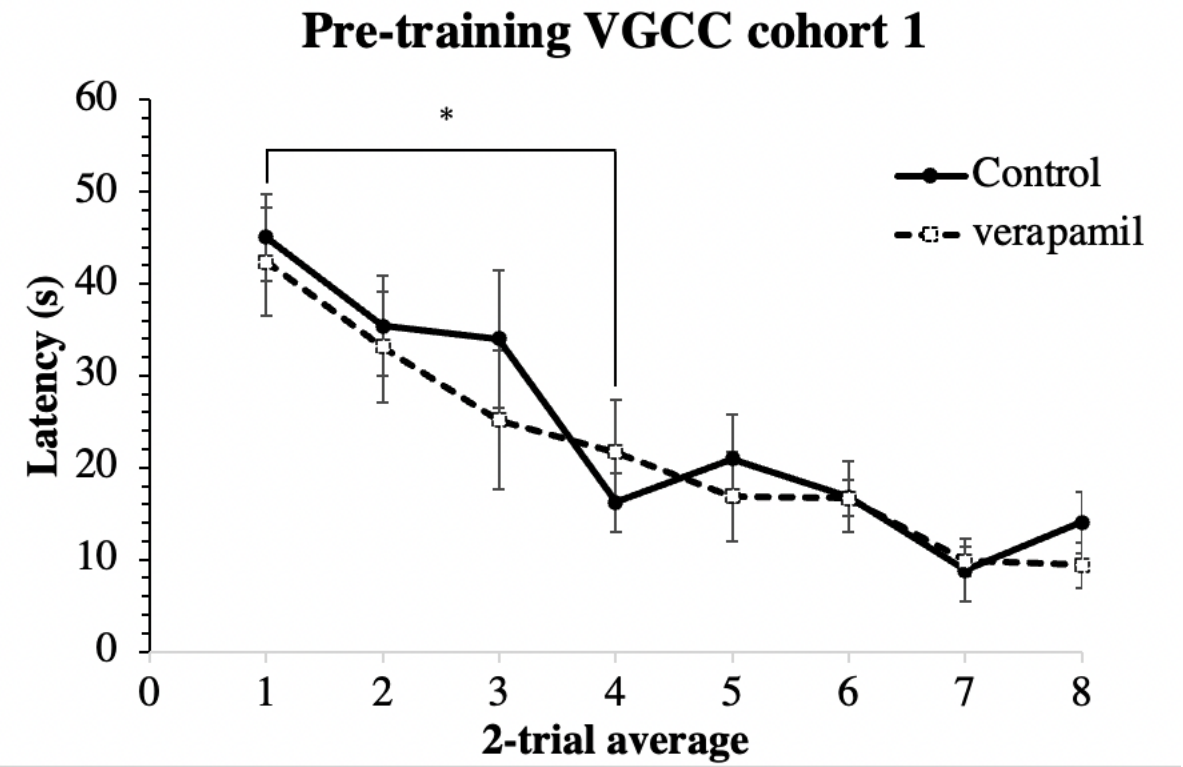

Figure S3: *Latency to platform as a 2-trial average during pre-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 days, 8 trials per day. Both groups learned. P = 0.004, Error bars: +/- 1 SE. Significance is denoted by "*".*

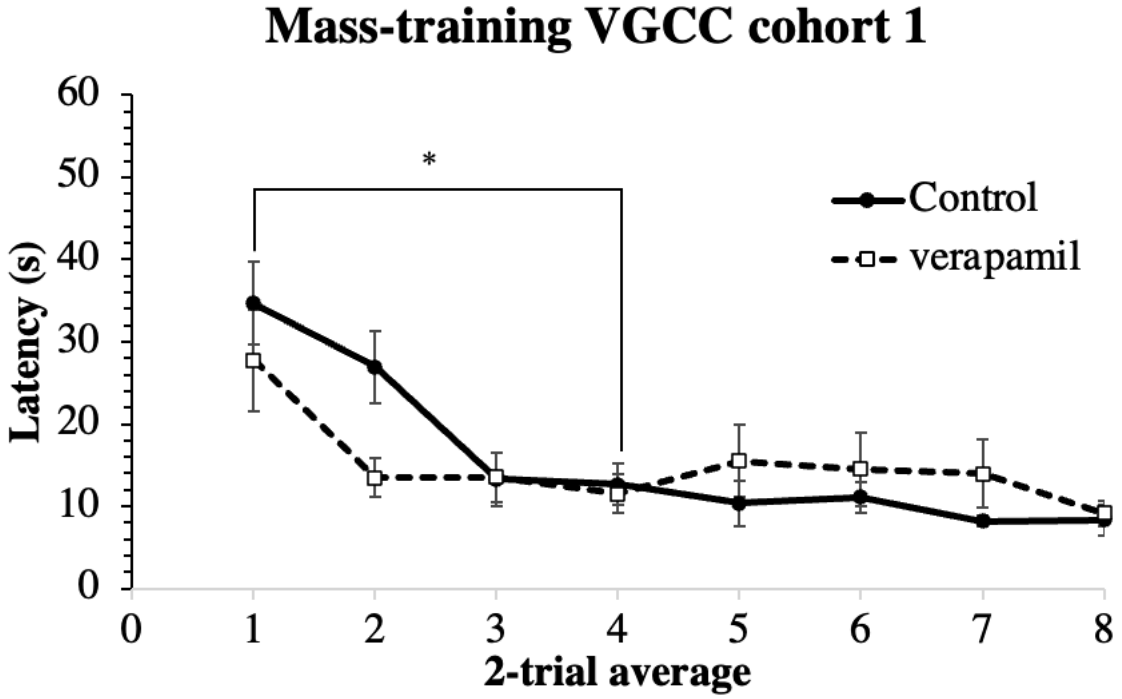

Figure S4: *Latency to platform as a 2-trial average during mass-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The control rats show significant learning whereas the verapamil rats do not. P = 0.029, Error bars: +/- 1 SE. Significance is denoted by "*".*

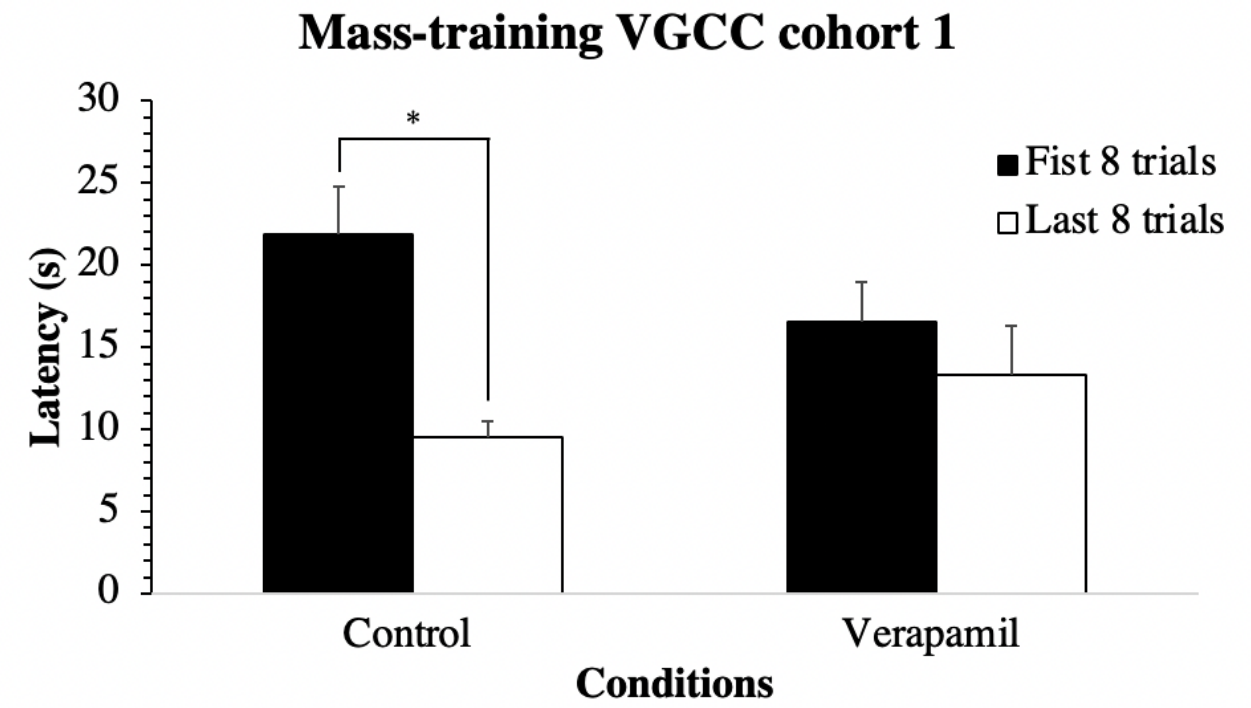

Figure S5: *Latency to platform early vs. late in mass-training of all the rats in cohort 1, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The control rats show significant learning whereas the verapamil rats do not. P = 0.002, Error bars: +/- 1 SE. Significance is denoted by "*".*

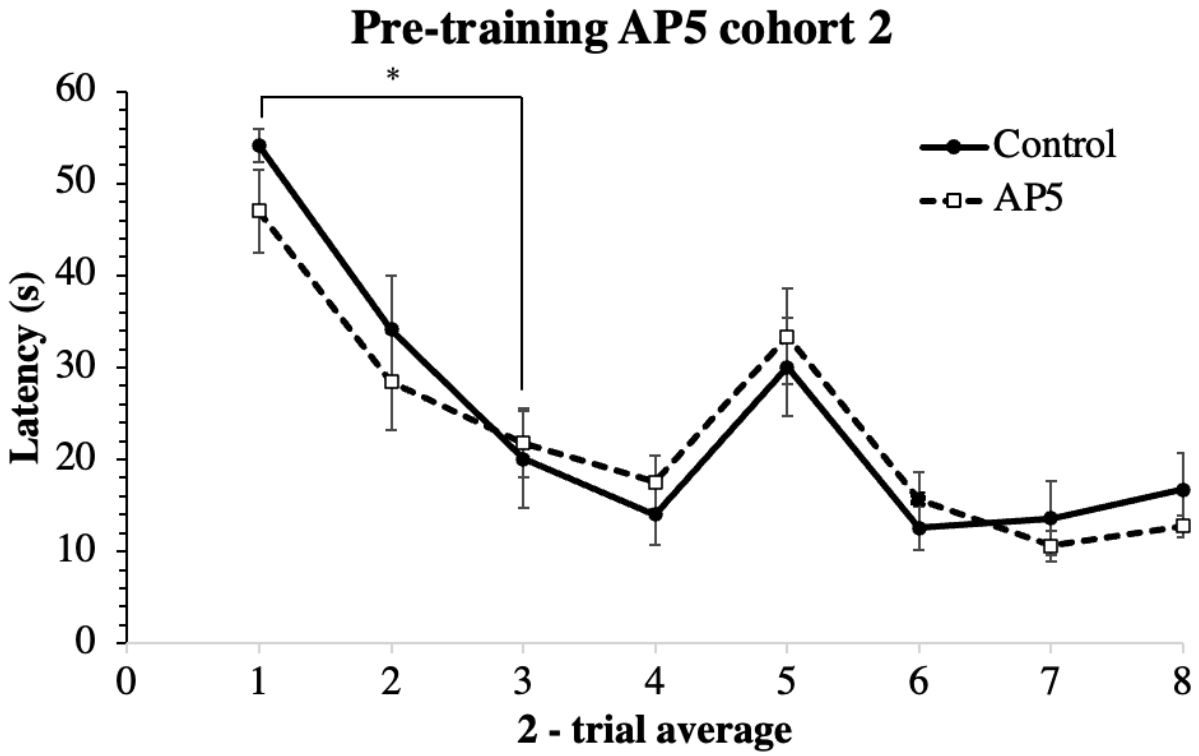

Figure S6: *Latency to platform as a 2-trial average during pre-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 days, 8 trials per day. Both groups learned. P < 0.001, Error bars: +/- 1 SE. Significance is denoted by "*".*

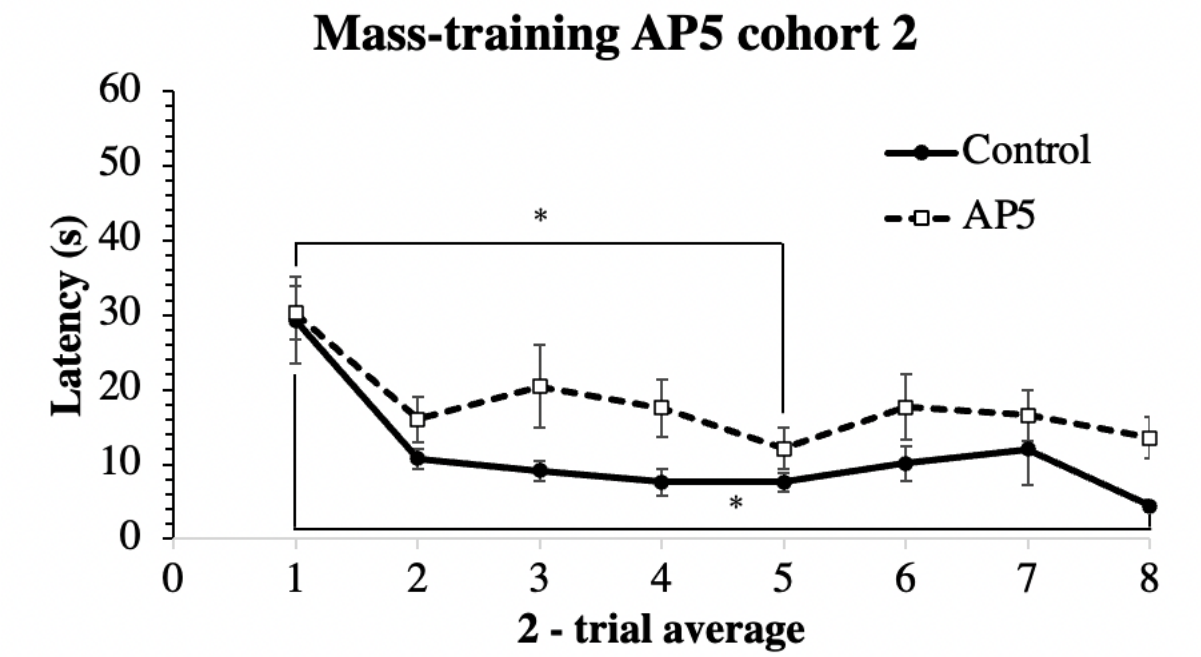

Figure S7: *Latency to platform as a 2-trial average during mass-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. All rats displayed learning. Control rats p = 0.04, AP5 rats p = 0.03, Error bars: +/- 1 SE. Significance is denoted by "*".*

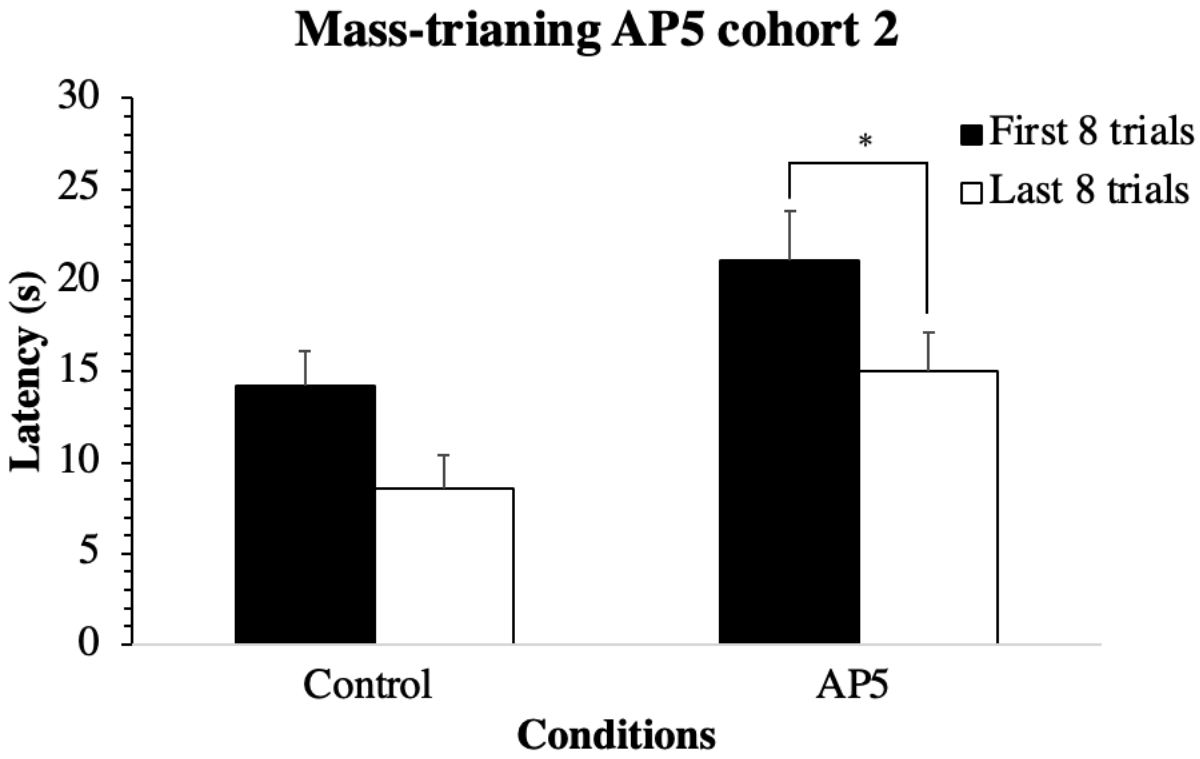

Figure S8: *Latency to platform early vs. late in mass-training of all the rats in cohort 2, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. The AP5 rats show significant learning whereas the control rats do not. P = 0.016, Error bars: +/- 1 SE. Significance is denoted by "*".*

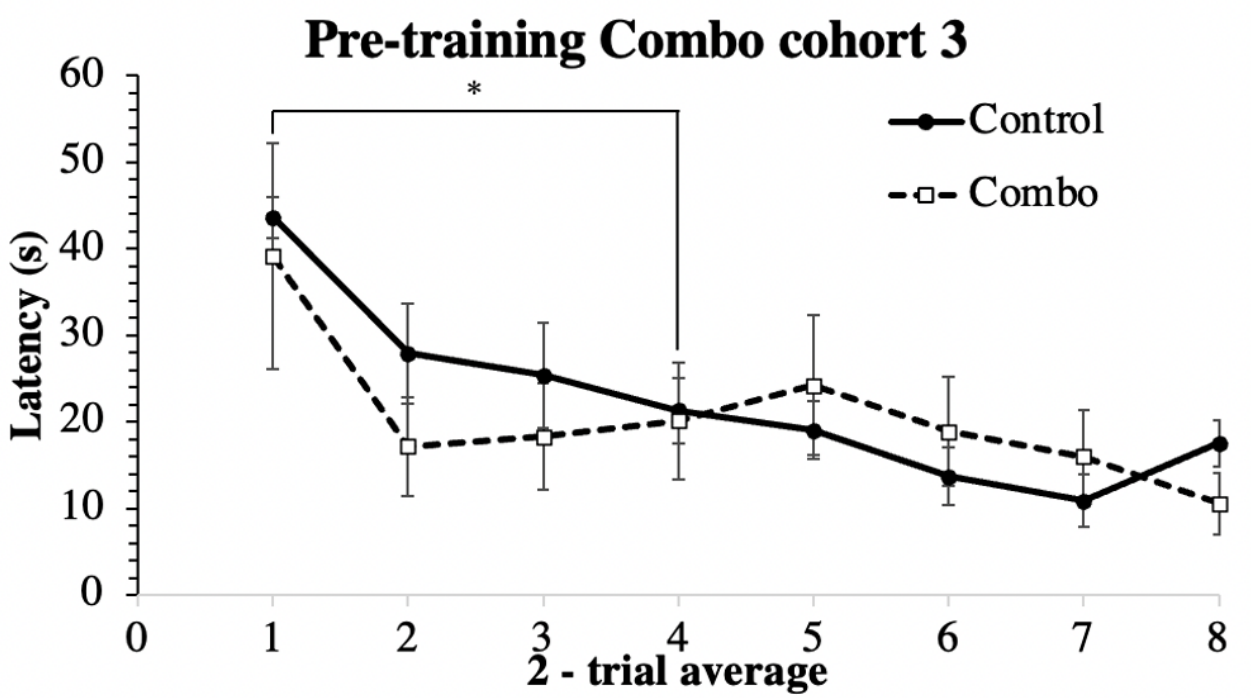

Figure S9: *Latency to platform as a 2-trial average during pre-training of all the rats in cohort 3, experiment 2 on the Morris Water Task. Rats were trained over 2 days, 8 trials per day. Both groups learned. P = 0.01, Error bars: +/- 1 SE. Significance is denoted by "*".*

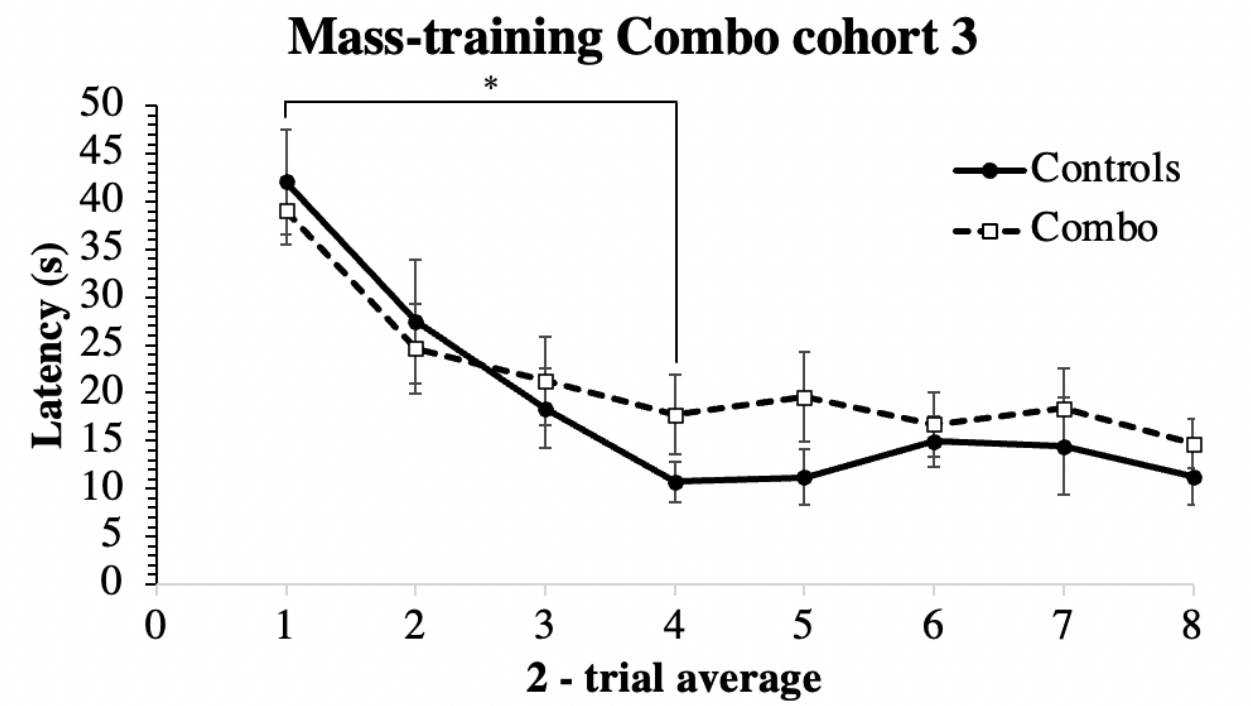

Figure S10: *Latency to platform as a 2-trial average during mass-training of all the rats in cohort 3, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. All rats learnt. P = 0.015, Error bars: +/- 1 SE. Significance is denoted by "*".*

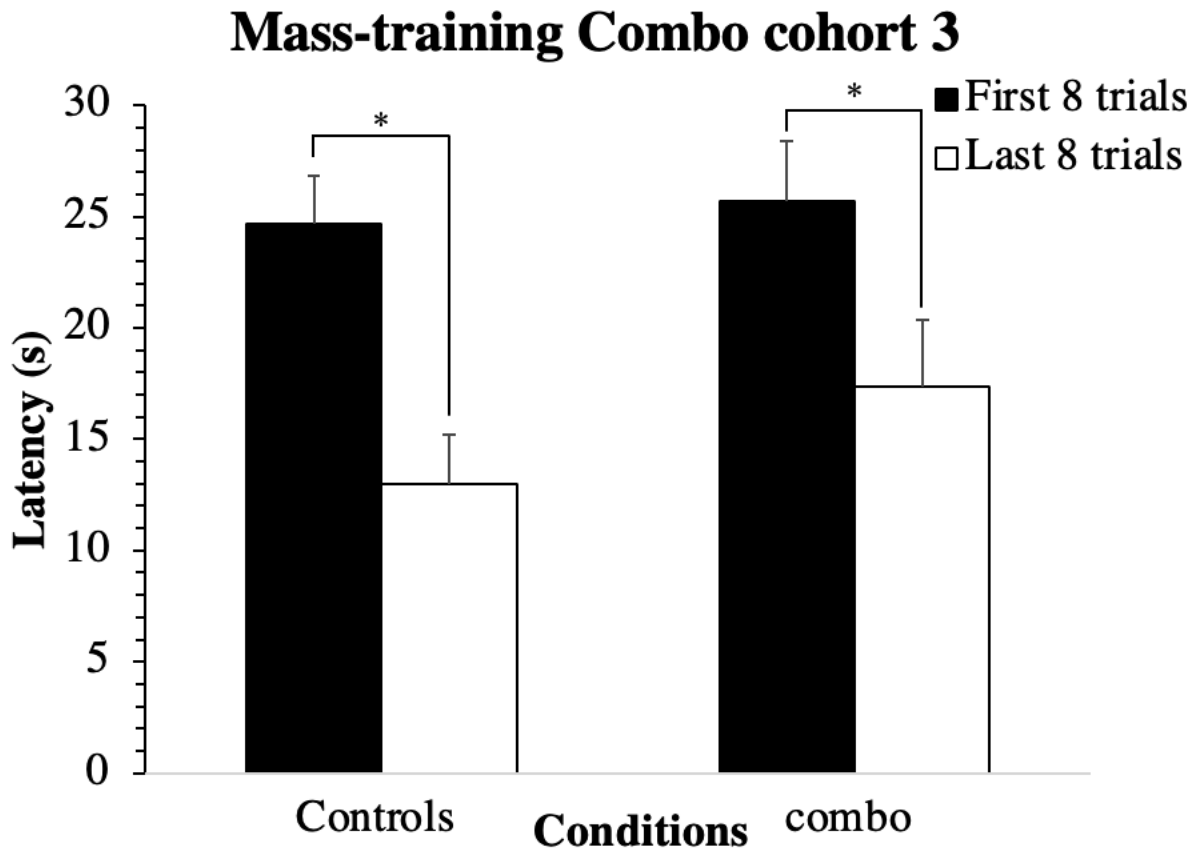

Figure S11: *Latency to platform early vs. late in mass-training of all the rats in cohort 3, experiment 2 on the Morris Water Task. Rats were trained over 2 hours, 16 trials to a platform location in the opposite quadrant where pre-training occurred. Both the control and combo rats show significant learning. P = 0.003, p = 0.02, Error bars: +/- 1 SE. Significance is denoted by "*"*