Université de Montréal

Effect of Spatial Learning on the Protein Tyrosine Phosphatase STEP

Par

Christina McAnulty

Département de pharmacologie et physiologie de l'Université de Montréal

Faculté de médicine

Mémoire présenté en vue de l'obtention

du grade de Maîtrise en pharmacologie, option neuropharmacologie

Avril 2020

Université de Montréal

Département de pharmacologie et physiologie de l'Université de Montréal

Faculté de médicine

Ce mémoire intitulé

Effect of Spatial Learning on the Protein Tyrosine Phosphatase STEP

Présenté par

Christina McAnulty

A été évalué(e) par un jury composé des personnes suivantes

René Cardinal
Président

Jonathan Brouillette
Directeur

Pierre-Paul Rompré
Membre du jury

Résumé

La protéine Striatal-Enriched Protein Tyrosine Phosphatase (STEP) joue un rôle important dans la régulation de la force synaptique, notamment par sa capacité à s'opposer au renforcement synaptique et à encourager la dépression à long terme. Des niveaux anormaux de STEP peuvent altérer l'apprentissage et la mémoire et ont été impliqués dans une variété de troubles neuropsychiatriques tels que la maladie d'Alzheimer. Bien qu'il existe de nombreux substrats et régulateurs connus de STEP, la gamme complète des molécules capables d'intéragir avec STEP reste à découvrir. Dans cette étude, nous avons utilisé deux méthodes complémentaires afin de trouver de nouveaux intéracteurs de STEP: l'identification par proximité à la biotine (BioID) et la purification par affinité couplée à la spectrométrie de masse (AP-MS). Nous avons ensuite utilisé le protocole de la piscine de Morris chez le rat afin de déterminer l'effet d'un apprentissage spatial sur les niveaux de STEP61, STEP non phosphorylé, le récepteur 1 de la neuromédine U (NMUR1) et la neurologine-1 (NLGN-1) dans l'hippocampe des rats. Nous avons observé qu'un environnement naturel riche en indices distaux radicalement différents les uns des autres était plus propice à l'apprentissage spatial qu'un environnement plus uniforme avec uniquement des images disponibles pour être utilisées comme indices distaux. Nous avons également constaté que la protéine STEP61 totale, la STEP non-phosphorylé et la NMUR1 n'ont pas changé à la suite d'un apprentissage spatial, mais que la NLGN-1 change dans l'un des protocoles utilisés. Enfin, nous n'avons pas été en mesure d'induire des changements dans les niveaux de STEP grâce à l'utilisation de NMDA ou de DHPG pour induire une dépression à long-term dans des cultures hippocampiques dissociées. Des recherches supplémentaires seront nécessaires afin de déterminer la nature des nouvelles interactions découvertes, ainsi que la façon dont celles-ci sont affectées par un apprentissage spatial, et le rôle de la dépression à long terme ou de la potentialisation à long terme dans ces processus.

Mots-clés : Mémoire, apprentissage, piscine de Morris, modèles animaux, STEP

Abstract

The Striatal-Enriched Protein Tyrosine Phosphatase (STEP) plays an important role in the regulation of synaptic strength, namely through its ability to oppose synaptic strengthening and encourage long term depression. Abnormal levels of STEP can impair normal learning and memory, and have been implicated in a variety of neuropsychiatric disorders such as Alzheimer's Disease. Though there are many known substrates and regulators of STEP, the full range of STEP interactions remains to be discovered. In this study, we used Proximity-dependent Biotin Identification (BioID) and affinity-purified mass spectrometry (AP-MS) in order to identify novel interactors of STEP. We then used the Morris water maze (MWM) protocol in rats to determine the effect of a spatial learning event on $STEP_{61}$, non-phosphorylated STEP, neuromedin U receptor 1 (NMUR1) and neurologin-1 (NLGN-1) levels in the hippocampus of rats. Throughout our experiments, we determined that a natural environment rich with dramatically different distal cues was more conducive to spatial learning than a more uniform environment with only images available to be used as distal cues. We found also that total $STEP_{61}$, non-phosphorylated-STEP, and NMUR1 did not change as a result of a spatial learning event, but that NLGN-1 was increased in one of the protocols used. Finally, we were unable to induce changes in STEP levels through the use of NMDA or DHPG to induce long-term depression (LTD) in dissociated hippocampal cultures. Further research is required in order to determine the nature of the novel interactions discovered, as well as how these are impacted by a spatial learning event, and the role of LTD or long-term potentiation (LTP) in these processes.

Keywords: Memory, Learning, Morris Water Maze, Animal Models, STEP

Table of Contents

List of Figures

Liste des Acronyms and Abbreviations

Aβ: Beta-amyloid

AD: Alzheimer's Disease

aMCI: Amnesic mild cognitive impairment

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANOVA: Analysis of variance

AP-MS: Affinity Purification Mass Spectrometry

BioID: Proximity-dependent Biotin Identification

CARD10: Caspase Recruitment Domain-Containing Protein 10

CO-IP: Co-immunoprecipitation

CPE: Cytoplasmic polyadenylation element

CREB: Cytoplasmic polyadenylation element binding protein

DHPG: (s)-3,5-Dihydroxyohenylglycine

EPSC: Excitatory postsynaptic current

ERK1/2: Extracellular Signal Regulated Kinases 1/2

FMRP: Fragile X mental retardation protein

GIMAP6: GTPase of Immunity-Associated Protein 6

GluA2: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subtype A2

GluN2B: N-methyl D aspartate receptor subtype 2B

HBSS: Hank's Balanced Salt Solution

HEK293: Human embryonic kidney 293

K^+: Potassium Ion

KIM: Kinase Interacting Motif

KO: Knock out

LC/MS/MS: Liquid chromatography tandem mass spectrometry

LTD: Long-term depression

LTP: Long-term potentiation

MAPK: Mitogen-activated protein kinase

MEM-HS: Minimum Eagle Essential Medium-Horse Serum

METTL26: Methyltransferase-like 26

Mg^{2+}: Magnesium ion

mGluR: Metabotropic glutamate receptor

MWM: Morris Water Maze

$Na^{+:}$ Sodium Ion

NLGN-1: Neurologin-1

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate receptor

NMUR1: Neuromedin U receptor 1

PER1: Period Circadian Regulator

PP: Polyproline-rich

PP1: Protein phosphatase 1

PTP: Protein tyrosine-phosphatase

PTPN4: Protein Tyrosine Phosphatase Non-Receptor Type 4

SAINT: Significance analysis of interactome

STEP: Striatal-Enriched Tyrosine Phosphatase

SUV39H1: Histone-lysine N-methyltransferase SUV39H1

For Juju

Acknowledgments

A special thanks to Benoit Coloumbe and his team from the Montreal Clinical Research Institute for performing the BioID and AP-MS screening, as well as to Maude-Éloïse Piché-Lemieux for her help in data collection and Julien Dufort-Gervais for teaching me the techniques required for my project.

Chapter 1 – Introduction

1.1 Learning and Memory

1.1.1 Overview

In the most general terms, learning and memory can be thought of as the ability of an organism to first obtain information from the environment and the subsequent retention of this information for future recall and use. The neurological basis of memory is complex and is still being explored, though it is well established that synaptic plasticity (the ability for synaptic transmission to change in response to stimuli) plays an important role.

The most well-studied forms of synaptic plasticity thought to be involved in the formation of memories include long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus. First proposed by Donald Hebb in 1949, the Hebbian theory proposed that the firing of a postsynaptic neuron while the presynaptic neuron is active could lead to changes within synapse that would strengthen the connection between the two. Evidence for this process, which we now refer to as LTP, was first provided decades later when it was shown that synapses in the hippocampus could be potentiated (i.e. signal transmission between neurons is increased) for days following repeated activation using high frequency stimulation (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). LTD was later described in 1982 by Ito and colleagues (Ito and Kano, 1982; Ito et al., 1982). It is thought that the strengthening and weaking of connections in the brain through LTP and LTD, respectively, likely play a role in the coding and storing of information (*.i.e.* memory) in the brain.

Various forms of LTP and LTD have been documented, with different brain regions exhibiting different forms. However, most of the research has focused on the hippocampus due to

strong evidence for its involvement in memory (Martin et al., 2000; Zola-Morgan and Squire, 1993, Voss et al., 2018). The exact role of the hippocampus in memory is still being explored, though it is known to have a role in episodic and spatial memory (Bird & Burgess, 2008, Voss et al., 2018).

1.1.2 Molecular Mechanisms of Memory

Amongst the various molecular mechanisms underlying LTP and LTD are N-methyl-D-aspartate receptor (NMDAR)-dependent LTP, NMDAR-dependent LTD and mGluR1-dependent LTD.

Perhaps the most well-known form of LTP in the hippocampus, NMDAR-dependent LTP relies on the activation of NMDA as well as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) in the postsynaptic neuron. Usually found colocalized on dendritic spines, these receptors function together to induce LTP. LTP is triggered by a burst of high frequency stimulation at the presynaptic neuron which causes it to release glutamate into the synaptic cleft. Following the binding of glutamate to AMPA receptors in the postsynaptic neuron, Na+ and K+ are allowed through the AMPAR ion channels. If there is sufficient cation influx, sufficient depolarization of the membrane occurs to allow for the removal of a magnesium ion that usually blocks the NMDAR channel, which then permits a subsequent influx of cations (Mayer et al., 1984; Nowak et al., 1984). If sufficient calcium enters the postsynaptic neuron through NMDAR channels, LTP is induced (Malenka, 1991; Malenka and Nicoll, 1993). The expression of NMDAR-dependent LTP in the CA1 hippocampal synapses appears to depend on alterations in AMPAR trafficking that lead to the increased insertion of calcium permeable AMPARs in the postsynaptic membrane (Bredt and Nicoll, 2003; Derkach et al, 2007; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir, 2002).

Interestingly, calcium influx through NMDARs is also involved in NMDAR-dependent LTD. While LTP requires large increases in postsynaptic calcium levels, LTD seems to occur when lower frequency stimulation is applied repetitively, thus still allowing calcium influx through NMDA receptors, though in a much smaller concentration (Cummings et al., 1996). This form of LTD involves, at least in part, removal of AMPA receptors from the postsynaptic membrane (Bredt and Nicoll, 2003; Collindgride et al., 2004; Derkach et al., 2007; Malenka and Bear, 2004; Malinow and Malenka, 2002). Metabotropic glutamate receptor-dependent LTD is less well studied, but also appears to involve the removal of AMPARs from the synapse (Snyder et al., 2001; Wang and Linden, 2000; Xiao et al., 2001).

Most research into LTP and LTD have taken place *in vitro*, and thus the way in which these processes affect behaviour are still poorly understood. There is, however, evidence to suggest a role for LTP in hippocampal memory. For instance, mice with increased NMDAR function had evidence of increased LTP in a subset of hippocampal synapses as well as improvements in spatial learning performance (Whitlock et al., 2006). In other research the abolition of LTP maintenance using a protein kinase Mzeta inhibitor was shown to cause the loss of a spatial memory in rats (Pastalkova et al., 2006). Though much evidence points to the involvement of LTP in memory, causality has not yet been firmly established (Stuchlik, 2014).

Although the involvement of LTP in memory formation and retention has long been suspected, research also suggests a role for hippocampal LTD. Dong et al., for instance, showed in 2012 that memory enhancements induced by novelty exploration in rats was increased when NMDAR-dependent LTD was facilitated, and blocked when LTD was inhibited, giving evidence that LTD has its own role to play in memory acquisition. Later, they also demonstrated a role for LTD in spatial reversal learning during the Morris water maze (Dong et al., 2013). Thus, the

formation and maintenance of memories through synaptic plasticity appears to be dynamic in nature, requiring coordinated changes within the synapse that involve both long-term depression and long-term potentiation. Importantly, these studies and others have demonstrated that a behavioural experience can induce the formation of LTP and LTD (Dong et al., 2012; 2013; Kemp and Manhan-Vaughan, 2004; Manaha-Vaughan and Braunewell, 1999).

1.1.3 The Hippocampus and Spatial Memory

Memory disturbances due to hippocampal damage have long been known to occur, though the precise nature of its role in various types of memory remains debated. What is clear, however, is that it plays an important role in the formation and maintenance of long-term memories (Baddeley and Warrington, 1970; Cave & Squire, 1992). In addition, the hippocampus is known to be implicated in spatial memory, and damage to the area frequently causes issues with forgetting where an object has been placed or with the ability of an individual to properly navigate (Bird and Burgess, 2008). It comes at no surprise, then, to learn that the hippocampus is one of the first areas of the brain to be affected by Alzheimer's disease (AD) (Frisoni et al., 2010), where the first symptoms individuals display often include a tendency to forget directions and to misplace objects. It is thought that the importance of the hippocampus in spatial memory is, at least in part, due to the presence of "place cells," that is, cells that have been shown to specifically fire when an animal is in a specific location in an environment or "place field." Place cells were first discovered in rodents but were later also discovered in primates as well as in humans (O'Keefe, 1971; Ekstrom AD, 2003; Ono, 1991). Due to the relative ease with which spatial memory can be assessed in laboratory animals, spatial memory tasks are a widely used tool to study hippocampal-dependent memory in rodents.

1.2 The Striatal-Enriched Protein Tyrosine Phosphatase (STEP)

1.2.1 Overview

First discovered nearly 30 years ago, the Striatal-Enriched Protein Tyrosine Phosphatase (STEP) is, as its name suggests, a tyrosine-specific phosphatase that is found in large quantities within the striatum in the brain (Lombroso et al., 1991; 1993). In addition to its high concentration in the striatum, it is also found in the other structures of the brain, excluding the cerebellum. STEP isoforms are not only differentially expressed throughout different tissues (Lombroso et al., 1991; 1993), but through developmental time as well (Raghunathan et al., 1996). STEP can be found in both excitatory and inhibitory neurons (Oyama et al., 1995; Choi et al., 2007). Depending on the brain region under observation, STEP can be found either throughout the neurons (Boulanger et al., 1995), or in some cases only in neurites (Kim et al, 2008), and in some cases, such as under ischemic conditions or optic nerve damage, expression has been seen in astroglia (Hasewaga et al., 2000; Lorber et al., 2004). Abnormal levels of STEP have been linked to various neuropsychiatric disorders and problems with memory and cognition, such as Alzheimer's Disease and Schizophrenia, amongst others.

1.2.2 Structure

The STEP protein is encoded by the *ptpn5* gene and alternative splicing leads to four distinct isoforms. Two of these isoforms (Fig. 1) contain an active phosphatase domain which allows STEP to dephosphorylate specific tyrosine residues on its substrates ($STEP_{61}$ and $STEP_{46}$), while the other two are lacking a phosphatase domain ($STEP_{38}$ and $STEP_{20}$) (Sharma et al., 1995; Bult et al., 1996, 1997). The functions of $STEP_{38}$ and $STEP_{20}$ are not currently known, though it has been proposed that they may have a regulatory function and compete with active STEP variants for binding sites (Goebel-Goody, 2012). All of the splice variants of STEP contain a kinase interacting motif (KIM)

domain that allows for binding to substrates, and a polyproline-rich (PP) present in some isoforms ($STEP_{61}$ and $STEP_{38}$) appears to confer substrate specificity (Nguyen et al., 2002). $STEP_{61}$ contains an extra sequence that targets it to cell membranes, including the postsynaptic density (Oyama et al., 1995) and the endoplasmic reticulum, while $STEP_{46}$ lacks this sequence and is instead found in the cytosol (Lombroso et al., 1993; Bult et al., 2006).

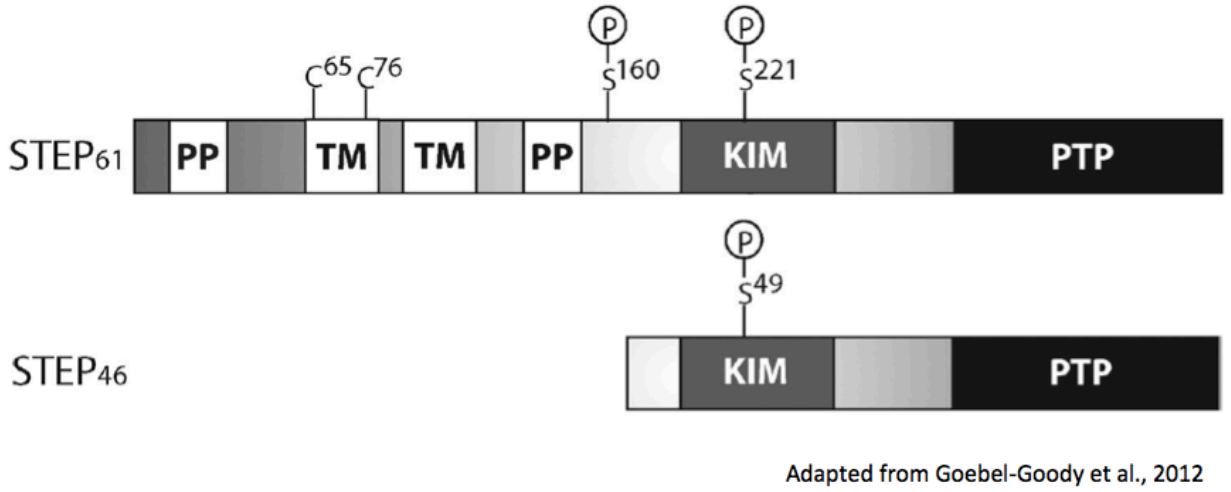


Figure 1 - Structure of $STEP_{61}$ and $STEP_{46}$ Isoforms. These main isoforms of STEP both contain a kinase interacting motif (KIM) domain, as well as a tyrosine phosphatase (PTP) domain. Phosphorylation of Ser^{221} on $STEP_{61}$ or Ser^{49} on $STEP_{46}$ inactivates STEP by sterically interfering with substrate binding. $STEP_{61}$ contains a transmembrane domain (TM) as well as popypropoline-rich domains (PP) which are hypothesized to have a role in substrate specificity.

1.2.3 Substrates

Known STEP substrates include ERK1/2, Pyk2, p38 and Fyn, as well as AMPA and NMDA receptor subunits (Fig. 2).

Extracellular Signal Regulated Kinases 1/2 (ERK1/2)

ERK1/2 is a member of the mitogen-activated protein kinase (MAPK) family that is known to have an important role in the induction and maintenance of synaptic plasticity (Sweatt, 2004),

and is a known substrate of STEP (Munoz et al., 2003; Paul et al., 2003). Following NMDAR stimulation, STEP deactivates ERK1/2 by dephosphorylation of two tyrosine residues, which limits the amount of time that the enzyme stays active (Paul et al., 2003; Valjent et al., 2005), suggesting a place for STEP in the opposition of synaptic strengthening. In addition, ERK1/2 phosphorylation is increased in the hippocampus and several other brain structures in STEP KO mice, further supporting the important of STEP in ERK1/2 regulation (Venkitaramani et al., 2009, 2011).

p38

Another member of the MAPK family, p38, is also a substrate of STEP and opposes the actions of ERK1/2. While ERK1/2 encourages synaptic strengthening and promotes cell survival, p38 is implicated in glutamatergic excitotoxicity and cell death pathways (Hardingham et al., 2002; Semenova et al., 2007; Ivanov et al., 2009; Poddar et al., 2010). Prolonged stimulation of extrasynaptic NMDARs leads to cleavage and inactivation of $STEP_{61}$. STEP becomes unable to dephosphorylate Tyr^{182} of p38, which promotes its activity and leads to neuronal death (Xu et al., 2009).

GluN2B

NMDARs contain four subunits, two of which are obligatory GluN1 subunits, and two of which are usually either both GluN2 units or a GluN2 and GluN3 subunit (Paoletti et al., 2013, Regan et al., 2015). Different splice variants of the subunits exist, one of which (GluN2B) is regulated by STEP. STEP regulates phosphorylation of the NMDAR subunit GluN2B both directly and indirectly. STEP regulates GluN2B directly by dephosphorylating the subunit at Tyr1472

(Snyder et al., 2005; Kurup et al., 2010). This is correlated with an increased association of GluN2B with clathrin adapter proteins, which promotes endocytosis of the GluN2B-containing NMDAR (Nakazawa et al., 2006), and in fact, we see an increase in surface GluN2B receptors in STEP KO mice (Zhang et al., 2010; Venkitaramani et al., 2011). In addition to directly regulated GluN2B phosphorylation, STEP also indirectly regulates it by dephosphorylating and inactivating of Src-family kinase Fyn (Nguyen et al., 2002). Active Fyn phosphorylates GluN2B on three tyrosine residues, and STEP inhibits this activity by dephosphorylating a tyrosine residue (Tyr^{420}) on Fyn (Nguyen et al., 2002). Consistent with the evidence that STEP mediates endocytosis of GluN2B-containing NMDARs is the reduction in excitatory postsynaptic currents (EPSCs) and LTP following the application of STEP to hippocampal slices and in contrast, inhibition of tonic levels of STEP enhances NMDAR EPSCs and prevents LTP (Pelkey et al., 2002). This supports a role for STEP in the tonic opposition of synaptic strengthening, which implies that STEP must be inactivated or removed in order for LTP to occur, and that higher amounts of STEP contribute to LTD (Zhang et al., 2008; Gladding et al., 2009; Chen et al., 2013).

GluA2

AMPARs contain four possible subunits (GluA1-GluA4) that form heterotetramers, most commonly found as GluA1/GluA2 or GluA2/GluA3 within the hippocampus (Park, 2018). In addition to its role in the regulation of NMDAR trafficking, STEP also regulates synaptic plasticity through dephosphorylation of a tyrosine residue on the AMPAR subunit GluA2 following group I mGluR stimulation using (s)-3,5-Dihydroxyohenylglycine (DHPG) (Zhang et al., 2008). This dephosphorylation results in internalization of the AMPA receptors and is reduced by a substrate-trapping STEP mutant (Zhang et al., 2008), suggesting that STEP is a requirement for this process.

In addition, STEP KO mice had higher levels of GluA2 containing AMPA receptors at the surface of neurons (Zhang et al., 2008, Venkitaramani et al., 2011). Recently, Won et al. (2019) demonstrated that the interaction between GluA2 and STEP is direct, and does not take place through an intermediary protein.

Pyk2

Finally, the focal adhesion kinase Pyk2 also appears to be a substrate of STEP (Xu et al., 2010; Venkitaramani et al., 2011). When activated Pyk2 phosphorylates and activates Fyn, which is then available to phosphorylate GluN2B, thereby increasing the surface expression of GluN2B-containing NMDARs (Besshoh et al., 2005; Le et al., 2006). In addition, Pyk2 activates ERK1/2, further promoting LTP (Nicodemo et al., 2010). By dephosphorylating and inactivating Pyk2, STEP again opposes the formation of LTP by reducing the activity of ERK1/2 and Fyn.

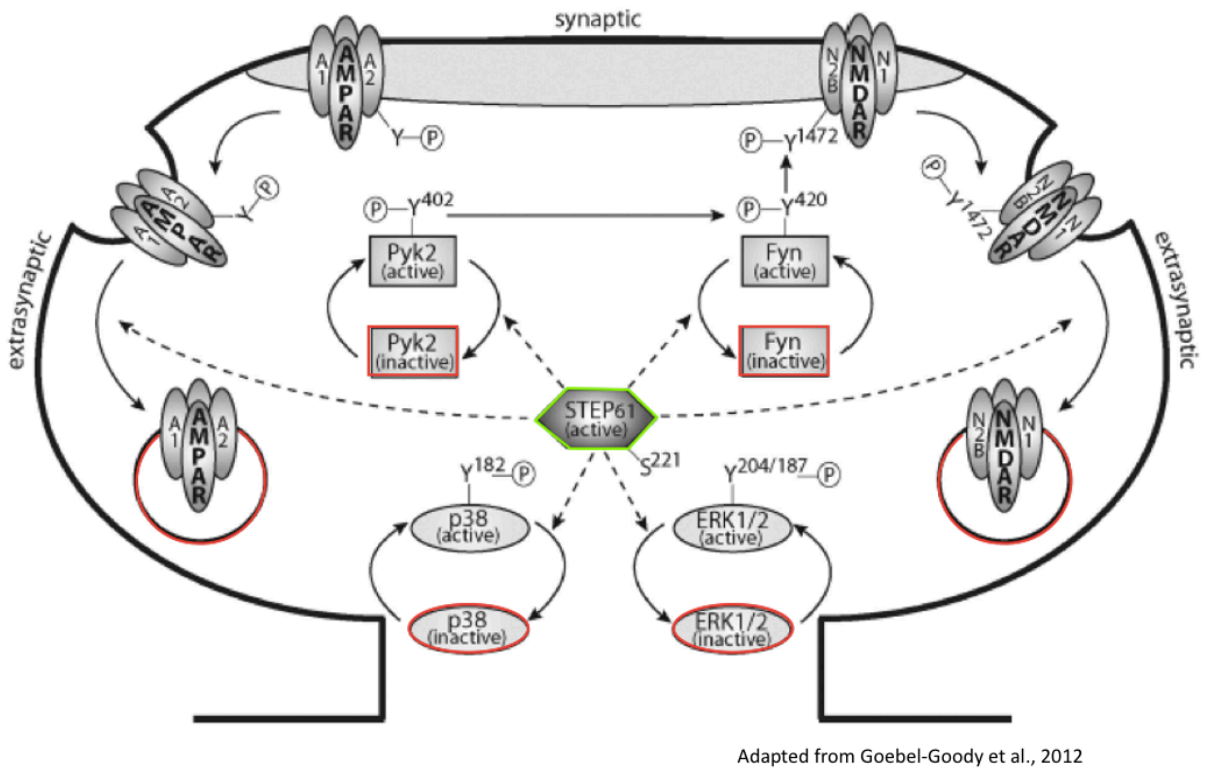


Figure 2 - STEP$_{61}$ Regulation of Substrates. Well known substrates of STEP$_{61}$ include Pyk2, Fyn, p38, ERK1/2, as well as AMPA and NMDA receptors. When phosphorylated at Ser^{221}, STEP$_{61}$ dephosphorylates regulatory tyrosine residues on Pyk2, Fyn, p38 and ERK 1/2 and consequently inactivates them. In addition, STEP$_{61}$-mediated phosphorylation of regulatory tyrosine residues on AMPA and NMDA receptors lead to their endocytosis, further opposing synaptic strengthening.

1.2.4 Regulation

STEP is regulated through a variety of mechanisms including phosphorylation, ubiquitination, proteolytic cleavage, dimerization and local translation.

Phosphorylation of Ser^{221} of STEP$_{61}$ or Ser^{49} in STEP$_{46}$ within the KIM domain reduces STEP activity by sterically hindering its ability to bind to substrates (Paul et al., 2000; 2003). Protein kinase A (PKA) is one enzyme known to phosphorylate (and deactivate) STEP following DR1-like dopamine receptor stimulation (Paul et al., 2000). In addition to directly phosphorylating

STEP, PKA also activates DARPP-32, which regulates STEP through the inhibition of protein phosphatase 1 (PP1) (Valjent et al., 2005). An opposing process occurs following the activation of NMDARs, which leads to increased PP1 activity via calcineurin, and hence, increased STEP phosphorylation and activity (Paul. et al., 2003; Snyder et al., 2005; Valjent et al., 2005). Interestingly, though NMDAR activation can lead to increased STEP activity through PP1, it has also been shown to lead to rapid degradation of STEP through ubiquitination (Xu et al., 2009). Extrasynaptic NMDAR stimulation has also been shown to reduce STEP activity through a mechanism involving calpain-mediated proteolytic cleavage within the KIM domain (Xu et al., 2009). This cleavage results in a $STEP_{33}$ fragment which is no longer capable of binding to substrates. Consequently, the MAPK p38 (which is usually inactivated by STEP) becomes activated and triggers an apoptotic signaling cascade; blocking the proteolytic cleavage of $STEP_{61}$ protects against glutamate neurotoxicity (Xu et al., 2009). Dimerization of $STEP_{61}$ occurs through cysteine residues within the amino terminus in low amounts basally, and in higher amounts upon the induction of oxidative stress, and this dimerization is also associated with a reduction in phosphatase activity (Deb et al., 2011).

Local dendritic translation of $STEP_{61}$ is thought to play a role in mGluR-dependent LTD (Zhang et al., 2008), and the presence of cytoplasmic polyadenylation elements (CPEs) within the 3' UTR of STEP suggests that this translation may be regulated by the CPE binding protein (CREB) (Goebel-Goody et al., 2012; Piqué et al., 2008). The fragile X mental retardation protein (FMRP) is also thought to regulate translation of STEP through a G-quartet sequence in the 3' UTR (Darnell et al., 2011). The current hypothesis is that CREB and FMRP bind and repress STEP mRNA, and that the dissociation of these proteins following synaptic stimulation leads to the rapid translation of STEP (Goebel-Goody et al., 2012).

1.2.5 Role in Memory/Psychiatric Disorders

The dysregulation of STEP is linked to a large number of neuropsychiatric disorders. Numerous investigations have found abnormal levels of STEP in neurological conditions such as Alzheimer's disease, schizophrenia, Fragile X, as well as substance abuse disorders, amongst others.

Evidence suggests that at least some of the cognitive decline observed in patients who have Alzheimer's disease (AD) is due to abnormally elevated levels of STEP. This is apparently due to modulation of STEP by beta-amyloid (Aβ) protein that is increased in AD. Aβ increases STEP activity by inhibiting the ubiquitin-proteasome system, which therefore slows down the rate of STEP degradation (Kurup et al., 2010). In addition to this mechanism, Aβ also activates calcineurin and PP1, which dephosphorylate and activate $STEP_{61}$ (Snyder et al., 2005). The subsequent increase in NMDAR endocytosis leads to cognitive decline (Kurup et al., 2010; Zhang et al., 2010). Consistent with this hypothesis, several animal models of AD as well as post-mortem samples of prefrontal cortex from AD patients show increased STEP activity (Chin et al., 2005; Kurup et al., 2010; Zhang et al., 2010). Recent research has shown that STEP is also increased in the hippocampus of animal models and humans with amnesic mild cognitive impairments (Castonguay et al., 2018), which are part of the prodromal phase of AD.

STEP also appears to have a role in alcohol-induced memory loss, which prevents the formation of new memories while intoxicated (McIntosh and Chick 2004). STEP is required for ethanol-induced inhibition of NMDAR function and LTP, as well as for ethanol's ability to inhibit fear learning (Hicklin et al., 2011). Treatment of wild type and STEP KO brain slices with ethanol back up these findings by demonstrating that ethanol only leads to reduced phosphorylation of the GluN2B subunit in the wild type (Hicklin et al., 2011).

1.2.6 STEP and Learning

Much of the research on STEP has examined the effect of STEP on memory. There is very little research, however, that examines the effect of learning on STEP. One paper that looked at the effect of fear conditioning on STEP found that fear induction in rats resulted in the *de* novo translation of both $STEP_{61}$ and $STEP_{46}$ in the amygdala within 10 min, and infusion of a STEP substrate-trapping mutant into the amygdala interfered with fear conditioning memory and LTP induction (Paul et al., 2007). Paul et al. demonstrated that experiences can affect STEP regulation, and that at least in some circumstances, STEP may be required for LTP induction in the lateral amygdala, despite its well-known role in opposing the strengthening of synapses in the hippocampus. The effect of learning a hippocampal-dependent spatial task on STEP has, to the best of our knowledge, not yet been studied. In our study, we determined the effect of training in a spatial task (the Morris water maze) on the level of total $STEP_{61}$ protein and on the active form of $STEP_{61}$ in the rat hippocampus. In addition, we examined the effect of DHPG and NMDA treatments (meant to induce LTD) on $STEP_{61}$ in primary hippocampal neuron cultures.

The negative consequences of elevated STEP levels suggest that the reduction of STEP may in fact improve certain aspects of cognition. In fact STEP KO mice have shown improved performances in both hippocampal-dependent (Venkitaramani et al., 2011) and amgydala-dependent learning (Olausson et al., 2012). For instance, though STEP KO mice were able to learn the Morris water maze task (which is hippocampal-dependent) as well as their wild type counterparts, they also performed better than the wild type when it came to a reversal learning task where the platform was moved (Venkitaramani et al., 2011). This suggests that they had greater cognitive flexibility, or perhaps improved extinction learning. In addition, the STEP KO mice

committed fewer reference and working memory errors in the radial-arm water maze, a much more difficult spatial learning task.

More recent evidence shows a role for STEP in age-related cognitive decline in several different species. STEP levels were found to be higher in cognitively impaired aging mice, rats, monkeys, as well as post-mortem amnesic mild cognitive impairment (aMCI) human hippocampi, compared to non-cognitively impaired controls (Castonguay et al., 2018). In addition, the overexpression of STEP in the CA1 region of the hippocampus of young mice was able to induce similar declines in performance during MWM acquisition (Castonguay et al., 2018); subsequent injection of the small molecule STEP inhibitor TC-2153 also restored cognitive performance in mice overexpressing STEP as well as in aged rats. Unfortunately, TC-2153 is difficult to dissolve and has an unstable sulfur ring structure, which consequently make it not ideal for the purpose of drug development and has led to hesitancy to test it in clinical trials. However, it has helped to support STEP as a potentially powerful drug target (Lombroso et al., 2019). To date, however, there are no commercially available drugs that selectively target STEP. Alternatively, it might be possible to affect the pathways that STEP is involved in either through the targeting of STEP regulators or its substrates.

1.2.7 The Search for Interacting Proteins

Though many substrates of STEP have been identified and studied, the full scope of STEPs interactions are unknown. Recently, however, Won et al. (2019) used liquid chromatography tandem mass spectrometry (LC/MS/MS) to identify proteins that co-immunoprecipitated with $STEP_{61}$ in mouse cortex and hippocampus lysate. The co-immunoprecipitation yielded several hundred potential interactors, and a major finding of this study was that $STEP_{61}$ binds directly to the GluA2 and GluA3 subunits in the hippocampus, which contradicts previous findings (Pelkey

et al., 2002). Importantly, this highlights the possibility that novel interactors of STEP might be found through the use of more modern research techniques and/or equipment.

Proximity-dependent Biotin Identification (BioID) is a recent advance to the interactome discovery toolkit. The process requires the fusion of a promiscuous biotin ligase to the end of a protein of interest. The protein of interest can then be expressed in living cells and, upon addition of biotin, it will biotinylate proteins that pass within 10 μm of the biotin ligase region. The method allows for the detection of proteins that are found in close proximity at any point in time throughout the experiment and does not require direct interaction of the proteins. As such, BioID helps to identify proteins that are localized within the same space as the bait protein as well as potential interactors of the bait protein that may interact only transiently or weakly, and therefore are unlikely to show up when using other screening methods such as co-immunoprecipitation. Affinity-purified mass spectrometry (AP-MS) is a complementary method to screen proteins that relies on the identification of proteins through the analysis of co-precipitated proteins through the chemical analysis of their components identified through mass spectrometry. We used results from both BioID and AP-MS in order to uncover a list of potential STEP-interacting proteins. We assessed the level of one of these proteins (neuromedin-U receptor 1, NMUR1) in the hippocampus of rats following MWM training. NMUR1 is primarily expressed in the peripheral nervous system, though it is also found in the hippocampus (Zhang et al., 2010). In addition, neuromedin U is capable of inhibiting memory impariments in mice and neuronal death in cultured neurons exposed to inflammation (Iwai et al., 2008). Furthermore, neuromedin exposure in neuromedin U neurons of the dorsal root ganglion has been linked to increases in ERK1/2, a known substrate of STEP (Zhang et al., 2012), making it an interesting potential STEP-interacting protein.We also assessed the level of neurologin-1 (NLGN-1), a synaptic anchoring protein recently shown to interact with $STEP_{61}$

(Won et al., 2019), in addition to $STEP_{61}$ total protein and active $STEP_{61}$ (not phosphorylated on Ser^{221}).

Chapitre 2 – Hypotheses & Objectives

2.1 Hypotheses

Our main hypothesis was that a spatial learning task would increase the level and/or activity level of STEP protein in the hippocampus of rats. We also hypothesized that these changes would be associated with changes in LTD and/or LTP in the hippocampus. Finally, we expected that there are a variety of STEP substrates and/or regulators that have not as of yet been discovered that may play a role in spatial learning, LTD and/or LTP.

2.2 Objectives

2.2.1 Objective 1

Our first objective was to develop a list of possible novel interactors of STEP using proximity-dependent biotin identification (BioID) and affinity purification mass spectromery (AP-MS).

2.2.2 Objective 2

Our second objective was to examine the effect of a spatial learning task (the Morris water maze) on $STEP_{61}$ total protein and on $STEP_{61}$ phosphorylation on Ser_{221} in the hippocampus of rats. At the same time, we looked at the effect on neuroligin-1 (NLGN-1) and neuromedin U receptor 1 (NMUR1).

2.2.3 Objective 3

Our third objective was to develop an *in vitro* protocol that could chemically induce LTD in order to study the effect of LTD on STEP and potentially interacting proteins in rat primary hippocampal neuron cultures

Chapitre 3 – Materials and Methods

3.1 Objective 1

Proximity-dependent Biotin Identification (BioID) and affinity-purified mass spectrometry (AP-MS) were used in order to identify potentially novel substrates or regulators of STEP. BioID and AP-MS, as well as all protein expression and cultures required in order to perform the these experiments were carried out by the team of Dr. Benoit Coulombe at the Montreal Clinical Research Institute. Significance analysis of interactome (SAINT) scores were assigned by Dr. Coulombe's team to the proteins identified using BioID and FLAG AP-MS. Our team used the SAINT scores provided to help narrow down which proteins were the most likely to be interacting with STEP.

3.1.1 Proximity-Dependent Biotin Identification (BioID)

BioID was used in order to identify proteins existing in the same cellular environment as $STEP_{61}$ and $STEP_{46}$, as previously published (Cloutier et al., 2017). In short, BioID consists of a technique in which a promiscuous biotin ligase (BirA*) is fused to a protein of interest. The fusion protein can then be expressed in living cells and will biotinylate proteins that are within a 10 μm distance of the protein of interest (or more specifically, within range of the biotin ligase) when biotin is added to the cells. The cells are later lysed and biotinylated proteins are pulled down and identified using mass spectrometry. Thus, potential interacting proteins may be revealed as well as proteins that are found in the near environment, with which protein of interest does not necessarily directly interact. The experiments were repeated 3 times in HEK293 cells. False positive interactions were reduced through the use of the Decontaminator computational approach (Lavallee-Adam et al., 2010).

3.1.2 Affinity-purifiation Mass Spectrometry

Affinity-purification mass spectrometry was used in order to identify potential directly-interacting STEP substrates and/or regulators, according to previously published protocols (Thiffault et al., 2015, Cloutier et al., 2017). In short, $STEP_{46}$ was fused with a FLAG-tag and expressed in human embryonic kidney 293 (HEK-293) cells. The FLAG-tagged $STEP_{46}$ was then immunoprecipitated and proteins bound to STEP were eluted and identified using mass spectroscopy. The experiment was repeated 3 times. False positive interactions were reduced through the use of the Decontaminator computational approach (Lavallee-Adam et al., 2010).

3.2 Objective 2

3.2.1 Animals

3-month old male Long-Evans rats (N=54) were purchased from Charles River (Kingston, On). Rats were kept on a 12-hour light/12-dark cycle (lights on at 8am) at an ambient temperature between 22°C and 25°C. They had *ad libitum* access to food and water for the entire duration of the study. Rats were single-housed and allowed to acclimate to the animal facility for one week before undergoing 5 days of handling habituation. All procedures were approved by the appropriate ethics committee (Le comité d'éthique de l'expérimentation du centre de recherche de l'Hôpital du Sacré-Coeur de Montréal).

3.2.2 Experimental Design

In order to determine the effect of a spatial learning task on STEP, we first trained rats on the Morris water maze (MWM) task (N=54). Those rats were separated into two groups, that is, one group that completed a typical probe test (n=22) without the platform at the end of training,

and those who essentially underwent a final training session with the platform still available (n=23), rather than the probe test. THE NUMBER OF ANIMALS PER GROUP VARIED. INSERT HERE. A control group consisted of 9 rats who underwent all of the same handling as the test rats but were never released into the water. One hour following the probe test the rats were euthanized by decapitation, and the brains were flash frozen on dry ice and stored at -80°C until further processing. The protocol for the Morris water maze was changed several times throughout the study (see **Fig. 3A, 5A, 7A, 9A**), as described below.

3.2.3 Morris Water Maze

The Morris water maze consists of three main components : 1) An acquisition phase (lasting 4 to 5 days), in which rats are trained to use distal cues to find a hidden platform, 2) A series of cued trials (3 over the same day) where the platform is made visible and moved around the pool in order to ensure that the rodents have adequate vision and the motivation to escape onto the platform, and 3) A probe trial (on the final day), in which the platform is removed and learning through the use of distal cues can be inferred through the amount of time the rodent spends in the area where the platform was previously located.

The Morris water maze (MWM) task was carried out in a pool 180 cm in diameter that was filled with a mixture of water and white non-toxic paint in order to avoid visibility of the submerged platform. One hour prior to testing, the rats were brought into the testing room in order to acclimate to the environment. The lights were left on until immediately prior to testing, when a solitary light was left on above the pool only. Any time a rat was removed from the water, it was immediately toweled dry and then remained in a clean cage on top of a heating pad for several min before being returned to its cage, in order to avoid hypothermia. Control rats were brought to the water but were only touched to the surface of the water before being towelled off and brought to the heating pad.

At all times, the room was kept at 25°C, and the water in the pool was kept between 23°C-25°C. Distal cues present around the pool were changed several times throughout the study (**Fig. 3B, 5B, 7B, 9B**).

During the acquisition phase (4 or 5 days), the platform (10.5 cm or 15 cm) was located in the centre of the North-West quadrant of the pool, 0.5 cm below the surface of the water. The rats were placed gently in the water at semi-random locations around the edge of the pool (**Supplemental Figures 4, 6, 8, 10**), and were released facing the outer edge of the pool. Rats were given a maximum of 90 sec to find the hidden platform, after which they were guided to through the water towards the platform. After all acquisition trials, rats were left on the platform for 10 sec in order to give them time to observe the surroundings. These trials were repeated 3 times per acquisition day, with a minimum interval of 45 min between each trial.

The cued trials consisted of a series of trials where the platform was made visible by raising it 0.5 cm above the surface of the water and covering the sides with black tape. The cued trials consisted of 3 x 30 sec trials, each trial being at least 45 min apart. The cued trials took place either in the morning on the day before acquisition training or in the afternoon after the third acquisition training day. The platform was moved to a different quadrant for each cued trial (**Fig. 3A**). Rats that failed to find the platform after 30 sec were led to it.

The probe trial took place on the final day (Day 6 or Day 7). One group of rats underwent a standard probe trial where the platform was removed from the water and the rats were allowed to search for it for 30 sec (probe group). Another group was also given 30 sec to find the platform, but in this group the platform was not removed (platform group). Rats were euthanized one hour following the probe trial.

For all Morris water maze manipulations, the movements of the rats were captured with a camera and analyzed using SMART software (Harvard Apparatus). Using the SMART software, we were able to determine the speed of displacement, latency to target, distance travelled, and the percentage of time spent in different zones of the pool.

3.2.4 Protein Extraction and Western Blot

Rats were euthanized by decapitation 1 hour following the probe test. Their brains were rapidly removed and flash frozen on dry ice and then kept at -80°C. Later, one hemispere was sliced 500 μm thick at -20°C, and the dorsal hippocampus was excised. The protein from these sections was extracted for analysis by Western blot. The hippocampi were kept on ice and were homogenized in 1X Cell Lysis Buffer (Cell Signaling, Cat. No 9803), 0.5% CHAPS (Fisher BioReagents, Cat. No. BP571-1) and a protease and phosphatase inhibitor (Thermo Scientific Cat. No. A32961). The samples were then sonicated at an amplitude of 20% (18 x 1 s on, 1 s off), following which they were rotated for 1 hour at 4°C. Finally, the samples were centrifuged at 14 000 rcf for 20 min, still at 4°C. Following centrifugation the supernatant containing the protein was stored in clean tubes and the pellet was discarded. The proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific Cat. No. 23225).

Proteins were diluted to a final concentration of 1μg/ μl. LDS sample buffer (Thermo Fisher, Cat. No. NP 007) and Bolt reducing agent (Invitrogen, Cat. No. B0009) were added. This solution was heated to 70°C for 10 min. 20 μl of each sample was loaded onto 4-12% Bis-Tris Plus 15 well gels (Thermo Fisher, Cat. No. NW04125BOX), and an intergel control was loaded onto each gel in order to allow for comparison between samples that were not on the same gel. The gels underwent electrophoresis for 90 min at 100V in MOPS running buffer with 500μl Bolt antioxidant (Invitrogen, BT0005). The proteins were later transferred to PVDF membranes (Immobilon-P^{sq},

Cat. No. ISEQ00010, 0.2µm pored) in NuPage-Methanol Transfer Buffer with antioxidant for 90 min at 100V. The membranes were then blocked using either Odyssey Blocking Buffer (PBS) (LiCor, Cat. No. 927-40000) or Intercept Blocking Buffer (PBS) (LiCor, Cat. No. 927-70001) for an hour at room temperature, and later allowed to incubate with the primary antibodies anti-STEP(D9H3) (Cell Signaling, Cat. No. 9069), anti-non-phospho-STEP(Ser221) (Cell Signaling, Cat. No. 5659), anti-GAPDH (Santa Cruz, Cat. No. sc-25778), anti-NMUR1 (Abbexa, Cat. No. abx217197), anti-neuroligin-1 (Santa Cruz, sc-365110) overnight at 4°C. Primary antibodies were all diluted to 1:1000 in Odyssey Blocking Buffer (PBS) and 0.2% Tween 20 (Fisher BioReagents, Cat. No. BP337-500). The following day the membranes were rinsed for 4 times in PBS-T 1X (0.1% Tween 20) for 5 min, after which they were kept in the dark and incubated with secondary antibodies IRDye 800CW (LiCor, Cat. No. 926-32211) or IRDye 680RD (Licor, Cat. No. 926-68070) for an hour at room temperature. The membranes were again rinsed 4x 5 min in PBS-T 1X, after which they were rinsed for 5 min with PBS 1X in order to remove residual Tween. The membranes were allowed to dry and were later scanned using the Odyssey CLx apparatus (Licor, Cat. No. 9140) and Image Studio 3.1 software. Band quantification was performed using ImageJ software.

3.2.5 Statistical Analysis

Latency, distance, and speed data for the acquisition and cued trials was analyzed out using one-way repeated measures analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. In the cases where data was non-spherical, the Greenhouse-Geisser F value correction was applied. In the event of a statistically significant result ($p<0.05$) pairwise comparisons were performed as appropriate. Latency to the platform target during the probe trial was assessed using the two-tailed independent samples t-test and % time spent in the target

quadrant versus other quadrants as well as platform crossings in the target quadrant *vs* other quadrants was analyzed using a two-tailed paired-samples t-test. All statistical analyses were carried out using IBM SPSS Statistics software version 25. Western blots were analyzed using one-way ANOVA. All results are reported as group mean +/- s.e.m.

3.3 Objective 3

3.3.1 Animals

Three Female Long Evans rats (Charles River, Kingston, ON) were mated at our facility. Matings were timed so that cultures occurred on embryonic day 18 or 19. All procedures were approved by the appropriate ethics committee (Le comité d'éthique de l'expérimentation du centre de recherche de l'Hôpital du Sacré-Coeur de Montréal).

3.3.2 Neuron Cultures

Primary hippocampal cell cultures were prepared from female Long-Evans rats on E18 or E19. Females were decapitated and the embryos were rapidly removed by caesarean section and stored in a HBSS buffer containing: Hank's Balanced Salt Solution (HBSS, Sigma Cat. No. H1641), HEPES 1M (Gibco, Cat. No. 15630-080), 1X Amphotericin B, Penicillin and Streptomycin (Gibco, Cat. No. 15240-062), 7.5% $NaHCO_3$ (Gibco, Cat. No. 15630-080). Embryos were immediately transferred to a biological safety cabinet, and all solutions and procedures were carried out under sterile conditions. Embryonic hippocampi were dissected as rapidly as possible and stored in groups of 6 hippocampi for 1mL HBSS at 37°C. Once all hippocampi had been dissected, 400 µl of 2.5% trypsin (Life Technologies, Cat no. 15090-046) was added to each of the tubes, which were then rotated at 900 rpm for 15 min, still at 37°C. Following trypsinization, the

hippocampi were washed twice with HBSS, following which they were dissociated in 1 mL HBSS with the aid of a 21G and 28G needle. Between 150 µl and 200 µl (depending on the culture) of the resulting solution was used to inoculate each well. The 12-well plates (Costar, Cat. No. 3513) contained 1 mL MEM-HS (MEM (Sigma, Cat. No. M0275), Horse Serum (Sigma, Cat. No. H1138), $NaHCO_3$, D-Glucose (Sigma, Cat. No. BP-250), GlutaMax (Sigma, Cat. No. RNBD9302), 1X Amphotericin B, Penicillin and Streptomycin) and were previously coated with poly-D lysine hydrobromide (Sigma, Cat. No. P6407-5MG). Cultures were kept at 5% oxygen, 37°C for approximately 24 hours, when the MEM-HS was replaced by 2 mL of Neurobasal Medium (Neurobasal Medium (Gibco, Cat. No. 21103-049), B27 supplement (Gibco, Cat. No. 17504-044), Glutamax, 1X Amphotericin B, Penicillin and Streptomycin). Half of the Neurobasal medium was removed and replaced with fresh media on day 7. On day 13, the volume of Neurobasal medium was reduced to 1 ml per well in order to simplify treatments, which took place on day 14.

3.3.3 Neuron Culture Treatments

We used both DHPG treatments and NMDA treatments in order to chemically induce LTD. Picrotoxin ($GABA_A$ receptor antagonist; 50µm, Tocris Cat. No. 1128, 0.1% DMSO) and L-689,560 (NMDA antagonist; 5µM, Tocris, Cat. No. 0742, 0.2% DMSO) were added 20 min before DHPG treatments, after which 100 µM DHPG (Tocris. Cat. No. 0342) was added for 10 min according to previousy published protocols (Gladding et al., 2009). Control wells were treated with DMSO. NMDA treatments (20µM, Tocris, Cat. No. 0114) lasted 3 min (protocol from Lee et al., 1998, Holman et al., 2007), and the control wells were treated with sterile water. All treatments took place on culture day 14. At the end of the treatment, media was removed from the wells and washed with ice cold PBS 1X. 150µl ice cold cell lysis buffer was added to each well. Cells were scraped off the bottom of the wells into the lysis buffer, and then removed and stored on ice in

clean microfuge tubes. The proteins were then extracted using the same protocol as the brain tissue samples (see section 3.2.4), with the exception that the cells were not incubated at 4°C for an hour. The protein was quantified and western blots were performed using STEP(D9H3) and GAPDH antibodies.

Chapitre 4 – Results

4.1 Objective 1

4.1.1 Interactome Screenings

BioID and FLAG AP-MS screening was carried out by Dr Coulombe and his team at the IRCM and the results were provided to us with accompanying SAINT confidence scores. Due to potential difficulties in the purification of membrane proteins, BioID was performed using both the transmembranous $STEP_{61}$ and cytosolic $STEP_{46}$ fusion proteins as bait. The $STEP_{46}$ BioID yielded a much higher number of results that $STEP_{61}$. A large number of proteins found during the BioID and FLAG AP-MS screening (**Supplemental Fig. 1-3**). In order to help narrow these down to a manageable list of potential interactors, we considered only those proteins that had a SAINT confidence score of 1 (the highest possible score, indicating the highest probability that the interaction is real) for the $STEP_{46}$ results, and a SAINT score of 0.9 for $STEP_{61}$. The BioID screenings resulted in eighty-nine proteins having a SAINT score of 1 using $STEP_{46}$ as bait (**Supplemental Fig. 1**), as compared to fourteen when using $STEP_{61}$ (**Supplemental Fig. 2**.). Lowering the SAINT score cutoff to 0.9 for $STEP_{61}$ added another thirteen proteins to our list. Eleven proteins obtained through AP-MS had SAINT scores of 1 (**Supplemental Fig. 3).** We then narrowed the list of proteins down further, base primarily on the localization of the protein in hippocampal neurons, and the commercial availability of antibodies. Additional criteria considered were having a tyrosine residue known to be regulated by phosphorylation, known expression in the hippocampus and previous evidence supporting a role in learning and memory, though these were not strict criteria. We obtained commercial antibodies for ten of these proteins and retained the most reliable antibody, which targeted Neuromedin U receptor-1 (NMUR1) (Data not shown).

4.2 Objective 2

4.2.1 Morris Water Maze

We performed various analyses in order to ensure that the Morris water maze protocol induced a spatial learning event in the rats. First, we assessed latency to the target, distance travelled to the target, and mean speed of the rats during the acquisition trials. In the event that learning has taken place, we expect to see a decrease in the latency and distance travelled to the target, and sometimes an increase in the mean speed. We analyse the cue latency in order to ensure that the animals have the physical ability to see the platform, swim to it in a timely manner, and climb onto it, as well as the motivation to do so. We expect that the rat should succeed at this task at least once during the three cued trials. Finally, in order to differentiate spatial learning from other types of learning, we determine the percentage of time the rats spend in the target 'platform' quadrant versus the other three quadrants. In the event that the rats have used a platform searching strategy other than the use of distal cues, we expect that the percentage of time spent in the target quadrant will not be significantly higher than in the other three quadrants. Four different protocols were tested throughout the experiments, and these analyzed separately below. For the acquisition and cued trials, data for the probe and platform groups were combined for analysis after we ensured that there were no significant differences for the results between each group (for analysis between groups, see **Supplemental Figures 5, 7, 9, 11**). The probe group consists of the rats that underwent the typical MWM probe trial with platform removed, while the platform group did not have the platform removed on the day of the probe trial.

Following the MWM, we performed western blots using protein from the hippocampus and probed for total $STEP_{61}$, non-phosphorylated-$STEP_{61}$ and NMUR1 (in the last three protocols), as

well neuroligin-1 (NLGN-1). Outliers that were 2 SD from the mean were identified by SPSS and removed before data analysis. Protein levels are expressed as a ratio of protein over GAPDH.

4.2.1.1 Protocol 1

The first MWM protocol (probe n = 4, platform n = 4) (**Figure 3**) consisted of 3x 30 sec cued trials on the first day of training, followed by five days of acquisition training (3x 90 sec/day), with a 30 sec probe test on the final day (**Fig. 3A**). We placed the cued trials before the acquisition training in order to ensure that changes to STEP and other proteins would be due to a long term learning effect, rather than a short term learning that would occur had the cued trials been placed at the end of training. The platform was 15 cm in diameter. Curtains were hung on all sides of the pool, from which were hanging four different small (28 x 21.5 cm) rectangular 'distal cues' (**Fig. 3B**). The starting locations of the rats for each trial are shown in **Supplemental Figure 4**. There was a reduction in latency to the platform and distance during the acquisition training ($F_{4,28} = 3.838$, p= .022 and $F_{4,28} = 3.3069$, p= .032; **Fig. 3D & 3E**) with the reduction in acquisition latency between day 1 and day 4 being statistically significant (p=.038), but no increase in swim speed ($F_{4,28} = 2.184$, p= .097; **Fig. 3F**). Latency did not change from one cued trial to another ($F_{2,14} = .962$, p= .406; **Fig. 3G**). During the probe trials, the rats did not spend more time in the NW (target) quadrant as compared to the other three quadrants, nor did they cross the NW platform area more frequently than the equivalent areas in the other three quadrants (p>.05; Fig. **3H & 3I**).

There were no significant differences in $STEP_{61}$ ($F_{2,13} = .171$, p=.844) , NLGN-1 ($F_{2,13} = 1.691$, p=.222) or non-phospho-STEP ($F_{2,13} = .458$, p=.642) total protein levels or in the ratio of non-phosphorylated to total $STEP_{61}$ ($F_{2,13} = .148$, p =.864) between the probe (n=3), platform (n=4) or control group (n=9) (**Fig. 4**). When combining the two MWM groups, NLGN-1 was significantly

($F_{1,13}$=5.021, p=.043) lower in the MWM conditions (79.9% +/- 5.3) as compared to controls (100% +/-7.19) (**Supplemental Fig. 12)**.

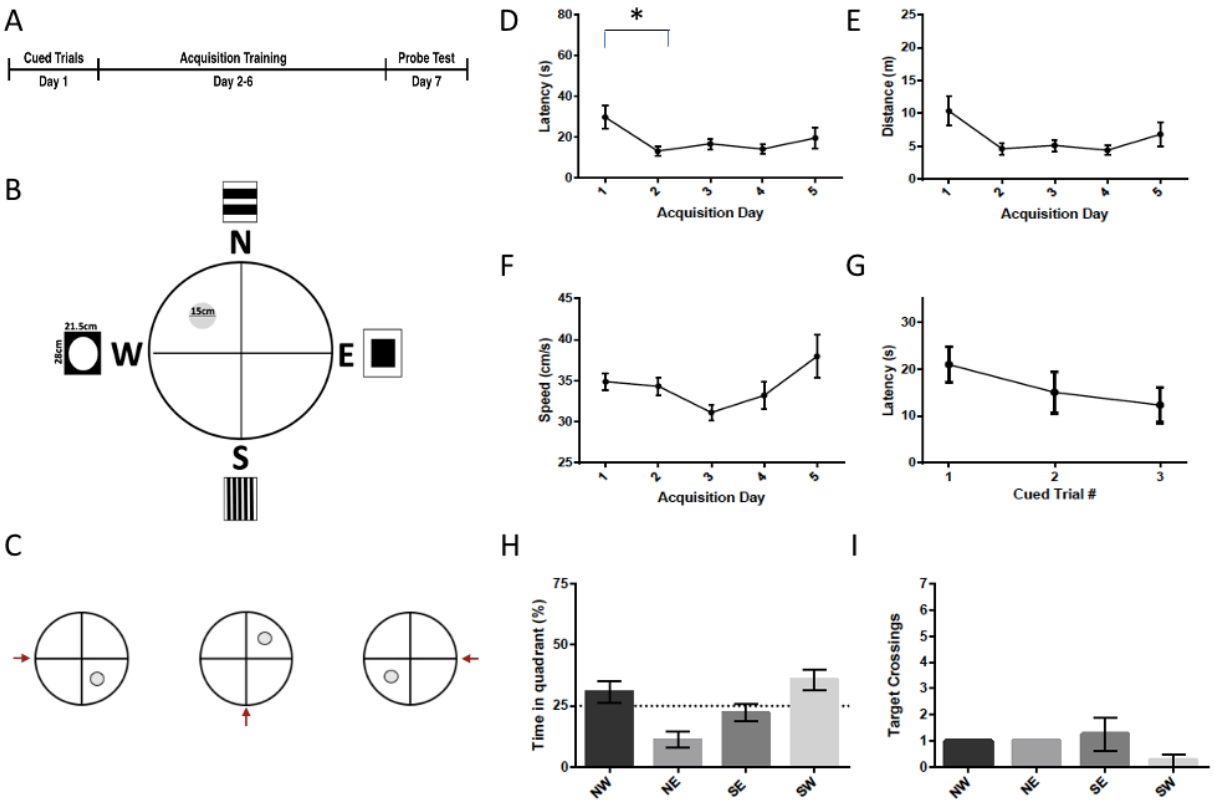


Figure 3 - Morris Water Maze Protocol 1. (A) Timeline of the Morris water maze protocol 1. In this protocol, the cued trials took place on day 1, acquisition took place from day 2-6, and the probe test took place on day 7. **(B)** Setup of the tank during the acquisition phase. Rats must find their way to the 15 cm platform located in the NW quadrant using the 21.5 cm x 28 cm distal cues. During the probe phase, the platform is removed. **(C)** Set up of the tank during the cued trials. The platform is placed in a different location for each of the 3 trials. The red arrow indicates the location where the rat was placed into the water. Distal cues are still present, though not shown in the schematic. **(D)** Latency to the platform during acquisition. There was a significant reduction in latency to the platform during acquisition training. The reduction was statistically significant on day 4 relative to day 1. **(E)** Distance travelled to the platform during acquisition training. There was a significant reduction in the distance travelled to the platform. **(F)** Swim speed was not significantly changed throughout the acquisition training. **(G)** Latency during the cued trials. Rats were able to find the platform within 30 sec during the cued trials. The latency did not change significantly throughout the cued trials. **(H)** Percent time spent in the target quadrant during the probe trial. The rats did not spend significantly more time in the target (NW) quadrant than in any of the other quadrants (p>.05) **(I)** Target crossings during the probe trial. The rats did not spend more time in a zone of 30 cm in diameter located in the center of the NW quadrant than in the equivalent areas in the other three quadrants (p>.05)

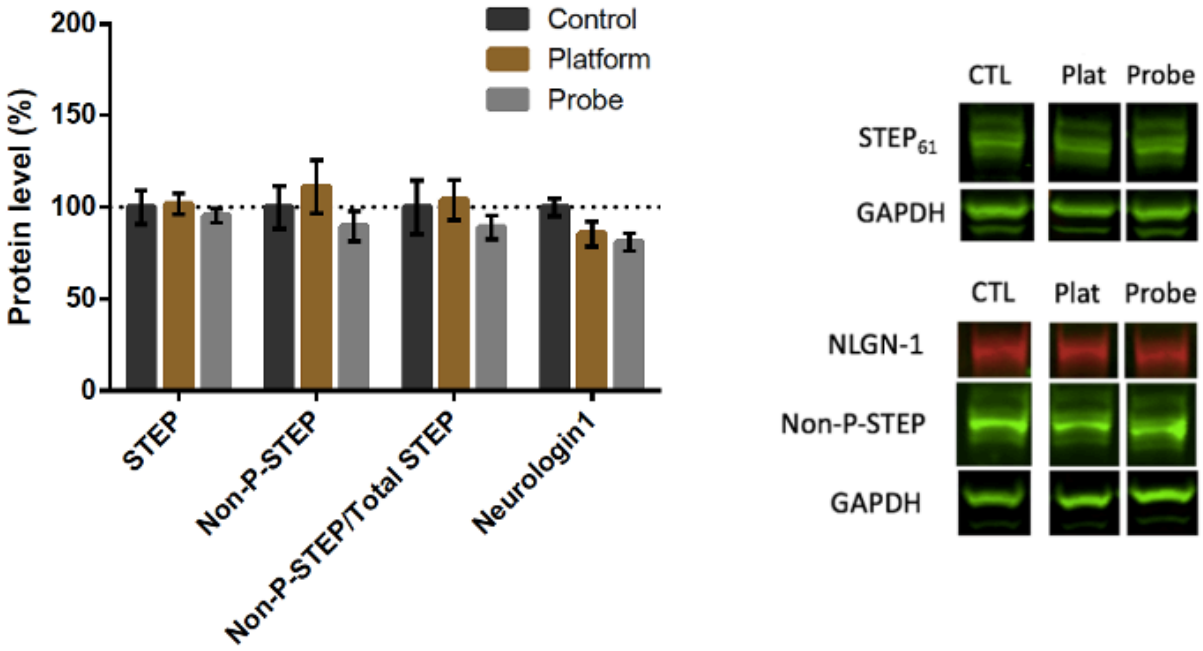


Figure 4 - Effect of MWM Protocol 1 on $STEP_{61}$, NLGN-1, and non-phospho-STEP protein
There were no significant differences in $STEP_{61}$, NLGN-1 or non-phospho-STEP between the probe group, the platform group, or the control group.

4.2.1.2 Protocol 2

During the second water maze protocol (probe n = 7, platform n = 8) (**Figure 5**), the smaller signs that served as distal cues surrounding the pool were removed and were replaced by larger signs (56 cm x 71 cm), in an effort to improve their visibilty to increase the likelihood that the rats would see them and use them in order to spatially navigate. These were placed in the same location on the curtains as in the previous protocol. Since the acquisition performance appeared to plateau towards the end of acquisition training, the acquisition period was shortened from five days to four. All other aspects of the test remained the same as in the first protocol. The rats did not start in the South-West until the probe trial, in order to discourage the memorization of a route to the platform. The starting locations of the rats for each trial are shown in **Supplemental Figure 6**. As in group

1, there was a reduction in latency and distance to the platform during acquisition training ($F_{3,42}$ = 3.267, p = .03 and $F_{3,42}$ =3.879, p = .016; **Fig. 5D & 5E**), and no change in swim speed during the acquisition training ($F_{3,42}$ = 1.113, p = .354, **Fig. 5F**). However, there was a decrease in the latency during the cued trials ($F_{2,28}$ = 11.039, p <.001), from a mean of 29 +/- 1s during the fist cue to a mean of 13 +/- 3 s in the last trial (**Fig. 5G)**. During the probe trial, there was not a significant difference in the percentage of time spent in the target quadrant (NW) versus the other quadrants, nor in the amoun of times the target platform area was crossed versus the analogous area in other quadrants (p>.05; **Fig 5H & 5I**).

There was a significant difference in NLGN-1 protein expression in the hippocampus ($F_{2,21}$ = 4.633, p =.022) during the second MWM protocol (**Fig. 6**). Post hoc analysis showed significantly higher expression of NLGN-1 in the platform group (n=8, 135% +/- 5.4) *vs* the control group (n=9; 100% +/- 10%, p = .017). There was no significant difference between NLGN-1 expression in the probe group (n=7) vs the platform (p= .229) or control group (p = .476). There were no significant differences between the groups with regards to level of $STEP_{61}$, NMUR1, non-phosphorylated-STEP, or the ratio of non-phosphorylated STEP to total $STEP_{61}$ ($F_{2,20}$ = 3.049, p = .070; $F_{2,17}$ = .264, p = .771; $F_{2,21}$ =.512, p =.606; $F_{2,20}$ =.156, p = .857) (**Fig. 6**). When the MWM conditions were combined **(Supplemental Fig. 13)** NLGN-1 expression was also higher in the MWM training group (126% +/- 5.7) *vs* the control (100% +/- 10.1) ($F_{1,22}$ = 5.875, p =.024).

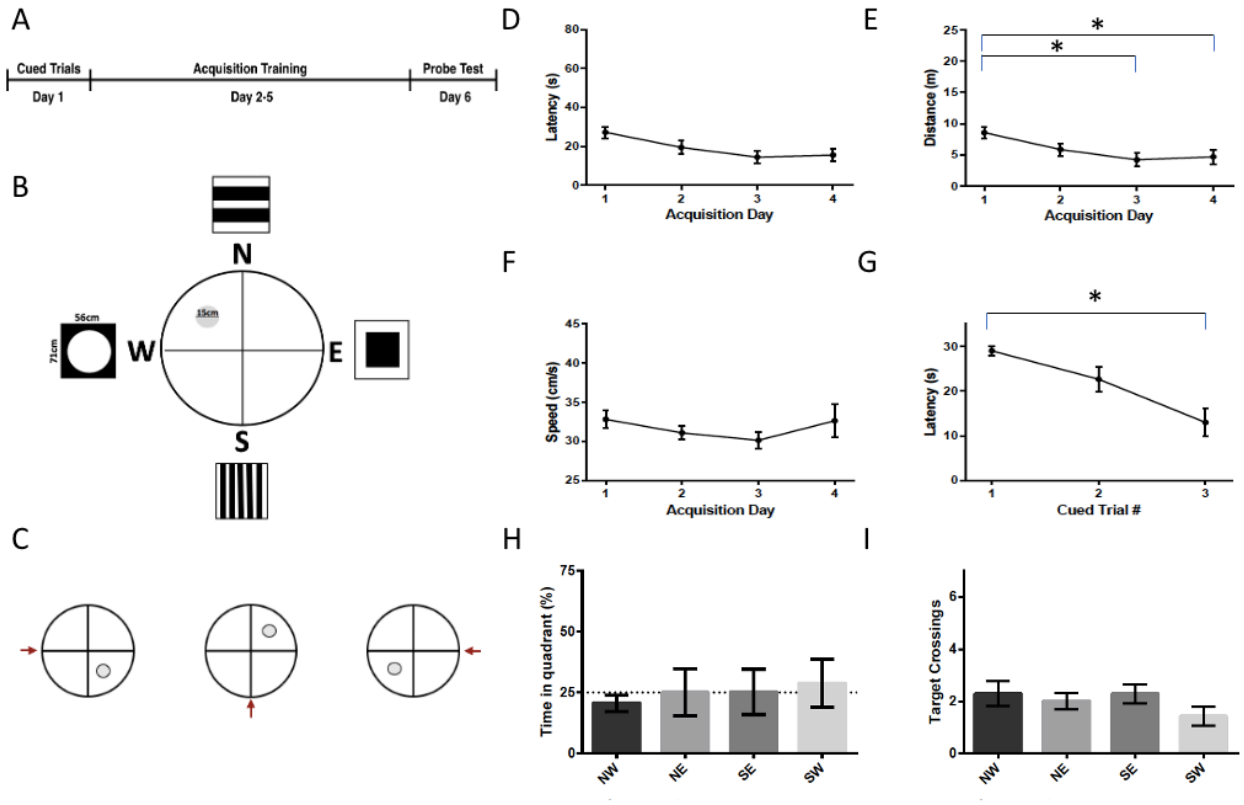


Figure 5 - Morris Water Maze Protocol 2 (A) Timeline of the Morris water maze protocol 2. In this protocol, the cued trials took place on day 1, acquisition training took place on day 2-5, and the probe test took place on day 6. **(B)** Setup of the tank during the acquisition phase. The distal cues surrounding the pool were larger for this protocol (56cm x 71cm). **(C)** Set up of the tank during the cued trials. The platform is placed in a different location for each of the 3 trials. The red arrow indicates the location where the rat was placed into the water. Distal cues are still present, though not shown in the schematic. **(D)** Latency to the platform during acquisition. There was a significant reduction in latency during the acquisition phase. **(E)** Distance travelled to reach the target during acquisition. There was a significant reduction in the distance travelled to reach the target during the acquisition phase. **(F)** Average swimming speed during the acquisition phase. The average swimming speed did not change significantly during acquisition training. **(G)** Latency during the cued trials. There was a significant reduction in latency during the cued trials. **(H)** Percent time spent in the target quadrant during the probe trial. The rats did not spend significantly more time in the NW quadrant as opposed to the NE, SE, or SW quadrant ($p>.05$) **(I)** Target crossings during the probe trial. The rats did not spend more time in a zone of 30cm in diameter located in the center of the NW quadrant than in the equivalent areas in the other three quadrants ($p>.05$)

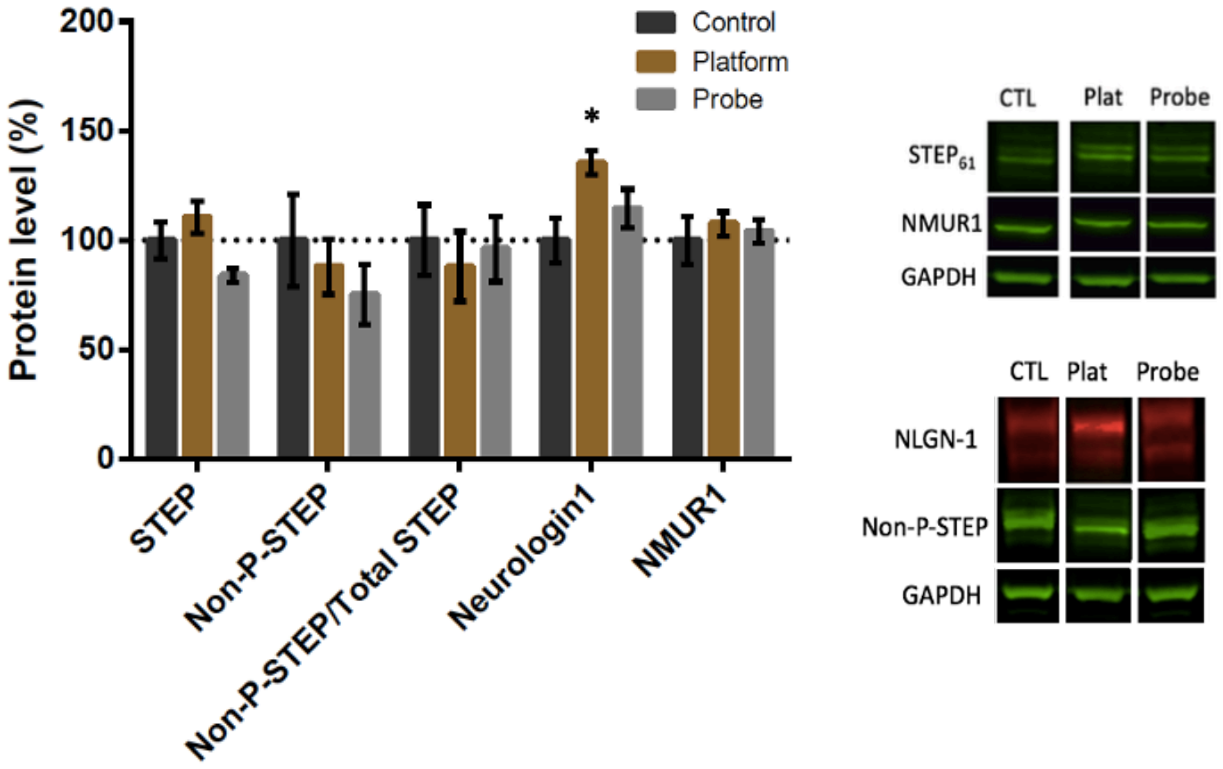


Figure 6 - Effect of MWM Protocol 2 on $STEP_{61}$, NLGN-1, NMUR1 and non-phospho-STEP protein. NLGN-1 expression differed between groups ($F_{2,21}$ = 4.633, p =.022), which can be attributed to a higher level of NLGN-1 in the platform group relative to the control (p=.017). $STEP_{61}$, NMUR1, non-phosphorylated-STEP and the ratio of non-phosphorylated STEP to total $STEP_{61}$ did not differ significantly between groups.

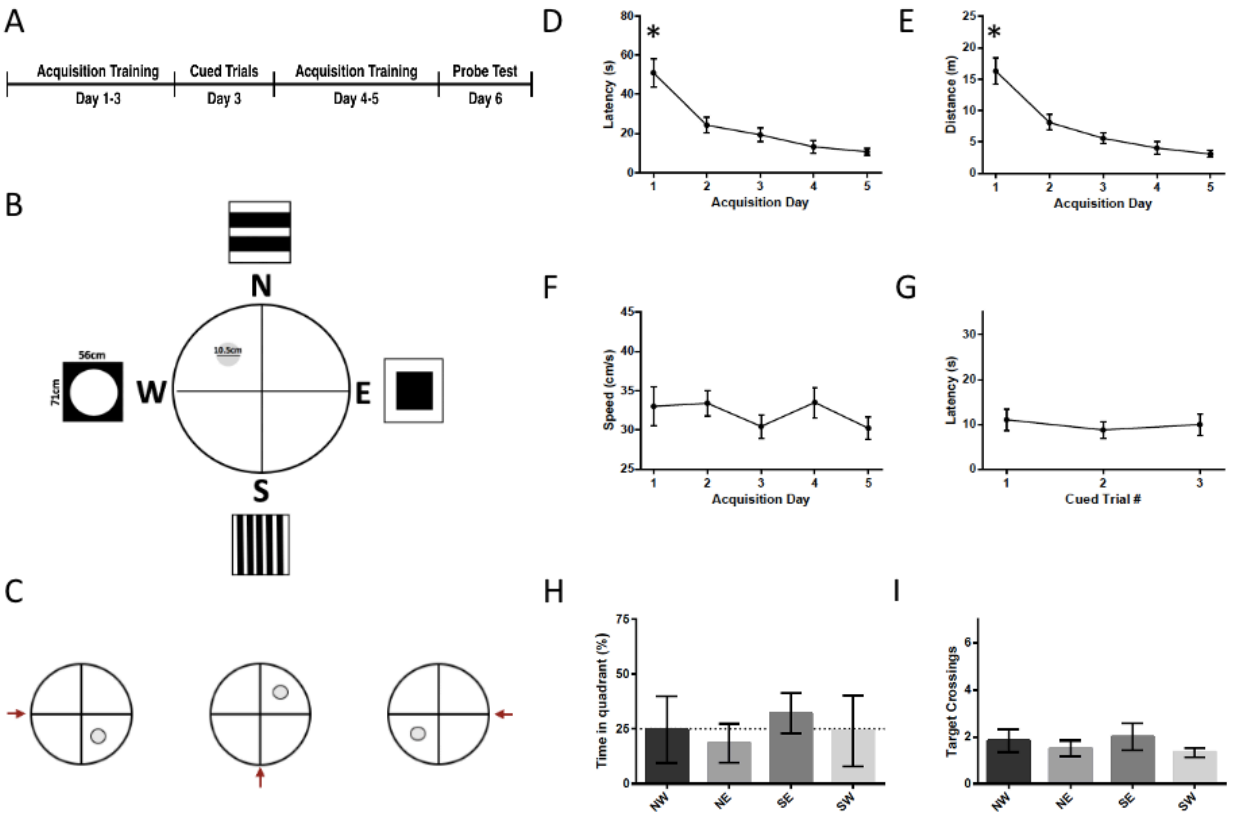


Figure 7 - Morris Water Maze Protocol 3 (A) Timeline of the Morris water maze protocol 2. In this protocol, the cued trials took place on day 1, acquisition training took place on day 2-5, and the probe test took place on day 6. **(B)** Setup of the tank during the acquisition phase. Rats must find their way to the 10.5 cm platform located in the NW quadrant using the 21.5cm x 28cm distal cues. During the probe phase, the platform is removed. **(C)** Set up of the tank during the cued trials. The platform is placed in a different location for each of the 3 trials. The red arrow indicates the location where the rat was placed into the water. Distal cues are still present, though not shown in the schematic. **(D)** Latency to the platform during acquisition. The reduction in latency was statistically significant. Pairwise interactions showed that latency was lower in the last four acquisition days as compared to the first day. **(E)** Distance travelled to the platform during acquisition training. Distance to the platform was significantly reduced during acquisition. Pairwise analysis showed significant reductions on days 2-5 as compared to day 1. **(F)** Swim speed. There was no difference in swimming speed throughout acquisition training. **(G)** Latency during the cued trials. There was no significant difference in latency to find the platform between different cued trials. **(H)** Percent time spent in the target quadrant. Rats did not spend significantly more time in the target (NW) quadrant than in the other quadrants during the probe trial ($p>.05$) **(I)** Target crossings during the probe trial. There was no difference in the number of platform crossings in the NW quadrant as compared to the other quadrants ($p>.05$).

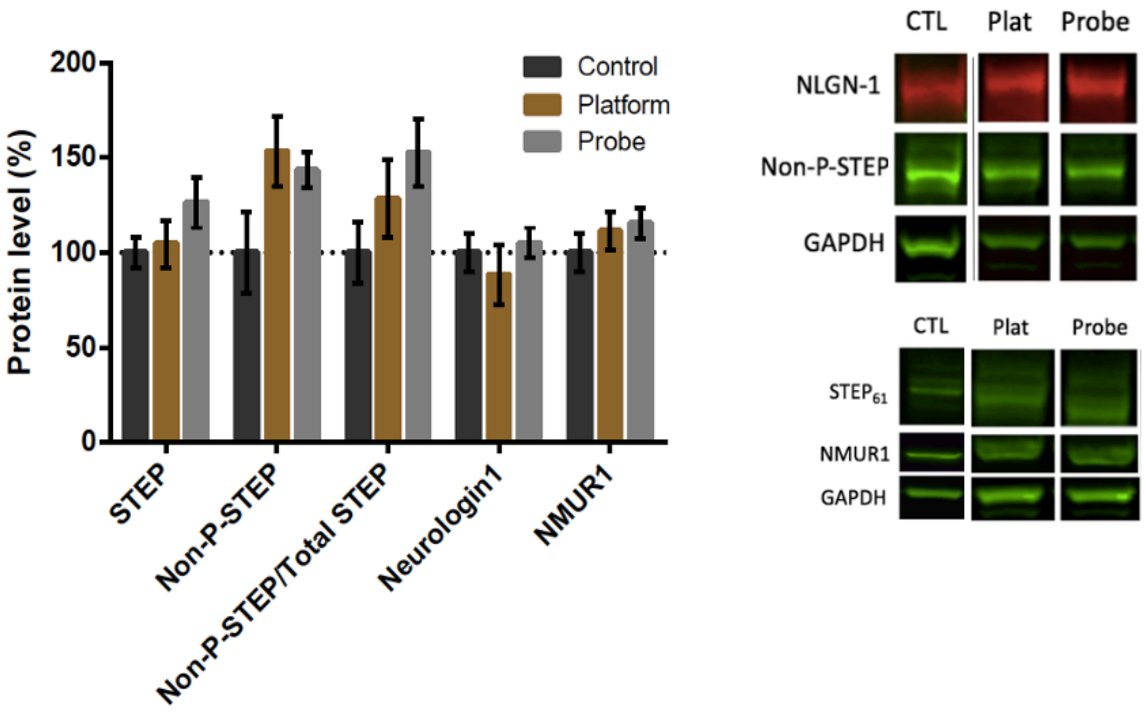


Figure 8 - Effect of MWM Protocol 3 on $STEP_{61}$, NLGN-1, NMUR1 and non-phospho-STEP protein. There were no significant differences in the level of $STEP_{61}$, non-phosphorylated-STEP, NMUR1, or NLGN-1 between groups.

4.2.1.3 Protocol 3

During the third MWM protocol (N=11) (**Fig. 7**), the 15 cm platform was exchanged for a 10 cm platform, in order to increase the difficulty of finding the platform, to increase the chances that the rats used distal cues to find the platform, rather than accidentally happening upon it. In addition, the cued trials were moved from the morning of day 1 to the afternoon of the third acquisition day, in order to provide a more accurate learning curve as measured through acquisition latency and distance to the platform. An extra acquisition day was added, so that the total number of acquisition days was one again 5, to ensure that the cued trials on the third day did not negatively impact performance on the probe trial. The probe trial took place on day 6. All other aspects of the experiment remained the same is in protocol 2. The rats did not start in the South-West until the

probe trial. The starting locations of the rats for each trial are shown in **Supplemental Figure 8**. Latency to the platform were reduced during the acquisition phase ($F_{2,20}$ = 16.810, p <.001; **Fig. 7D**), with each of the four last acquisition latencies being significantly shorter than the first (p=.016, p=.002, p=.012, p=.004). Distance to the platform was also significanlty reduced during acquisition ($F_{2,20}$ = 19.288, p <.001; **Fig. 7E**), with the first day again being significantly different from days 2-5 (p =.013, p=.002, p=.008, p=.002). Once again, swim speed during acquisition did not change ($F_{4,40}$ = 1.354, p = .267; **Fig. 7F**). There were no differences in latency during the cued trials ($F_{2,20}$ = .235, p =793; **Fig. 7G**), nor in time spent in the NW quadrant as compared to the other quadrants or number of times the NW platform was crossed as compared to the other quadrants (p>.05; **Fig 7H & 7I**).

Though there was no difference between groups for total $STEP_{61}$ ($F_{2,17}$ = 1.924, p = .117) protein or non-phosphorylated STEP ($F_{2,17}$ = 2.486, p = .113), or for the ratio of non-phosphorylated STEP to total $STEP_{61}$ ($F_{2,17}$ = 2.309, p=.130) (**Fig. 8**). There were no significant differences in NLGN-1 ($F_{2,17}$ = .438, p = .625) or NMUR1 ($F_{2,14}$ = .711, p = .508) protein levels (Figure 6). The control samples for this group came from protocols 1 & 2. When the MWM groups were combined **(Supplemental Fig. 14)** non-phosphorylated-STEP expression was significantly higher ($F_{1,18}$=5.186,p=.035) in the MWM training group (148.4% +/- 9.4) *vs* the control (100% +/- 21.1)

4.2.1.4 Protocol 4

During the fourth MWM protocol (N=11) (**Fig. 9**), we dramatically altered the distal cues, once again with the hope that this would encourage the rats to use the distal cues in order to spatially navigate. We removed the curtains from all sides of the pool except for the South side, and removed the large signs previously used as distal cues from all sides of the pool except for the West side,

where we affixed the sign to the wall (rather than the curtain). The North wall contained a large window, though the glass was covered with black cardboard in order to avoid interference from outside light. The East side contained two large shelving units. The rats were sometimes started in the South-West during acquisition, and the cued trials remained on day 3 as this had a large positive impact on the acquisition learning curves in protocol 2 *vs* protocol 1. All other aspects of the experiment remained the same as in protocol 3. The starting locations of the rats for each trial are shown in **supplemental figure 10**. Acquistion latency again decreased significantly ($F_{4,40} = 47.990$, $p < .001$; **Fig 9D**), with a significantly lowered latency in the last four days of acquisition as compared to the first ($p < .001$ for each). Acquisition distance to the platform was also reduced ($F_{4,40} = 12.223$, $p = .002$; **Fig 9E**), with the distance travelled on day 1 being significantly higher than on day 4 ($p = .023$) and day 5 ($p = .018$). Once more, swim speed was unchanged throughout the acquisition phase ($F_{4,40} = 1.467$, $p = .230$; **Fig. 9F**). Latency was reduced during the cued trials ($F_{2,20} = 7.150$, $p = .005$; **Fig. 9G**), with both the second and third trial being significantly lower than the first ($p = .012$, $p = .018$). For the first time, rats spent more time in the NW quadrant as compared to the NE, SE and SW quadrant during the probe trials ($p = .012$, $p = .025$, $p = .030$; **Fig. 9H**), and the rats crossed the NW target area significantly more often than the other analogous areas in the three remaining quadrants (NE, $p = .004$; SE, $p = .002$; SW, $p = .005$; **Fig. 9I**).

There were no between group differences for $STEP_{61}$ ($F_{2,17} = 2.423$, $p = .119$), NMUR1 ($F_{2,14} = 5.866$, $p = .081$), NLGN-1 ($F_{2,17} = 1.35$, $p = .284$) or non-phosphorylated STEP ($F_{2,17} = 1$, $p = .906$), nor was there any difference in the ratio of non-phosphorylated STEP to total $STEP_{61}$ protein ($F_{2,17} = 0.299$, $p = .745$) (**Fig. 10**). When the MWM groups were combined **(Supplemental Fig. 15)**, NMUR1 expression was significantly higher ($F_{(1,15)}=6.281$, $p = .024$) in the MWM group (154.5% +/- 14.9) *vs* the control (100% +/- 9.9).

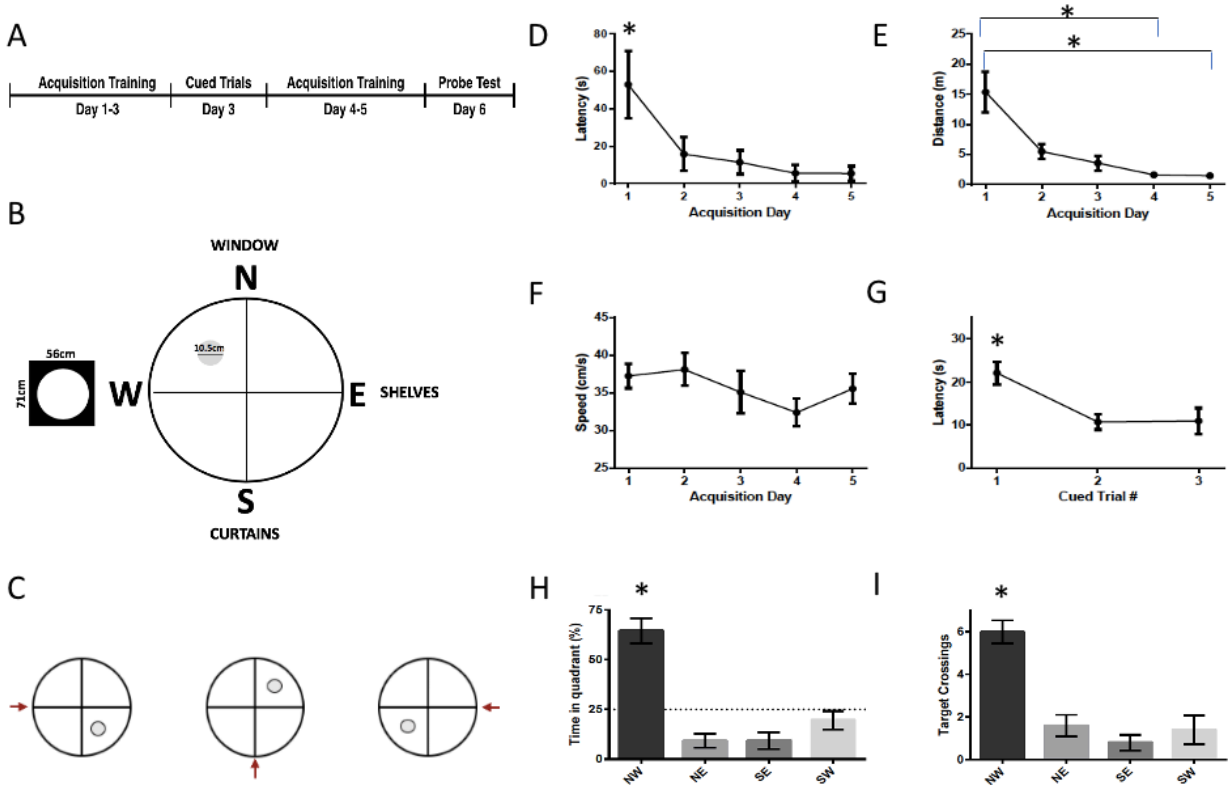


Figure 9 - Morris Water Maze - Protocol 4

(A) Timeline of the Morris water maze protocol 2. In this protocol, the cued trials took place on day 1, acquisition training took place on day 2-5, and the probe test took place on day 6. **(B)** Setup of the tank during the acquisition phase. Rats must find their way to the 10.5 cm platform located in the NW quadrant distal cues present in the room. During the probe phase, the platform is removed. **(C)** Set up of the tank during the cued trials. The platform is placed in a different location for each of the 3 trials. The red arrow indicates the location where the rat was placed into the water. Distal cues are still present, though not shown in the schematic. **(D)** Latency to the platform during acquisition. There was a reduction in latency during acquisition, with latency on the first day of acquisition being significantly longer than the others. **(E)** Distance travelled to the platform during acquisition training. Distance to the platform was significantly reduced during acquisition, with the distance travelled on day 1 being significantly higher than on day 4 and day 5. **(F)** Swim speed during acquisition. Swim speed did not increase throughout the acquisition phase. **(G)** Latency during the cued trials. Latency was reduced in the cued trials. Latency was increased on the first cue trial as compared to the second and third. **(H)** Percent time spent in the target quadrant during the probe trial. The rats spent significantly more time in the NW target quadrant as compared to the NE, SE, and SW quadrants during the probe trial. **(I)** Target crossings during the probe trial. Rats spent significantly more time in the NW quadrant as compared to the NE, SE and SW quadrant.

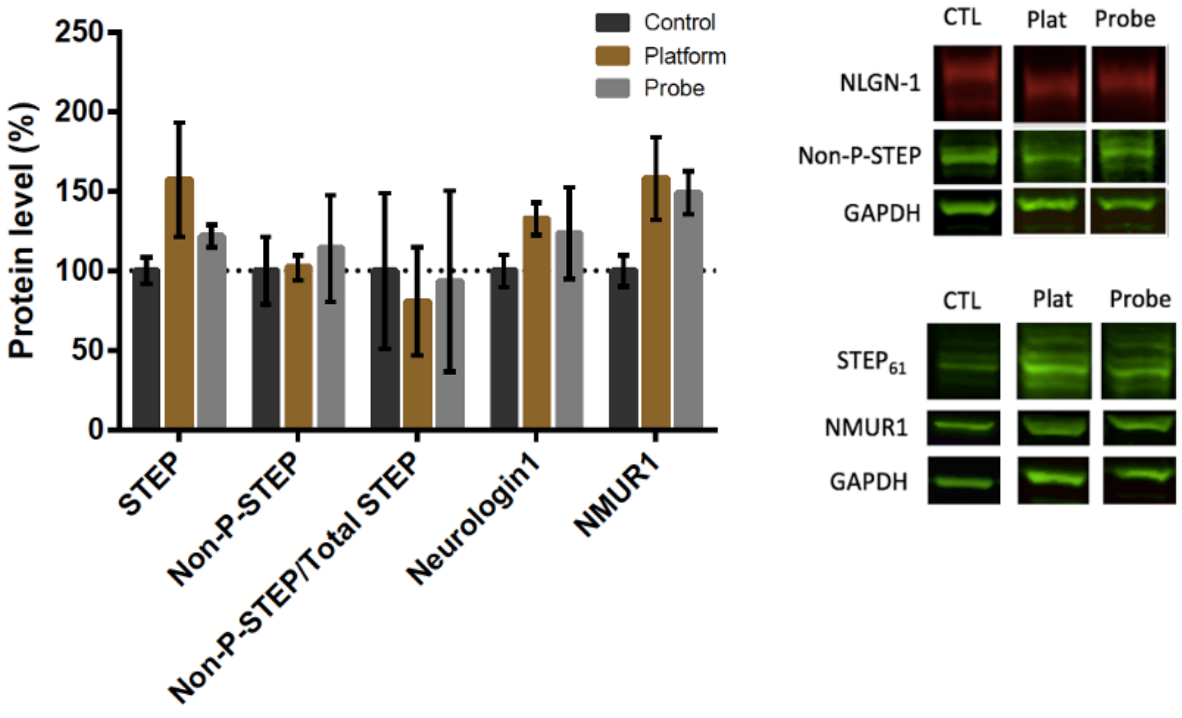


Figure 10 - Effect of MWM Protocol 4 on $STEP_{61}$, NLGN-1, NMUR1 and non-phospho-STEP protein. There were no significant changes in total $STEP_{61}$, NMUR1, NLGN-1 or non-phosphorylated STEP protein levels, nor in the ratio of non-phosphorylated STEP to total STEP protein.

4.3 Objective 3

We used two different protocols to chemically induce LTD in cultured hippocampal neurons, using NMDA or DHPG.

4.3.1 NMDA

Treatment of primary hippocampal neuron cultures with 20μM NMDA on culture day 14 did not result in any significant changes in $STEP_{61}$ total protein levels (Figures 9 & 10). The first culture (control n=5, treatment n=8, p=.231) was performed using NMDA that had previously been frozen and stored for 3 months while the second (control n=4, treatment =6, p=.328) used freshly prepared media.

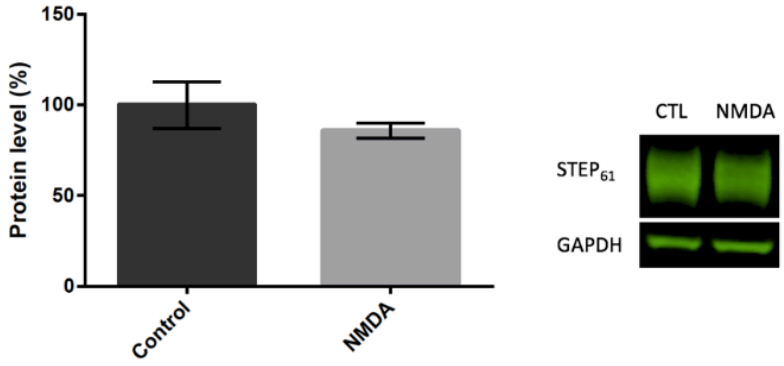


Figure 11 - Effect of Previously Frozen 20µM NMDA treatment on Total $STEP_{61}$ in Primary Hippocampal Neuron Cultures

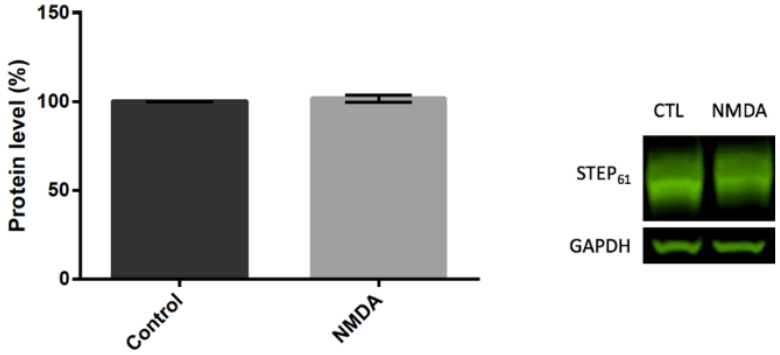


Figure 12 - Effect of 20 µM of Freshly Prepared NMDA treatment on Total $STEP_{61}$ in Primary Hippocampal Neuron Cultures

4.3.2 DHPG

Treatment of primary hippocampal neuron cultures with 100µM DHPG on culture day 14 did not result in any significant changes in $STEP_{61}$ total protein levels (control n=4, treatment n=6, p=.701) (Figure 11).

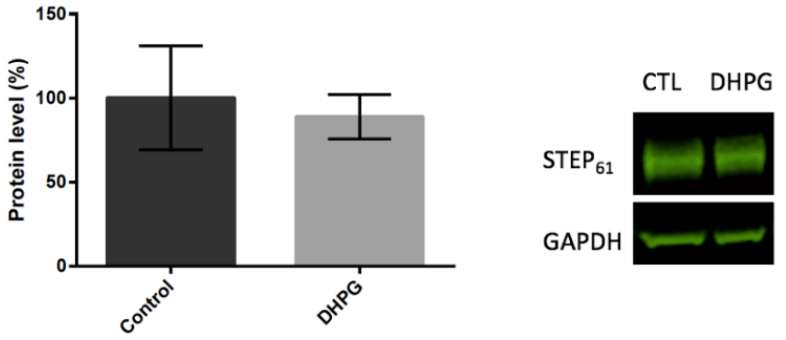


Figure 13 - Effect of 100µM DHPG treatment on Total $STEP_{61}$ in Primary Hippocampal Neuron Cultures

Chapter 5 – Discussion

5.1 BioID and AP-MS Screening

It is unsurprising that $STEP_{46}$ yielded a higher number of potential interacting proteins, as this is the soluble form of STEP, and hence, it is more likely to be able to move around within a cell and come into contact with a larger array of proteins. $STEP_{61}$, on the other hand, is membrane bound, and likely has a much more limited ability to move throughout the cell and interact with other proteins. Interestingly, the only protein that had a high SAINT confidence score in both BioID and FLAG-tag AP-MS screenings was ERK2 (MAPK1), a known interactor of STEP (Munoz et al., 2003; Paul et al., 2003). In addition, ERK1 (MAPK3) was also found to have a high SAINT score using AP-MS (Supplemental Fig. 3). The finding of well-known STEP substrates in these screenings increases our confidence in the screening methods that we have used. Given that affinity purification results are highly likely to uncover directly interacting proteins, we also chose BNIP2 and NMUR1 to investigate, despite them being found only through AP-MS and not through BioID. Four proteins not previously suspected of interacting with STEP and having a SAINT score of over 0.9 were found in both BioID screenings, and also fit our selection criteria (GIMAP6, PTPN4, SUV39H1, and TXN). As we were primarily interested in $STEP_{61}$ for the purpose of this study, since it is the only isoform found in the hippocampus, we also selected another three proteins that showed up only with this isoform (CARD10, PER1, METTL26). In addition to the reduced ability of $STEP_{61}$ to travel throughout a cell and interact with other proteins, it is also possible that the lower number of results for $STEP_{61}$ may be partially explained by specificity confered by the PP domain in this isoform (Nguyen et al., 2002). The harsher buffers required in order to extract membrane proteins are also more likely to interrupt protein-protein interactions, which could also

contribute to the lower number of interactors found with $STEP_{61}$. It cannot be discounted that the creation of a fusion STEP may change the way it interacts with regulators and substrates. As such, it is important to confirm the results of the BioID and AP-MS screenings using other methods. In this study, we simply determined if there was a correlation between $STEP_{61}$, non-phospho-STEP, and NMUR1 following a MWM task. Recently, Won et al. (2019) also found that NLGN-1 could be co-immunoprecipitated with $STEP_{61}$ in mouse hippocampal tissue. Given that NLGN-1 is a synaptic anchoring protein, we decided to include it in our study, despite it not having been found in our screenings. It is likely that NLGN-1 was not found in our particular screenings because they were carried out in HEK 293 cells rather than in hippocampal tissue, which likely reduced the amount of NLGN-1 available for STEP to interact with, and also did not provide the unique intracellular environment found in a neuron.

Though the BioID and AP-MS screenings were an important exploratory step, much remains to be done in order to better comprehend the results of our screenings. For instance, the performance of CO-IP experiments in brain tissues (especially in those where STEP dysregulation has been linked to cognitive dysfunction) as well as in neuron cultures would be helpful in order to determine whether STEP is directly interacting with the newly discovered proteins in hippocampal tissue. This can be carried out using endogenous proteins, though more difficult to detect interactions might be confirmed only through overexpression of the protein(s) or with the use of a substrate-trapping mutant in the case of weak or transient interactions. Further exploration of the results may include activity assays, as well as mutation analysis. It will also be important to examine the tyrosine phosphorylation in these new interactors, as changes in STEP may affect tyrosine phosphorylation in some of these proteins without affecting the overall protein level. Continued experiments will lead to a better understanding of the array of proteins that STEP

interacts with, and will open the door to better understanding STEP's effect on memory and cognition.

5.2 Morris Water Maze

Overview

Four different MWM protocols were used throughout the experiments, involving changes in the distal cues, platform size, as well as a different experimental timeline. As expected, there was a significant reduction in latency and distance during the acquisition trials for all four MWM protocols, indicating that some form of learning took place. That is, in all protocols, finding the platform required less time spent searching and less unproductive swimming on the last day of acquisition training versus the first day. Swim speed, however, remained unchanged for most groups. Percent time spent in the target quadrant as well as number of target zone crossings was significantly higher only in the fourth version of the MWM protocol. The results for each protocol are discussed in more detail below.

Protocol 1

The first MWM protocol (**Fig. 3A**) consisted of 3 x 30 sec cued trials (**Fig. 3C**) on the first day with a minimum intertrial interval of 45 min, 3 x 90 sec acquisition trials on days 2-6, followed by a 30 sec probe trial on day 7. A 15 cm target platform was used and small distal cues were hung on curtains surrounding the pool (**Fig. 3B**).

In this protocol, latency during the cued trial did not change significantly from one cued trial to another (**Fig. 3G**). This is interesing, considering that one might have expected that the latency to the platform might be less once the rats had understood the task. Though a decrease in

latency and distance swam to get to the target during acquisition (**Fig. 3D & 3E**) indicates that some learning did take place, the rats did not spend significantly more time in the NW target quadrant as compared to the other quadrants (**Fig. 3H**), nor did they cross the NW target platform area more often than they crossed the analogous area in the other three quadrants during the probe trial (**Fig. 3I**). This would suggest that the rats did not learn the task through the use of distal cues, but instead used some other strategy to find the platform. Though the use of pseudo-random starting positions is supposed to encourage the rats to use the distal cues available to solve the task (Vorhees and Williams, 2006), it is likely that the rats in this experiment used strategies such as swimming a fixed distance from the wall of the pool, or swimming in small circles until they happened to land on the platform. Such methods would require a learned strategy that would allow them to find the platform more quickly than swimming in purely random directions or swimming along the outsides of the pool (thigmotaxis), for instance, swimming in small circles a fixed distance from the wall.

The MWM protocol 1 did not lead to any significant changes between groups in the level of $STEP_{61}$, non-phospho-STEP, or NLGN-1 in the hippocampus 1 hour following the probe test (**Fig. 4**). We did not examine NMUR1 in this group. When the two MWM trained groups were combined (probe & platform) and compared to the non-trained controls, NLGN-1 was significantly reduced (**Supplemental Fig. 12)**. However, as the protocol did not appear to induce a spatial learning event, it is impossible to extrapolate the effect of spatial learning on these proteins in the hippocampus. In addition, the reduction in NLGN-1 was not correlated with a change in the level of $STEP_{61}$ or non-phospho STEP.

Protocol 2

For the second MWM protocol we changed the smaller distal cue signs (28 x 21.5 cm) for larger ones (56 x 71 cm) that were twice as wide and more than three times as high, to see if the

lack of a spatial learning strategy might have been a result of the distal cues being too small for the rats to see and/or notice (**Fig. 5B**). The distal cues were still hung on the curtains surrounding the pool. Given that there appeared to be a plateau in the latency and distance to the platform towards the end of acquisition training, we also shortened the acquisition training from 5 days to 4 days (**Fig. 5A**). The rest of the protocol remained the same as in the previous version.

As with the previous protocol, latency and distance travelled to the platform decreased throughout the acquisition period (**Fig. 5D & 5E**), and there was no change in the mean speed during acquisition (**Fig. 5F**). In contrast to the first protocol, however, there was a significant decrease in the latency to the platform during the cued trials (**Fig. 5G**). This discrepancy might be explained by the higher number of animals undergoing the second protocol as compared to the first. Alternatively, the results may be due to an initial improved performance in the first group during the first trial (21s +/- 4 sec), as compared to the performance during the second protocol (29s +/- 1 sec). This might again be explained by the limited number of subjects in the first protocol, or perhaps might be related to the changed distal cues. For instance, if the rats were able to see the distal cues better, they might intitially swim towards them rather than the platform being the most prominent visual attractant, or they might be more likely to attempt a strategy that involved the distal cues before abandoning the strategy in the later trials. The rats in both groups reached a similar latency by the third cued trial (12s +/- 4 sec for the first protocol versus 13s +/- 3 sec for the second), indicating sufficient ability and motivation to reach the platform in order to escape from the water.

Unfortunately, there was again no significant difference during the probe trial in regards to time spent in the target quadrant (**Fig. 5H**), nor in number of times the platform area was crossed (**Fig. 5I**). This would suggest that, despite the presence of larger distal cues and improvement in

performance during the acquisition phase, the rats were still not using spatial memory strategies in order to locate the platform.

Interestingly, there was an increase in the level of NLGN-1 protein in the hippocampus of rats 1 h following the MWM probe in the group that performed the probe trial with the platform still available versus the control rats that were not trained on the MWM task (135.0% +/- 5 of the control), though this was not seen in the rats that underwent the traditional probe trial without a platform (**Fig. 6**). One possible explanation might be that a traditional probe trial is in essence an extinction trial, where the rats learn that the platform is not where they expected it to be. It is possible that this extinction led to reduced NLGN-1 levels as compared to the platform group, whose probe trial was essentially another acquisition trial. Previous research in mice has indicated that NLGN-1 is a required for normal spatial learning and memory as assessed using the MWM (Blundell et al., 2010), and we know that NLGN-1 is able to induce the expression of LTP (Kim et al., 2008). NLGN-1 levels may have remained higher in the group that continued to have the platform available due to continued LTP, while the rats that experienced a "failure" during the probe trial had altered levels of NLGN-1 as a response to. Further research is required to confirm this finding, as well as to determine the variation of NLGN-1 through time. In addition, since we again were unable to induce spatial learning through the use of distal cues, we cannot attribute the results to a spatial learning event. Why NLGN-1 levels were increased in this particular protocol versus the others is unclear, though it may again relate to a higher number of animals that underwent the second protocol as opposed to the other three protocols. As with the first protocol, there were no differences in $STEP_{61}$, non-p-STEP, or in the ratio of non-p-STEP to total $STEP_{61}$ protein (**Fig. 6**). In addition, there were no differences in NMUR1 protein levels (**Fig. 6**). When the two MWM trained groups were combined (probe & platform), NLGN-1 was significantly

higher in the trained rats relative to the controls (**Supplemental Fig. 13)**. This effect, however, seems to have been driven by the significant increase in NLGN-1 in the MWM-trained platform group seen when the three groups were analyzed separately.

Protocol 3

Given that some research suggests that the ratio of the platform to total pool area can influence MWM results (Vorhees and Williams, 2006) we decided to reduce the size of the platform for the third protocol (from 15cm to 10cm in diameter) (**Fig. 7B**). We also moved the cued trials to the afternoon of the third acquisition day (rather than on the first day) and returned to 5 days of acquisition rather than 4 (**Fig. 7A**), in order to see if we might have a steeper and more easily interpretable latency and distance travelled curve throughout the acquisition period. We continued to use the larger cues hung on curtains around the pool (**Fig 7B**).

Latency to the platform during acquisition was significantly shorter in days 2-5 as compared to the first day for this protocol (**Fig. 7D**). This is to be expected, as on the first day of acquisition, rats are more prone to try to swim around the edge of the pool, and then following this, to swim in random directions. As the rats learn that there is no escape around the outside of the pool, they abandon this strategy, and typically first find the platform through random movements. Through time, the rats learn other strategies (such as swimming in tight circles a fixed distance from the wall, or navigating through the use of distal cues). In the previous two protocols, the cued trials came before the acquisition trials, providing the rats the opportunity to learn that escape is possible through the finding of a platform, but not around the edges of the pool. This explains why latency and distance to the platform (**Fig. 7E**) on the first day of acquisition was longer in the third protocol as opposed to the first and second protocols. Once again, mean swimming speed did not change throughout the acquisition period (**Fig. 7F**). As opposed to the second protocol, there were no

significant differences in the latency during the cued trials (**Fig. 7G**). Given that the rats in this protocol were exposed to three acquisition days before the cued trial, they had already been given ample opportunity to become accustomed to the concept of searching for a platform. The results support this interpretation, as the rats following the third protocol had a similar mean latency throughout all three cued trials as the rats undergoing protocol one and two did in their final trials. Changing the platform size did not appear to improve performance in the probe trials (**Fig. 7H & 7I**), suggesting that ratio of the platform to the pool was not responsible for the lack of spatial learning observed.

There was no significant difference in $STEP_{61}$ or non-phospho-STEP protein levels between groups, nor in the ratio of non-phosphorylated-STEP to total $STEP_{61}$ (**Fig. 8**). NLGN-1 and NMUR1 total protein levels were unchanged (**Fig. 8**). When the two MWM trained groups were combined (probe & platform), non-phospho STEP expression was significantly higher in the MWM trained group relative to the controls, an effect that was not significant when the groups were analyzed separately. This suggests that the MMW protocol may have induced an increase in STEP activity. However, once again, as the protocol did not induce a spatial learning event, it is impossible to determine how this effect might relate to spatial learning.

Protocol 4

After failing to achieve the desired spatial learning effect using three different MWM protocols, we tried a fourth and final protocol (**Fig. 9**). For this protocol, we drastically changed our distal cues. Rather than surrounding the pool with curtains upon which signs were affixed, we removed the curtains from all but one side of the pool and used the natural environment of the room

as distal cues (**see Section 4.2.1.4**). In addition, we reintroduced a SW starting point for some of the acquisition trials as in protocol 1.

As with all of the other protocols, we saw a reduction in latency and distance to the platform during the acquistion period (**Fig. 9D-E**). The mean swimming speed during acquisition remained unchanged (**Fig. 9F**). Interestingly, latency was reduced in the second and third cued trials in this experiment, as opposed to the third protocol where rats performed well from the first cued trial (**Fig. 9G**). This may be an indication of increased reliance on distal cues within this group, such that the rats first attempted to navigate spatially before noticing the visible platform. The results of the probe trial support a spatial learning through distal cues, as for the first time we demonstrated a much higher percentage of time spent in the NW target quadrant relative to controls as well as a higher number of target crossings in the NW target area relative to the analogous zones in the other quadrants (**Fig. 9H-I**). These findings indicate that a natural environment filled with drastically different distal cues may be more useful for MWM training than a more uniform environment with relatively small indicators.

We found no significant differences between groups for NLGN-1, $STEP_{61}$, non-phospho-STEP, NMUR1, or the ratio of non-phospho-STEP to total $STEP_{61}$ protein in the hippocampus within this group (**Fig. 10**). This may be due to the small number of animals tested, or may reflect a lack of effect of MWM training on these proteins. Alternatively, it may be that levels of these proteins change in a time frame that we have not looked at, and it may be interesting to observe the effects over a larger timescale. Use of this protocol with a larger group of rats, and the comparison of changes in protein expression over multiple time points should be considered in the future. Our preliminary findings based on this protocol is that the levels of NLGN-1, $STEP_{61}$, non-phosphorylated-STEP and NMUR1 are unchanged in the hippocampus of rats that underwent a

spatial learning training one hour after the probe trial. Interestingly, NMUR1 expression was significantly higher in the combined MWM trained groups (probe & platform) compared to the non-trained controls **(Supplemental Fig.15**), suggesting that spatial learning may lead to increased NMUR1 expression in the hippocampus, though this does not seem to be related to STEP protein levels or STEP activity.

5.3 Hippocampal Neuron Cultures

We performed primary hippocampal neuron cultures from E18 Long-Evans embryos with the hopes to induce a change in STEP levels using chemical LTD induced by either NMDAR or DHPG for further use as an experimental tool. We could then use this procedure to confirm that the interactions found in HEK293 cells were also taking place in hippocampal cells, and determine if changes in STEP resulted in changes in the total protein levels or tyrosine phosphorylation levels of the novel interactors. Previous research found an increase in STEP in the hippocampal slices following DHPG-induced LTD (Zhang et al., 2008), though we were unable to replicate this finding in dissociated hippocampal neuron cultures. Neither were we able to increase the levels of STEP in culture using an NMDA-dependent LTD induction protocol, which to our knowledge had not previously been reported on. Given that we lacked the tools to confirm the production of LTD in our cultures, the reason for the failure is unclear. There is a possibility that our protocol did not effectively produce LTD in cultures, or that the effect on STEP might be more easily seen in hippocampal slices. Further studies in collaboration with researchers skilled in electrophysiology are needed in order to ensure that we are succesfully inducing LTD.

5.4 Conclusion

In conclusion, we identified a list of novel STEP interactors using BioID and Flag-tag AP-MS screening methods. We then optimized a MWM protocol in order to determine the effect a spatial learning task would have on STEP, non-phospho-STEP, NMUR1 and NLGN-1 protein levels. We found that, though rats learned to find the platform more quickly in all protocols, they only used a spatial learning strategy that involved the use of distal cues when the natural environment of the laboratory was used, and the distal cues were drastically different and easy to distinguish. We found no changes in the levels of any of the proteins except an increase in NLGN-1 as compared to controls in the second protocol, for reasons which are not clear, but may be explained by the larger number of animals in this group. When analyzing all MWM-trained animals as a group *vs* the non-trained controls, we saw a reduction in NLGN-1, an increase in NLGN-1, an increase in non-phospho STEP, and an increase in NMUR1 in protocols 1 through 4, respectively. We attempted to increase the level of STEP in primary hippocampal neuron cultures using chemical LTD-induction protocols, but were unable to do so. Further research is needed into the nature of the novel STEP intereactions, the effect of a spatial learning task on STEP and STEP interactors in the hippocampus, as well as the role of LTD and LTP in these processes is needed.

References

Baddely AD, Warrington EK (1970). Amnesia and the distinction between long and short-term memory. *J Verbal Learn. Verbal Behav.* 9: 176-189.

Besshoh S, Bawa D, Teves L, Wallace MC, Gurd JW (2005). Increased phosphorylation and redistribution of NMDA receptors between synaptic lipid rafts and post-synaptic densities following transient global ischemia in the rat brain. *J Neurochem.* 93: 186-194.

Bird CM, Burgess N (2008). The hippocampus and memory: insights from spatial processing. *Nature Reviews.* 9: 182-194.

Bliss TV, Gardner-Medwin AR (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit following stimulation of the perforant path. *J Physiol* 232: 357-374.

Bliss TV, Lomo T (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* 232: 331-356.

Blundell J, Blais CA, Etherton MR, Espinosa F, Tabuchi K, Walz C, Bolliger MF, Südhof TC, Powell CM. (2010) Neuroligin-1 Deletion Results in Impartial Spatial Memory and Increased Repetitive Behavior. J Neurosci. 30(6): 2115-2129.

Boulanger LM, Lombroso PJ, Raghunathan A, During MJ, Wahle P, Naegele JR (1995). Cellular and molecular characterization of a brain-enriched protein tyrosine phosphatase. *J Neurosci.* 15: 1532-1544.

Bredt DS, Nicoll RA (2003). AMPA receptor trafficking at excitatory synapses. *Neuron.* 40:361-379.

Bult A, Zhao F, Dirkx R, Jr., Sharma E, Lukacsi E, Solimena M, Naegele JR, Lombroso PJ (1996). STEP61: a member of a family of brain-enriched PTPs is localized to the endoplasmic reticulum. *J. Neurosci.* 16: 7821-7831.

Bult A, Zhao F, Drinkx R, Jr., Raghunathan A, Solimena M, Lombroso PJ (1997). STEP: a family of brain-enriched PTPs. Alternative splicing produces transmembrane, cytosolic and truncated isoforms. *Eur. J. Cell Biol.* 72: 337-344.

Castonguay, D, Dufort-Gervais J, Ménard C, Chatterjee M, Quirion R, Bontempi B, Schneider JS, Amsten AFT, Nairn AC, Norris CM, Ferland G, Bézard E, Gaudreau P, Lombroso PJ, Brouillette J (2018). The tyrosine phosphatase STEP is involved in age-related cognitive decline. *Curr Biol*. 28: 1079-1089.

Cave CB, Squire LR (1992). Intact verbal and nonverbal short-term memory following damage to the human hippocampus. *Hippocampus*. 2: 151-163.

Chen X, Lin R, Chang, Xu S, Wei X, Zhang J, Wang C, Anwyl R, Wang Q (2013). Enhancement of long-term depression by soluble amyloid β protein in rat hippocampus is mediated by metabotropic glutamate receptor and involves activation of p38MAPK, STEP and caspase-3. *Neuroscience.* 253: 435-43.

Chin J, Palop JJ, Puolivä, Massaro C, Bien-Ly N, Gerstein H, Scearce-Levie K, Masliah E, Mucke L (2005). Fyn kinase induces synaptic and cognitive impairments in a transgenic mouse model of Alzheimer's disease. *J Neurosci.* 25: 9694-9703.

Choi YS, Lin SL, Lee B, Kurup P, Cho HY, Naegele JR, Lombroso PJ, Obrietan K (2007). Status epilepticus-induced somatostatinergic hilar interneuron degeneration is regulated by striatal enriched protein tyrosine phosphatase. *J Neurosci*. 27: 2999-3009.

Cloutier P, Poitras C, Durand M, Hekmat O, Fiola-Masson É, Bouchard A, Faubert D, Chabot B, Coulombe B. 2017. R2TP/Prefoldin-like component RUVBL1/RUVBL2 directly interacts with ZNHIT2 to regulate assembly of U5 small nuclear ribonucleoprotein. *Nat. Commun.* 8(1).

Collingridge GL, Isaac JT, Want YT (2004). Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci.* 5: 952-962.

Cummings JA, Mulkey RM, Nicoll RA, Malenka RC (1996). Ca^{2+} signaling requirements for long-term depression in the hippocampus. *Neuron*. 16: 825-833.

Darnell JC, Van Briesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, Stone EF, Chen C, Fak JJ, Chi SW, et al. (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell*. 146: 247-261.

Deb I, Poddar R, Paul S (2011). Oxidative stress-induced oligomerization inhibits the activity of the non-receptor tyrosine phosphatase $STEP_{61}$. *J Neurochem.* 116: 1097-1111.

Derkach VA, Oh MC, Guire ES, Soderling TR (2007). Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat Rev Neurosci.* 8: 101-113.

Dong Z, Bai Y, Wu X, Li H, Gong B, Howland JG, Huang Y, He W, Li T, Wang YT (2013). Hippocampal long-term depression mediates spatial reversal learning in the Morris water maze. *Neuropharmacology.* 64: 65-73.

Dong Z, Gong B, Bai Y, Wu X, Huang Y, He W, Li T, Want YT (2012). Mechanisms of hippocampal long-term depression are required for memory enhancement by novelty exploration. *J Neurosci.* 32(35): 11980-11990.

Ekstrom AD, Kahana MJ, Caplan JB, Fields TA, Isham EA, Newman EL, Fried I (2003). Cellular networks underlying human spatial navigation. *Nature*. 425: 184-188.

Eswaran J, von Kries JP, Mardsen B, Longman E, Debreczeni JE, Ugochukwu E, Turnbull A, Lee WH, Knapp S, Barr AJ (2006). Crystal structures and inhibitor identification for PTPN5, PTPRR and PTPN7: a family of humn MAPK-specific protein tyrosine phosphatases. *Biochem J.* 395: 483-491.

Frisoni GB, Fox NC, Jack CR, Jr, Scheltens P, Thompson PM (2010). The clinical use of structural MRI in Alzheimer disease. *Nat Rev Neurol.* 6: 67-77.

Gladding CM, Collett VJ, Jia Z, Bashir ZI, Collingridge GL, Molnár E (2009). Tyrosine dephosphorylation regulates AMPAR internalization in mGluR-LTD. Mol. Cell. Neurosci. 40: 267-279.

Goebel-Goody SM, Baum M, Paspalas CD, Fernandez SM, Carty NC, Kurup P, Lombroso PJ (2012). Therapeutic Implications for Striatal-Enriched Protein Tyrosine Phosphatase (STEP) in Neuropsychiatric Disorders. *Pharmacol Rev.* 64: 65-87.

Hardinghan GE, Fukunaga Y, Bading H (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci.* 5: 405-414.

Hebb DO (1949). *The Organization of Behavior.* John Wiley: New York.

Hicklin TR, Wu PH, Radcliffe RA, Freund RK, Goebel-Goody SM, Correa PR, Proctor WR, Lombroso PJ, Browning MD (2011). Alcohol inhibition of the NMDA receptor function, long-term potentiation, and fear learning requires striatal-enriched protein tyrosine phosphatase. *Proc Natl Acad Sci USA.* 108: 6650-6655.

Holman D, Feligioni M, Henley JM (2007). Differential redistribution of native AMPA receptor complexes following LTD induction in acute hippocampal slices. Neuropharmacology. 52: 92-99.

Ito M, Kano M (1982). Long-lasting depression of parallel fiber-Purkinje cell transmission induced by conjunctive stimulation of parallel fibers and climbing fibers in the cerebellar cortex. *Neuroscience Letters:* 33(3):253-258.

Ito M, Sakurai M, Tongroach P (1982). Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. *J Physiol*. 324: 113-134.

Ivanov A, Pelligrino C, Rama S, Bumalska I, Salyha Y, Ben-Ari Y, Medina I. (2006). Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. *J physiol.* 572: 789-798.

Iwai T, Iinuma Y,Kodani R, Okay JI (2008). Neuromedin U inhibits inflammation-mediated memory impairment and neuronal cell-death in rodents. *J. Neurosci. Res.* 61(1): 113-9.

Kemp A, Manahan-Vaughan D (2004). Hippocampal long-term depression and long-termp potentiation encode different aspects of novelty acquisition. *Proc. Natl. Acad. Sci. U.S.A.* 101: 8192-8197.

Kim J, Jung S-Y, Lee YK, Park S, Choi J-S, Lee J, Kim H-S, Choi Y-B, Scheiffele P, Bailey CH, Kandel ER, Kim J-K (2008). Neurologin-1 is required for normal expression of LTP and associative fear memory in the amygdala of adult animals. PNAS. 105(26): 9087-9092.

Kim SY, Lee HJ, Kim YN, Yoon S, Lee JE, Sun W, Choi EJ, Baik JH (2008). Striatal-enriched protein tyrosine phosphatase regulates dopaminergic neuronal development via extracellular signal-regulated kinase signaling. *Exp Neurol*. 214: 69-77.

Kurup P, Zhang Y, Xu J, Venkitaramani DV, Haroutunian V, Greengard P, Nairn AC, Lombroso PJ (2010). Abeta-mediated NMDA receptor endocytosis in Alzheimer's disease involves ubiquitination of the tyrosine phosphatase $STEP_{61}$. *J Neurosci.* 30: 5948-5957.

Lavallee-Adam M, Cloutier P, Coulomb P, Blanchette M (2010). Modeling contaminants in AP-MS/MS experiments. *J Proteome Res.* 10: 886-895.

Lee HK, Kameyama K, Huganir RL, Bear MF (1998). NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 aubunit of AMPA receptors in the hippocampus. Neuron. 21: 1151-1162.

Le HT, Maksumova L, Wang J, Pallen CJ (2006). Reduced NMDA receptor tyrosine phosphorylation in PTPalpha-deficient mouse synaptosomes is accompanied by inhibition of four src family kinases and Pyk2: an upstream role for PTPalpha in NMDA receptor regulation. *J Neurochem*. 98: 1798-1809.

Lombroso PJ, Murdoch G, Lerner M (1991). Molecular characterization of a protein-tyrosine-phosphatase enriched in the striatum. *Proc Natl Acad Sci USA.* 88: 7242-7246.

Lombroso PJ, Naegele JR, Sharma E, Lerner M (1993). A protein tyrosine phosphatase expressed within dopaminoceptive neurons of the basal ganglia and related structures. *J Neurosci.* 13: 3064-3074.

Lombroso PJ, Ogren M, Kurup P, Nairn AC (2016). Molecular underpinnings of neurodegenerative disorders: striatal-enriched protein tyrosine phosphatase signaling and synaptic plasticity. *F1000Research*. F1000 Faculty Rev-2932. doi:10.12688/f1000research.8571.1

Lorber B, Berry M, Hendriks W, den Hertog J, Pulido R, Logan A (2004). Stimulated regeneration of the crushed adult rat optic nerve correlates with attenuated expression of the protein tyrosine phosphatases RPTPalpha, STEP, and LAR. *Mol Cell Neurosci.* 27: 404-416.

Malenka RC (1991). Postsynaptic factors control the duration of synaptic enhancement in area CA1 of the hippocampus. *Neuron.* 6:53-60.

Malenka RC, Nicoll RA (1993). NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. *Trends in Neurosci*. 16: 521-527.

Malenka RC, Nicoll RA (1999). Long-term potentiation--a decade of progress? *Science*. 285: 1870-1874.

Malinow R, Malenka RC (2002). AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci.* 25: 103-126.

Manahan-Vaughan D, Braunewell KH (1999). Novelty acquisition is associated with induction of hippocampal long-term depression. *Proc. Natl. Acad. Sci. U.S.A.* 23: 649-711.

Martin SJ, Grimwood PD, Morris RG (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci.* 23: 649-711.

Mayer ML, Westbrook GL, Guthrie PB (1984). Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nautre. 309(5965):261-3.

McIntosh C, Chick J (2004). Alcohol and the nervous system. *J Neurol Neurosurg Psychiatry*. 75 (Suppl 3):iii16-iii21.

Muñoz JJ, Tárrega C, Blanco-Aparicio C, Pulido R (2003). Differential interaction of the tyrosine phosphatases PTP-SL, STEP and HePTP with the mitogen-activated protein kinases ERK1/2 and p38alpha is determined by a kinase specificity sequence and influenced by reading agents. *Biochem J.* 372: 193-201.

Nakazawa T, Komai S, Watabe AM, Kiyama Y, Fukaya M, Arima-Yoshida F, Horai R, Sudo K, Ebine K, Delawary M, et al. (2006). NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity. *EMBO J.* 25: 2867-2877.

Nguyen TH, Liu J, Lombroso PJ (2002). Striatal enriched phosphatase 61 dephosphorylates Fyn at phosphotryosine 420. *J Biol Chem*. 277: 24274-24279.

Nicodemo AA, Pampillo M, Ferreira LT, Dale LB, Cregan T, Ribeiro FM, Ferguson SS (2010). Pyk2 uncouples metabotropic glutamate receptor G protein signaling but facilitates ERK1/2 activation. *Mol Brain.* 3:4.

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984). Magnesium gates glutamate-activated channels in mouse central neurones.

O'Keefe J, Dostrovsky J (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res.* 34: 171-175.

Olausson P, Vekitaramani DV, Moran TD, Salter MW, Taylor JR, Lombroso PJ (2012). The tyrosine phosphatase STEP constrains amygdala-dependent memory formation and neuroplasticity. *Neuroscience.* 225: 1-8.

Ono T, Nakamura K, Fukuda M, Tamura R (1991). Place recognition responses of neurons in monkey hippocampus. *Neurosci. Lett.* 121: 194-198.

Oyama T, Goto S, Nishi T, Sato K, Yamada K, Yoshikawa M, Ushio Y (1995). Immunocytochemical localization of the striatal enriched protein tyrosine phosphatase in the rat striatum: a light and electron microscopic study with a complementary DNA-generated polyclonal antibody. *Neuroscience.* 69: 869-880.

Paoletti P, Bellone C, Zhou Q (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat. Rev. Neurosci.* 14: 383-400.

Park M (2018). AMPA Receptor Trafficking for Postsynaptic Potentiation. *Front. Cell Neurosci.* 12: 361.

Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, Sacktor TC (2006). Storage of spatial information by the maintenance mechanism of LTP. *Science*. 313: 1141-1144.

Paul S, Snyder GL, Tokakura H, Picciotto MR, Nairn AC, Lombroso PJ (2000). The Dopamine/D1 receptor mediates the phosphorylation and inactivation of the protein tyrosine phosphatase STEP via a PKA-dependent pathway. *J Neurosci.* 20: 5630-5638.

Paul S, Nairn AC, Wang P, Lombroso PJ (2003). NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. *Nat Neurosci.* 6:34-42.

Paul S, Olausson P, Venkitaramani DV, Ruchkina I, Moran TD, Tronson N, Mills E, Hakim S, Salter MW, Taylor JR, Lombroso PJ (2007). The Striatal-Enriched Protein Tyrosine Phosphatase Gates Long-Term Potentiation and Fear Memory in the Lateral Amygdala. *Biol Psychiatry.* 61: 1049-1061.

Pelkey KA, Askalan R, Paul S, Kalia LV, Nguyed TH, Pitched GM, Salter MW, Lombroso PJ (2002). Tyrosine phosphatase STEP is a tonic break on induction of long-term potentiation. *Neuron.* 34: 127-138.

Piqué M, López JM, Foissac S, Guigó R, Méndez R (2008). A combinatorial code for CPE-mediated translational control. *Cell.* 132: 434-448.

Poddar R, Deb I, Mukherjee S, Paul S (2010). NR2B-NMDA receptor mediated modulation of the tyrosine phosphatase STEP regulates glutamate induced neuronal cell death. *J Neurochem.* 115: 1350-1362.

Raghunathan A, Matthews GA, Lombroso PJ, Naegele JR (1996). Transient compartmental expression of a family of protein tyrosine phosphatases in the developing striatum. *Brain Res Dev Brain Res.* 91: 190-199.

Regan MC, Romero-Hernandez A, Furukawa H (2015). A Strutural Biology Perspective on NMDA Receptor Pharmacology and Function. *Curr Opin Struct Biol.* 33: 68-75

Semenova MM, Mäki-Hokkonen AMJ, Cao J, Komarovski V, Forsberg KM, Koistinaho M, Coffey ET, Courtney MJ (2007). Rho mediates calcium-dependent activation of p38alpha and subsequent excitotoxic cell death. *Nat Neurosci.* 10(4): 436-43.

Sharma E, Zhao F, Bult A, Lombroso PB (1995). Identification of two alternatively spliced transcripts of STEP: a subfamily of brain-enriched protein tyrosine phosphatases. *Brain Res. Mol.* 32: 87-93.

Snyder EM, Philot BD, Huber KM, Dong X, Fallon JR, Bear MF (2001). Internalization of ionotropic glutamate receptors in response to mGluR activation. *Nat Neurosci*. 4: 1079-1085.

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK et al. (2005). Regulation of NMDA receptor trafficking by amyloid-beta. *Nat Neurosci.* 8: 1051-1058.

Song I, Huganir RL (2002). Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci*. 25: 578-588.

Stuchlik A (2014). Dynamic learning and memory, synaptic plasticity and neurogenesis: an update. *Front. Behav. Neurosci.* 8(106): 1-6.

Thiffault I, Wolf NI, Forget D, Guerrero K, Tran LT, Choquet K, Lavallée-Adam M, Poitras C, Brais B, Yoon G, et al. 2015. Recessive mutations in POLR1C cause a leukodystrophy by impairing biogenesis of RNA polymerase III. *Nat. Commun*. 6(1).

Sweatt JD (2004). Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr Opin Neurobiol*. 14: 311-317.

Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC, Stipanovich A, Caboche J, Lombroso PJ, Nair AC, et al. (2005). Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc Natl Acad Sci USA.* 102: 491-496.

Venkitaramani DV, Moura PJ, Picciotto MR, Lombroso PJ (2011). Striatal-enriched protein tyrosine phosphatase (STEP) knockout mice have enhanced hippocampal memory. *Eur J Neurosci.* 33: 2288-2298.

Venkitaramani DV, Paul S, Zhang Y, Kurup P, Ding L, Tressler L, Allen M, Sacca R, Picciotto MR, Lombroso PJ (2009). Knockout of striatal enriched protein tyrosine phosphatase in mice results in increased ERK1/2 phosphorylation. *Synapse*. 63: 69-81.

Voss JL, Bridge DJ, Cohen NJ, Walker JA (2018). A closer look at the hippocampus and memory. *Trends Cogn. Sci.* 21(8): 577-588.

Voorhees CV and Williams MT (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc.* 1(2): 848-858.

Wang YT, Linden DJ (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron*. 25: 635-647.

Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006). Learning induces long-term potentiation in the hippocampus. *Science.* 313: 1093-1097.

Won S, Incontro S, Li Yan, Nicoll RA, Roche KW (2019). The $STEP_{61}$ interactome reveals subunit-specific AMPA receptor binding and synaptic regulation. *PNAS.* 116(16): 8028-8037.

Xiao MY, Zhou Q, Nicoll RA (2001). Metabotropic glutamate receptor activation causes a rapid redistribution of AMPA receptors. *Neuropharmacology.* 41: 664-671.

Xu J, Kurup P, Zhang Y, Goebel-Goody SM, Wu PH, Hawasli AH, Baum ML, Bibb JA, Lombroso PJ (2009). Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP. *J Neurosci.* 29: 9330-9343.

Xu J, Kurup P, Bartos JA, Hell JW, Lombroso PJ (2010). Inhibition of pyk2 signaling by striatal-enriched tyrosine phosphatase (STEP). *Soc Neurosci Abstr.* 36: 452.13

Zhang Y, Jiang D, Zhang Y, Juan X, Wang F, Jun T (2012). Neuromedin U Type 1 Receptor Stimulation of A-type K+ Current Requires the βγ subunits of G_o Protein, Protein Kinase A, and Extracellular Signal-regulated Kinase 1/2 (ERK1/2) in Sensory Neurons. *J. Biol. Chem.* 287: 18562-18572.

Zhang Y, Jiang D, Zhang J, Wang F, Jiang X, Tao J (2010). Activation of Neuromedin U Type 1 Receptor Inhibits L-type Ca^{2}+ Channel Currents via Phosphatidylinositol 3-kinase-dependent Protein Kinase C Epsilon Pathway in Mouse Hippocampal Neurons. *Cell Signal.* 22(11): 1660-8.

Zhang Y, Kurup P, Xu J, Carty N, Fernandez SM, Nygaard HB, Pittenger C, Greengard P, Strittmatter SM, Nairn AC, et al. (2010). Genetic reduction of striatal-enriched tyrosine phosphatase (STEP) reverses cognitive and cellular deficits in an Alzheimer's disease mouse model. *Proc Natl Acad Sci USA*. 107: 19014-19019.

Zhang Y, Venkitaramani DV, Gladding CM, Zhang Y, Kurup P, Molnar E, Collingridge GL, Lombroso PJ (2008). The tyrosine phosphatase STEP mediates AMPA receptor endocytosis after metabotropic glutamate receptor stimulation. *J Neurosci.* 28: 10561-10566.

Zola-Morgan S, Squire LR (1993). Neuroanatomy of memory. *Annu Rev Neurosci.* 16: 547-563.

Supplemental Figures

ACBD5	CELSR2	GIMAP6	MACO1	RAB27B	TMEM131
ACSL3	CISD2	GOSR1	MAPK1	RAD50	TMEM131L
ADAMTSL2	CKAP4	GOSR2	MGRN1	RRBP1	TMX1
ADGRB1	CLCC1	GRIA2	MIA3	SCD5	TP53
ANKLE2	DCD	HIVEP2	MIB2	SLC35G2	TTC17
ANO6	DDRGK1	HIVEP3	MMGT1	SLC3A2	TXN
ATF6	DENND4B	HMOX2	MOSPD2	SNX19	UBE2J1
BAG4	DSG1	IKZF2	MYO15A	SRPRA	UBXN4
BCAP31	DVL1	ITPR1	MYOF	SRPRB	UNC79
BET1	EMC1	JAG2	NBN	SSR1	VANGL1
BNIP1	EMC10	KCNB2	NDC1	ST7	VAPA
C1orf68	EMC8	KPRP	PDZD8	STBD1	VAPB
C4orf54	FKBP8	KTN1	PKMYT1	STIM2	VXN
CDK5RAP3	FLG2	LEMD3	PTPN1	SUV39H1	ZFYVE27
CDKAL1	FNDC3A	LRRC59	PTPN4	TEKT4	

Supplemental Figure 1 - BioID results using STEP$_{46}$ as the bait protein. Due to the large number of proteins yielding a SAINT score over 0.9, only proteins yielding a SAINT confidence score of 1 were included. MAPK1 (ERK2) is a known STEP substrate. GIMAP6, PTPN4, SUV39H1, and TXN were chosen for subsequent testing based on known presence in neurons within the hippocampus and availability of commercial antibodies (Data not shown).

GeneID	SAINT confidence score	GeneID	SAINT confidence score
ZNF565	1	C7orf43	0.99
MAPK1	1	GLIS3	0.99
GIMAP6	1	SEMG2	0.99
STAB2	1	KLHL40	0.99
SUV39H1	1	MAPK14	0.98
GRIA2	1	PDGFRA	0.97
CARD10	1	PRR5	0.97
FLT1	1	C1orf68	0.94
KPRP	1	METTL26	0.92
PTPN4	1	KDR	0.92
CCDC93	1	TXN	0.91
PER1	1	RAB8B	0.91
PLEKHG4B	1	ZNF607	0.91
ITM2B	1		

Supplemental Figure 2 - BioID results using $STEP_{61}$ as the bait protein. MAPK1 (ERK1) is a known STEP substrate. GIMAP6, PTPN4, SUV39H1, and TXN were obtained using both $STEP_{61}$ and $STEP_{46}$ as a bait protein. CARD10, PER1 and METTL26 were found exclusively when using $STEP_{61}$. Green and blue highlighted proteins were chosen for subsequent testing based on known presence in neurons within the hippocampus and availability of commercial antibodies (Data not shown).

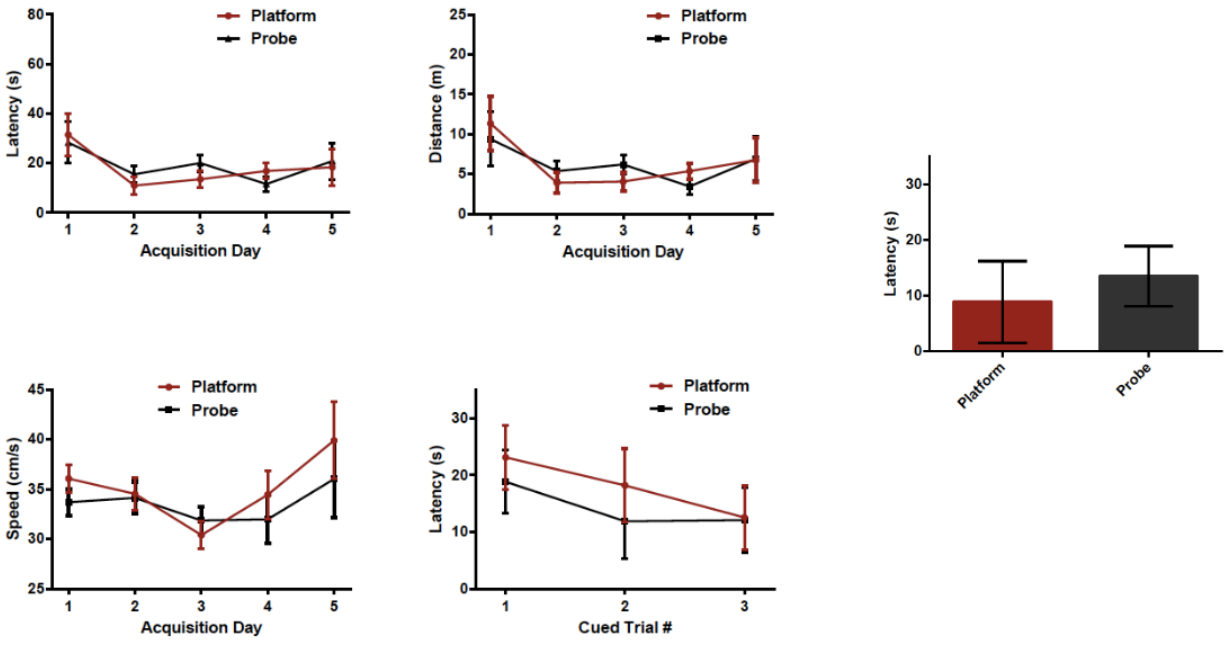
GeneID	SAINT confidence score
BNIP2	1
MAPK1	1
MAPK14	1
MAPK3	1
NCAPG2	1
NECTIN1	1
NMUR1	1
OC90	1
PTPN7	1
RGS22	1
TNR	1

Supplemental Figure 3- FLAG AP-MS results using $STEP_{46}$ as the bait protein. MAPK1(ERK2) and MAPK3 (ERK1) are known substrates of STEP. Green highlighted proteins were chosen for subsequent testing based on known presence in neurons within the hippocampus

and availability of commercial antibodies (Data not shown). NMUR1 was retained for use in the MWM experiments.

Acquisition Day	Trial #1	Trial # 2	Trial # 3
1	S	NE	SE
2	E	SW	SE
3	SW	E	S
4	NE	S	E
5	SW	SE	NE

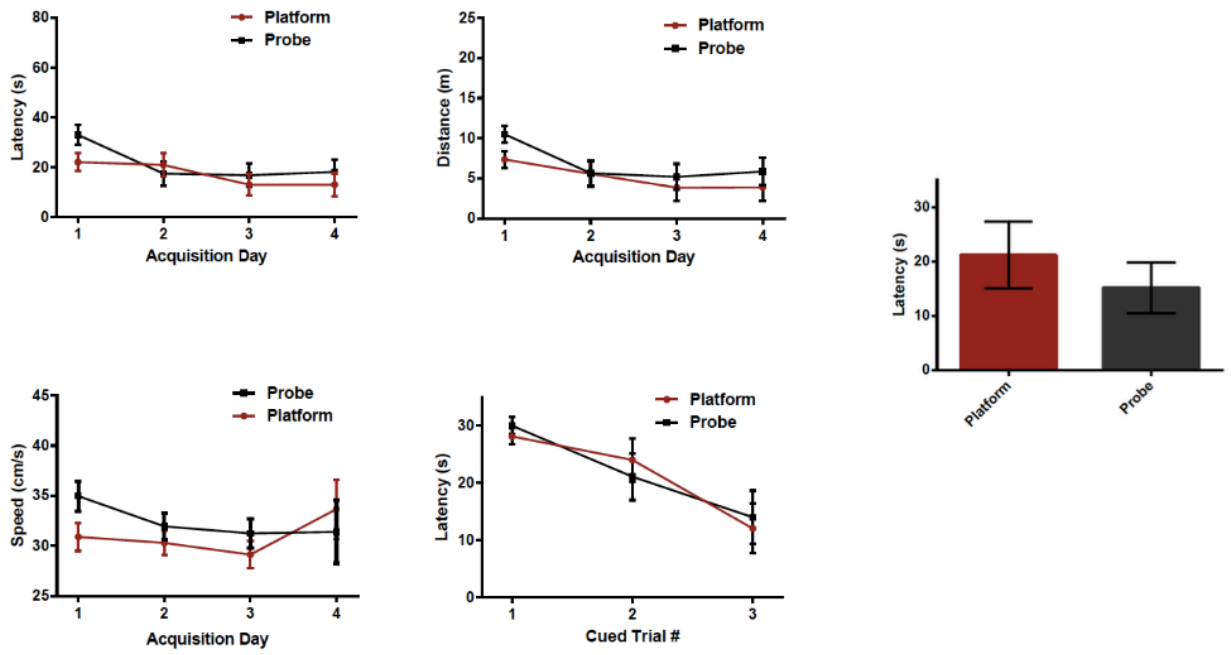
Supplemental Figure 4 - Starting Locations for the Morris Water Maze Acquisition Protocol 1



Supplemental Figure 5- Data for the MWM protocol 1 split by groups. **(A)** Starting positions during the acquisition phase **(B-F)** Latency, distance and speed to the platform during acquisition, as well as latency during the cued and probe trials were similar between groups ($p>.05$).

Acquisition Day	Trial #1	Trial # 2	Trial # 3
1	S	NE	SE
2	E	S	NE
3	SE	NE	S
4	NE	S	E

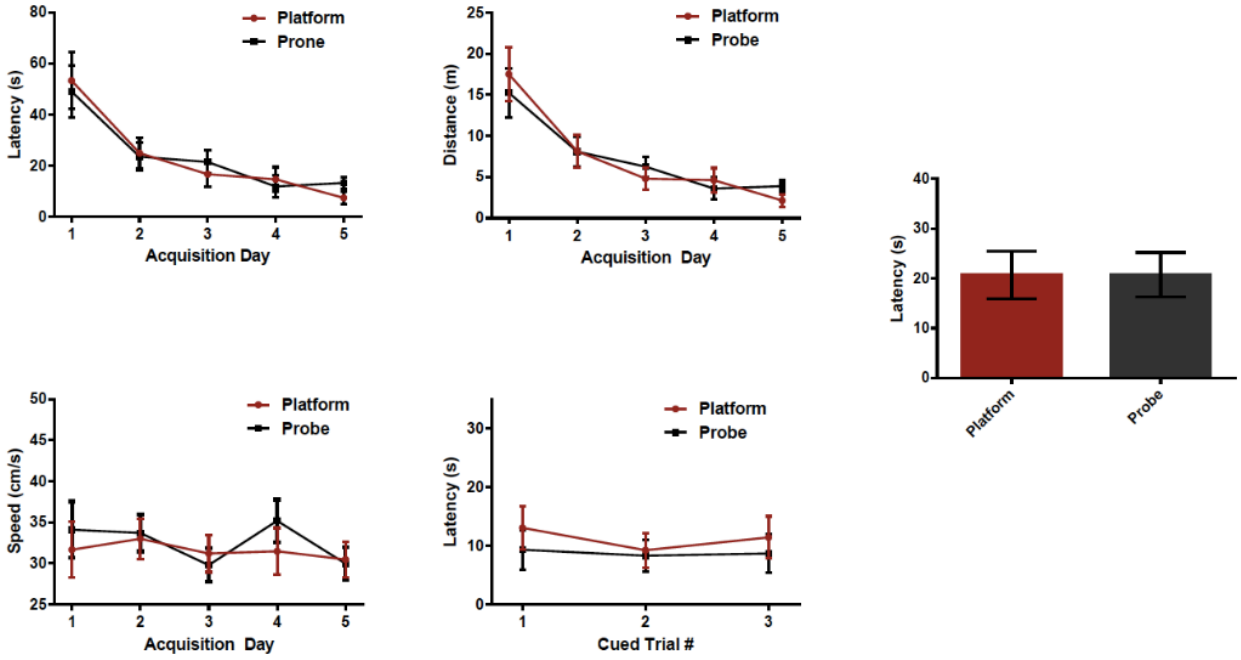
Supplemental Figure 6 - Starting Locations for the Morris Water Maze Acquisition Protocol 2



Supplemental Figure 7- Data for the MWM protocol 2 split by groups (A) Starting positions during the acquisition phase **(B-F)** Latency, distance and speed to the platform during acquisition, as well as latency during the cued and probe trials were similar between groups (p>.05).

Acquisition Day	Trial #1	Trial # 2	Trial # 3
1	S	NE	SE
2	E	S	NE
3	SE	NE	S
4	S	SE	E
5	NE	E	SE

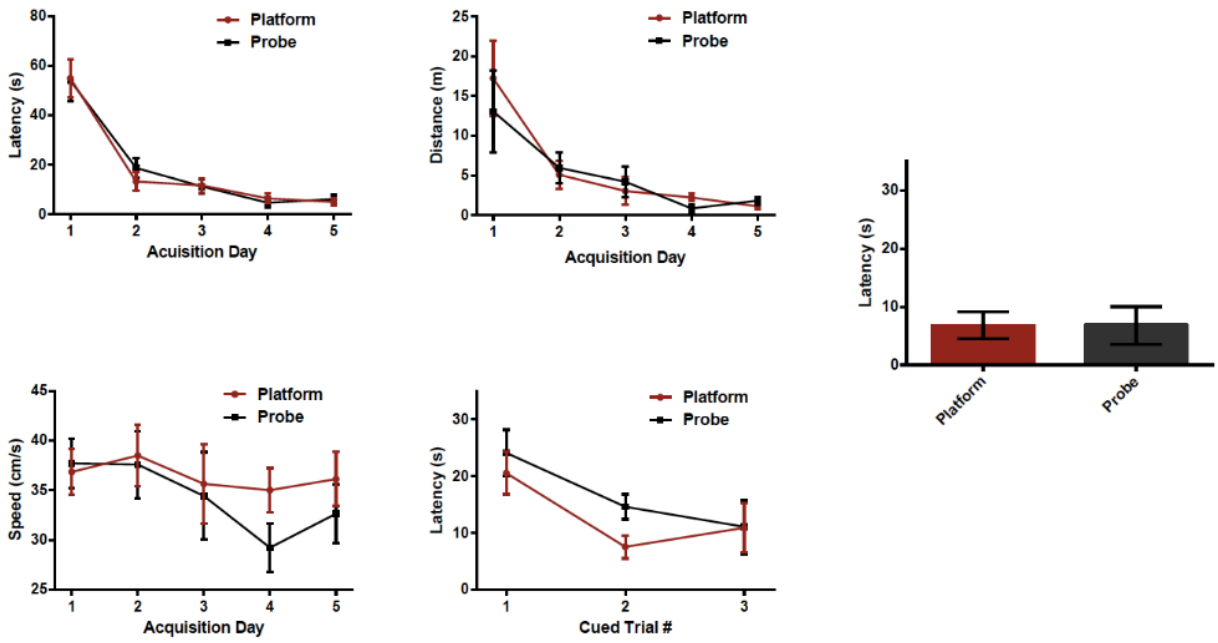
Supplemental Figure 8 - Starting Locations for the Morris Water Maze Acquisition - Protocol 3



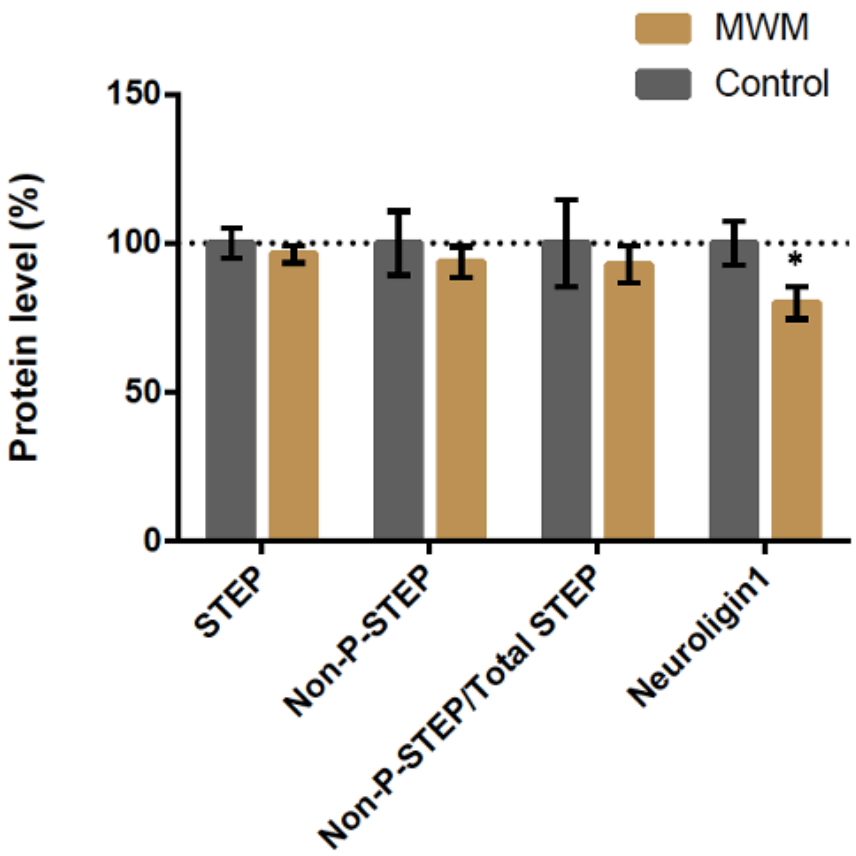
Supplemental Figure 9 - Data for the MWM protocol 4 split by groups (A) Starting positions during the acquisition phase **(B-F)** Latency, distance and speed to the platform during acquisition, as well as latency during the cued and probe trials were similar between groups (p>.05).

Acquisition Day	Trial #1	Trial # 2	Trial # 3
1	S	NE	SE
2	E	SW	SE
3	SO	E	S
4	SE	NE	SW
5	NE	S	E

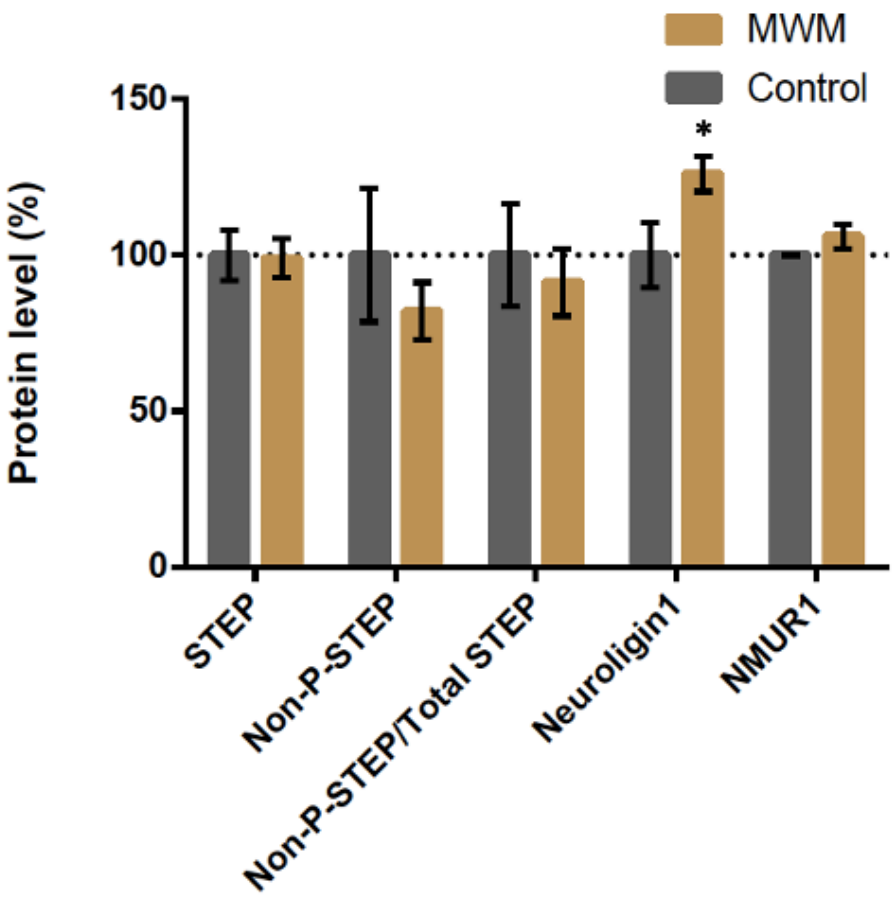
Supplemental Figure 10 - Starting Locations for the Morris Water Maze Acquisition - Protocol 4



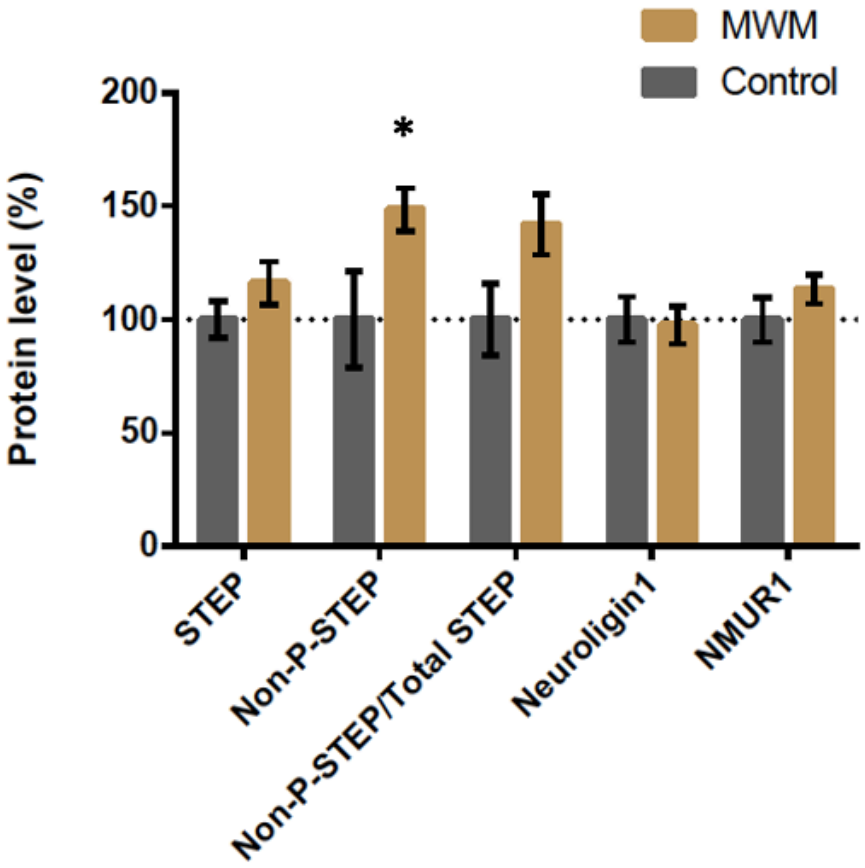
Supplemental Figure 11- Data for the MWM protocol 4 split by groups (A) Starting positions during the acquisition phase **(B-F)** Latency, distance and speed to the platform during acquisition, as well as latency during the cued and probe trials, were similar between groups (p>.05).



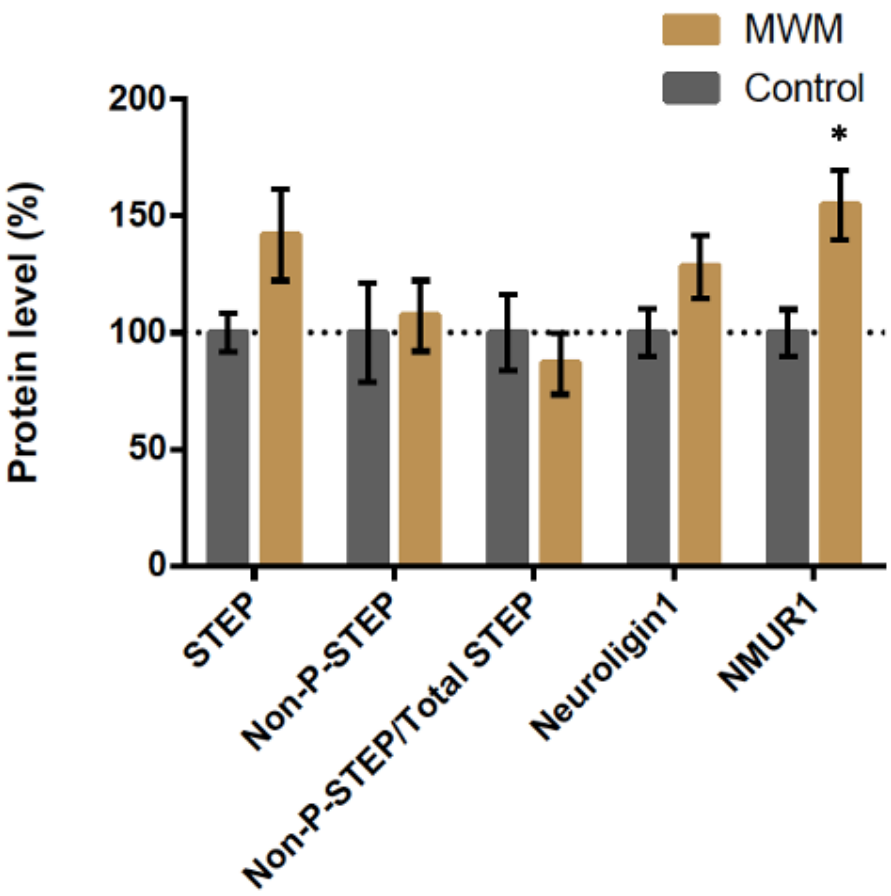
Supplemental Figure 12 - Effect of MWM Protocol 1 on $STEP_{61}$, NLGN-1, and non-phospho-STEP protein with probe and platform control groups combined. There were no significant differences in $STEP_{61}$ ($F_{1,13}$ = .232, p=.638), Non-P-STEP ($F_{1,13}$ = .177, p=.681), or Non-P-STEP/Total STEP ($F_{1,13}$ = .125, p=.730). There was a significant reduction in NLGN-1 ($F_{1,13}$ = 5.021, p=.043) when comparing the MWM trained rats (n=6, 79.9% +/- 5.3) to the control group (n=9, 100% +/- 7.19) that was not found when the MWM groups were analyzed separately.



Supplemental Figure 13 - Effect of MWM Protocol 2 on $STEP_{61}$, NLGN-1, NMUR1 and non-phospho-STEP protein with probe and platform control groups combined. NLGN-1 expression was higher in the MWM training group (126% +/- 5.7) *vs* the control (100% +/- 10.1) ($F_{1,22}$ = 5.875, p =.024), though this is attributed to a higher level of NLGN-1 in the platform group relative to the control **(Fig. 6).** There were no significant differences in $STEP_{61}$ ($F_{1,22}$ = .011, p=.919), non-phospho-STEP ($F_{1,22}$= 2.616, p=.120, non-phospho STEP/Total STEP ($F_{1,22}$ =.205, p=.655), or NMUR1 ($F_{1,18}$=.428, p=.521).



Supplemental Figure 14 - Effect of MWM Protocol 3 on $STEP_{61}$, NLGN-1, NMUR1 and non-phospho-STEP protein with probe and platform control groups combined. Non-P-STEP expression was significantly higher ($F_{1,18}$=5.186,p=.035) in the MWM training group (148.4% +/- 9.4) *vs* the control (100% +/- 21.1). There were no significant differences in $STEP_{61}$ ($F_{1,18}$ = 1.649, p=.215), non-phospho STEP/Total STEP ($F_{1,18}$ =3.952, p=.062), NMUR1 ($F_{1,15}$= 1.407, p=.254), or NLGN-1 ($F_{1,18}$=.040, p=.845).



Supplemental Figure 15 - Effect of MWM Protocol 4 on $STEP_{61}$, NLGN-1, NMUR1 and non-phospho-STEP protein with probe and platform control groups combined. NMUR1 expression was significantly higher ($F_{(1,15)}$=6.281, p=.024) in the MWM group (154.5% +/- 14.9) *vs* the control (100% +/- 9.9). There were no significant differences in $STEP_{61}$ ($F_{1,18}$ = 3.367, p=.083), non-phospho STEP ($F_{1,18}$= .084, p=.775), non-phospho STEP/Total STEP ($F_{1,18}$ =.423, p=.524) , or NLGN-1 ($F_{1,18}$=.119, p=.119).