The Role of Striatal Enriched Protein Tyrosine Phosphatase in NMDA Receptor Regulation

by

Eniola Funmilayo Aduke Yeates
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Abstract

*N*-methyl-D-aspartate receptors (NMDAR) form ionotropic channels through the postsynaptic membranes of neurons within the hippocampus. Previous studies have shown that the enzyme Striatal Enriched Tyrosine Phosphatase (STEP) is responsible for clathrin-mediated endocytosis of NMDARs, by the dephosphorylation of tyrosine residue 1472 on the NR2B subunit of the NMDAR. The objective of this study was to investigate the role of STEP in regulating NMDA receptor expression on hippocampal GABAergic interneurons maintained in primary neuronal cultures. The density of synapses and NMDA receptors on the primary dendrites of STEP *+/−* and STEP *−/−* interneurons were compared by the immunolabeling of hippocampal cultures grown for 21 days *in vitro* (DIV). However, no significant difference was found between the quantity of synapses, NR1 or NR2B containing clusters on the STEP *+/−* and STEP *−/−* interneuron dendrites.
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Chapter 1

The NMDA receptor

Glutamate is a key excitatory amino acid neurotransmitter in the Central Nervous System (CNS). At CNS glutamatergic synapses, the postsynaptic membranes contain receptors that can be classified into one of three families: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors (Watkins & Jane, 2006) (Figure 1). NMDA receptors form ionotropic channels composed of combinations of subunits encoded by different genes (Cull-Candy, 2002). Separate genes code for the functionally distinct NMDA receptor (NMDAR) subunits, including the NR1, the NR2 and the NR3 family.

Each NMDAR is comprised of four subunits. Typically, a functional receptor contains a pair of subunits from the NR1 family and a combination of two subunits that are from the four members of the NR2 family (NR2A-D) or the two members of the NR3 family (NR3A and B) (Villmann & Becker, 2007). Within the mammalian hippocampus, the pair of NR1 subunits are co-expressed with two NR2B subunits (Cull-Candy, 2002), a pair of NR2A subunits, or a combination of NR2A and NR2B (Figure 2a).

The NMDA receptor units are inserted in the plasma membrane such that the amino terminus (N) of each subunit resides extracellularly, while the carboxyl terminus extends into the cytoplasm. The N-terminus contains the glutamate and glycine binding domains. As this region is extracellular, antibodies generated to target the N terminus of the receptor can be used to specifically label NMDA receptors localized on the cell surface, as opposed to pools of receptors that are internalized within the cell cytoplasm.
**Figure 1. Glutamate Receptors.** The N-methyl-D-aspartate (NMDA) receptors are a class of ionotrophic glutamate receptor. The NMDA subunits include NR1, NR2A-D, and NR3A-B. Reprinted from (Villmann & Becker, 2007).
The carboxyl tail of the NR2 subunits interacts with scaffolding proteins within the postsynaptic density (PSD), a dense submembranous structure. The scaffolding proteins of the PSD facilitate the localization of the excitatory receptors at the synapse. Previous studies have demonstrated that NMDA receptors co-localize with scaffolding proteins including PSD-95 (Kornau, Schenker, Kennedy, & Seeburg, 1995), PSD93 and SAP102. PSD-95 has 3 protein-binding (PDZ) domains. The first and second domains are capable of binding to the carboxyl terminus of the glutamate receptors (Kornau, et al., 1995; Kornau, Seeburg, & Kennedy, 1997) as well as to PDZ domains of other proteins. Antibodies against PSD-95 can effectively be used to postsynaptic regions in dendritic spines that are sites of synaptic contact (Figure 2a). Similarly, markers for presynaptic components can be used to localize synapses. Synaptophysin is a synaptic vesicle trafficking protein that is embedded within the membrane of presynaptic vesicles (Wiedenmann & Franke, 1985). PSD-95 typically, but not always, colocalizes with synaptophysin (Figure 2a).

Although most NMDA receptors are localized at areas of synaptic contact, they are not stationary and they can diffuse laterally in the membrane to extrasynaptic sites. NMDA receptors are also removed from the plasma membrane via clathrin-mediated endocytosis. This activity-dependent process is facilitated by posttranslational modifications of the intracellular domains of the NR2 subunits. The NR2 carboxyl tails have multiple phosphotyrosine residues that provide target sites for post-translational modifications by kinases and phosphatases, the enzymes responsible for adding or removing phosphate groups. Phosphorylation of the tyrosine residue at position 1472 of the NR2B subunit has been implicated in the placement of the NR2B containing
NMDAR at the cell surface (Figure 2B). Conversely, dephosphorylation at the same site has been implicated in the endocytosis of the receptor. One phosphatase known to remove phosphotyrosine residues at position 1472 is STriatal Enriched protein tyrosine Phosphatase (STEP), an important intracellular phosphatase (Figure 2B, Figure 3).
Figure 2. The structure of vertebrate excitatory synapses in the CNS.

A, The intracellular region of NMDA receptors is associated with SAP102 and PSD95 within the postsynaptic density. For surface labeling, antibodies against NR1 and NR2B target the extrasynaptic region of the receptors. Presynaptic terminals can be labeled using antibodies developed against synaptophysin (syn), a component of presynaptic vesicles. B, The C-terminal tail of the NR2 subunits has multiple phosphotyrosine residues that may serve as targets for tyrosine kinases and phosphatases. Adapted from (Lau & Zukin, 2007; Salter & Kalia, 2004).
Figure 3. **NMDAR trafficking.** STEP removes phosphate groups from tyrosine 1472 of the NR2B subunit of the NMDA receptor resulting in receptor endocytosis. STEP inhibits ERK, as well as Fyn, inhibiting the ability of Fyn to direct trafficking of NMDA receptors into plasma membranes.

Adapted from (Braithwaite, Paul, Nairn, & Lombroso, 2006)
Striatal Enriched Phosphatase (STEP)

Striatal enriched protein tyrosine phosphatase (STEP) is a cytoplasmic tyrosine phosphatase that regulates trafficking of NMDA receptors (Braithwaite, Paul, et al., 2006) as well as, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, the most common type of glutamate receptor in the CNS (Zhang, et al., 2008). STEP is also termed protein tyrosine phosphatase, non-receptor type 5 (PTPN5) and is a member of the protein tyrosine phosphatase family (Eswaran, et al., 2006). Thus its enzymatic activity lies in its ability to dephosphorylate its substrates. The substrates of STEP include the NR2B subunit, extracellular signal-regulated kinase 1 and 2 (ERK 1/2), p38 kinase, Fyn kinase, and AMPA receptor GluR2 (Zhang, et al., 2008).

ERK 1/2 has an important role in signal transduction and also in memory formation (Paul, et al., 2007). STEP dephosphorylates ERK1/2 and inhibits its ability to phosphorylate its substrates within the classical mitogen-activated protein kinase, (MAPK) pathway (Paul, Nairn, Wang, & Lombroso, 2003). p38 is another substrate of STEP and is itself a kinase. p38 participates in signal transduction cascades and mediates the cellular responses to stress and apoptotic signals. STEP dephosphorylates p38, and this modification inhibits the activity of p38.

Another kinase, Fyn, phosphorylates the NR2B subunit at Tyr 1472 preventing the internalization of the receptor from the cell surface (Prybylowski, et al., 2005). Dephosphorylation of Fyn by STEP inhibits Fyn’s activity as a kinase (Nguyen, Liu, & Lombroso, 2002). Thus, STEP affects the density of the surface receptors both directly and indirectly.
STEP was first discovered by Lombroso and colleagues who isolated a cDNA clone from a subtractive library prepared from bovine striatum vs. cerebellum (Lombroso, Murdoch, & Lerner, 1991). They showed that the cDNA clone encoded a protein with an amino acid sequence similar to that of known protein tyrosine phosphatases (PTPs) (Lombroso, et al., 1991). It was discovered that mRNA coding for STEP was specific to the brain, when compared to other organs. Of the proteins in the PTP family, STEP was the only one discovered at that time to be so highly expressed in the nervous system (Lombroso, et al., 1991). Further studies utilizing antibodies against STEP in the rat brain were carried out in the Naegele laboratory by Boulanger and colleagues, and showed that medium spiny neurons of the striatum expressed high levels of STEP. Additionally, strong staining was found in the lateral septum, areas of the cerebral cortex, and the hippocampus (Boulanger, et al., 1995). Within the hippocampus, the pyramidal cells in region CA1-3 showed the most intense labeling (Boulanger, et al., 1995), but staining was also found in the hilus, entorhinal cortex, basal forebrain, amygdala, as well as cells in many other brain regions (Figure 4).

Further investigations in the mouse brain by Choi and colleagues, in collaboration with the laboratories of Drs. Naegele and Lombroso, showed that STEP expression in the hilar region of the hippocampus was localized to cell bodies and processes of GABAergic interneurons, especially those that expressed somatostatin (Choi, et al.,2007) (Figure. 5).
Figure 4. STEP immunoreactivity in the forebrain and thalamus of the rat.  A, B, Coronal sections of rat brain showing STEP-immunoreactive somata as dots and immunoreactive axons as crosshatching. STEP-immunostained neocortical pyramidal neurons show a distinct bi-laminar pattern. Scale bar equals 2mm. C, Intense STEP-immunoreactive pyramidal neurons in the hippocampus. Scale bar equals 10 µm. HP, hippocampus; IG, indusium griseum; RN, red nucleus; SN, substantia nigra. Subsequent studies confirmed similar patterns of expression in the mouse brain. Adapted from (Boulanger, et al., 1995)
Figure 5. STEP is expressed in GABAergic hilar interneurons. A, Diaminobenzidine-based immunohistochemical labeling revealed marked STEP expression in the hilus of the mouse hippocampus (Hil). Note the lack of STEP staining in the granule cell layer (GCL). The boxed region is magnified (right). Scale bar equals 100 µm in the low magnification image and 25 µm in the high magnification image. B, Immunofluorescent double-labeling for STEP and GAD65/67 revealed that STEP is expressed in GABAergic hilar interneurons. STEP is also expressed in GAD65/67-positive neurons in the stratum oriens (so) of CA1. Arrows indicate cells expressing STEP but not GAD65/67. Scale bars equal 20 µm. C, Double-labeling for STEP and somatostatin shows that somatostatin-positive interneurons express STEP in the hilus as well as in stratum oriens of CA1. Arrows denote somatostatin-negative cell weakly expressing STEP. Scale bars equal 20 µm. D, Quantitative analysis of STEP and somatostatin colabeled cells in the hilus. Denominator above bars indicates the double labeled cells out of total counted from 6 mice. Bar indicates mean ± SEM. Reprinted from (Choi, et al, unpublished).
GABAergic interneurons of the hippocampus.

The dentate gyrus is a region of the hippocampal formation that receives the first afferent synaptic connections from the entorhinal cortex (Amaral, Scharfman, & Lavenex, 2007). The dentate gyrus is composed of 3 layers, the molecular, granule cell and polymorphic layer, also known as the hilus.

Somatostatin-positive cells are a subset of GABAergic interneurons that appear predominately within the hilus and the CA1-3 of the hippocampal formation. GABAergic interneurons all produce GABA, but they can be further classified by differences in the expression of neuroactive substances and calcium binding proteins, as well as differences in dendritic and axonal morphology. Interneurons are not limited to a bipolar configuration. Many are multipolar, like the hilar perforant path-associated cell (HIPP) and the basket cells(Amaral, et al., 2007) (Figure 6).

GABAergic interneurons form inhibitory synapses onto other neurons, with two exceptions. During development, GABA has a depolarizing effect on immature neuroblasts and in the subgranular zone of the dentate gyrus. GABA also has a depolarizing effect. In mature circuits, however, when GABA is released onto the postsynaptic cell, it generally has the effect of decreasing the cell’s activity by causing hyperpolarization through the GABA$_A$ receptor, a chloride ion-selective membrane pore. The GABAergic interneurons of the hilus are innervated by the granule cells in the dentate gyrus and by projections from CA1. A particular subset of GABAergic interneuron in the hilus, called the HIPP cell (hilus interneuron projecting to perforant path), forms feedback inhibitory synapses on the distal dendrites of dentate gyrus granule cells. A second type of interneuron, the basket cell forms an extensive plexus
transversing 0.9 to 1.5 mm, and thus may inhibit as many as 1% of the excitatory granule cells in the dentate gyrus (Amaral, et al., 2007).
Figure 6. Examples of hippocampal interneuron morphology. A, Photomontage of GABAergic basket cell. B, line drawing of the same basket cell. C, Photomontage of HIPP interneuron in the hilus. D, line drawing of the same HIPP cell. Scale bar equals. ml, molecular layer; gl, granular layer; pl, polymorphic layer. Scale bars equal 25 µm. Adapted from (Amaral, et al., 2007)
**Temporal lobe epilepsy**

The hippocampus is perhaps most well known for its role in learning and for its role in memory formation. However, it is also a very significant structure in the pathology of epilepsy. In temporal lobe epilepsy (TLE), the most common type of epilepsy in humans, seventy percent of patients show hippocampal sclerosis, with neuronal loss occurring most prevalently in the CA1 and hilus of the dentate gyrus (Engel, Williamson & Weiser, 1997). The cells lost in the hilus are primarily GABAergic interneurons. Thus, it is not surprising that seizures, “uncontrolled, excess and hypersynchronous neuronal activity” (Buckmaster, 2004), occur as a result of disinhibition of neural circuits.

The hippocampus has been an area of interest due to its role in seizure initiation and propagation. To account for the imbalance of inhibition and excitation in the hippocampus observed under epileptic conditions, two hypotheses have been developed. The first suggests that there is a reduction in the number of inhibitory interneurons, while the second suggests that there is a loss of the excitatory input to these inhibitory interneurons (Dudek, Sutula, & Helen, 2007). The Naegele laboratory has investigated STEP and its relationship to the first hypothesis using several models of epilepsy in mice.

Several rodent models have been developed to study temporal lobe epilepsy, following induction of status epilepticus (SE). These include chemical induction with pilocarpine and kainic acid. Pilocarpine is a muscarinic acetylcholine receptor agonist, while kainic acid is a glutamate receptor agonist. Both are potent chemoconvulsants (Dudek, et al., 2007).
Previous experiments in C57Bl/6 mice have shown that after prolonged status epilepticus, (induced by pilocarpine but not kainic acid), there is a selective loss of GABAergic interneurons in the hilus, accompanied by the abnormal axonal sprouting of surviving excitatory neurons (Buckmaster & Dudek, 1997). This reduction in the number of GABAergic interneurons is associated with increased excitation within the hippocampus (Dudek, et al., 2007) and the development of recurrent spontaneous seizures within days to weeks.

STEP has been shown to be enriched in hilar GABAergic interneurons (Choi, et al., 2007), suggesting a link between the expression of STEP within these neurons and the increased susceptibility of these neurons to death after status epilepticus. Studies conducted by Choi and colleagues, showed that the excitotoxicity of the interneurons was indeed correlated with the presence of STEP (Choi, et al., 2007).

Seizure studies conducted as part of the BA/MA thesis by Stephen Briggs in the Naegele lab showed that STEP knockout mice generally require higher doses of pilocarpine to induce status epilepticus, compared to STEP wildtypes (Briggs, 2008). More recently, Drs. Goebel-Goody, Lombroso, and Naegele found that STEP deficiency is linked to a decreased incidence of audiogenic seizures in a mouse model of Fragile X syndrome (Goebel-Goody, et al., 2009). These findings suggest that STEP deficiency may increase inhibition in the brain, and thus indirectly increase the threshold for different types of seizures. While a number of alternative hypotheses might also account for these observations, I selected to test this hypothesis for my thesis work by examining STEP regulation of glutamate receptors on interneurons.
In the experiments conducted by Dr. Naegele and Briggs, mice between the ages of 6-8 weeks were injected with pilocarpine to induce status epilepticus. The results demonstrated that significantly fewer STEP knockout mice (KO) developed seizures that progressed to status epilepticus (SE) when compared to wildtype (WT) mice (75% SE in WT versus 35% SE in KO) (Briggs, 2008). These data were the first to suggest that there could be increased inhibition within the hippocampi of the STEP KO animals compared to the WT adult mice.

Subsequently, Stephen Briggs undertook a study in collaboration with Gloster Aaron, in which voltage sensitive dyes and calcium imaging were used to study the activity of granule cells and hilar interneurons in hippocampal slices from STEP KO mice vs. wildtype or heterozygous mice (Aaron, et al., unpublished). Briggs’s studies suggested that in STEP KO slices, a greater number of cells in the hilus area responded to an extracellular electrical stimulus in the Schaffer collaterals, compared with hilar cells in WT slices. Furthermore, the granular cell layer of the KOs showed less potentiation after 3 consecutive stimuli, compared to the wildtype.

These results were consistent with the hypothesis that there is increased inhibition in the KO hippocampus. These slices were generated from animals which had not been exposed to pilocarpine or undergone seizures. Moreover, the brain sections had similar numbers of cells in the hilar and dentate layers. These results seemed to indicate that an increase in the activity of the interneurons of the STEP KO hilus was responsible for suppressing the activity of granule neurons during potentiation. However, these results were obtained without the neuronal cell death of interneurons that usually accompanies SE, suggesting that STEP deficiency not only improved the chances of survival of an
interneuron after an insult (as was demonstrated by previous experiments), but also improved the ability of hilar interneurons to inhibit the hippocampal circuit a model of long term potentiation.

Based on additional findings that STEP regulates the endocytosis of NMDA receptors, and the enrichment of STEP in hilar somatostatin-positive interneurons, we hypothesized that an enrichment of glutamate receptors on the plasma membranes of hilar GABAergic interneurons might cause hyper-inhibition in the dentate gyrus and account for the seizure-resistant phenotype of the STEP KO mice. My thesis work has tested this hypothesis by examining the expression of synaptic NMDA receptors on hilar GABAergic interneurons from STEP KO mice compared with STEP WT interneurons.
Chapter 2

STEP Regulation of \( N \)-methyl-D-aspartate receptors in hippocampal interneurons

Summary:

**Purpose:** The objective of this study was to investigate the role of STEP in regulating NMDA receptor expression on hippocampal GABAergic interneurons maintained in primary neuronal cultures. **Methods:** The density of synapses and NMDA receptors on the dendrites of \( ^{+/+} \) (WT) and \( ^{-/-} \) (KO) interneurons were compared. This comparison was achieved by the immunolabeling of hippocampal cultures grown for 21 days in vitro (DIV). The interneurons, synapses, and NMDA receptor subunits were identified using immunofluorescent double labeling methods. **Results:** No significant difference was found between the quantitiy of synapses, NR1 or NR2B containing clusters on the dendrites of STEP WT and STEP KO interneurons.

Introduction:

\( N \)-methyl-D-aspartate receptors (NMDAR) form ionotropic channels through the post-synaptic membranes of neurons within the hippocampus. Previous research has shown that the enzyme Striatal Enriched Tyrosine Phosphatase (STEP) is responsible for clathrin-mediated endocytosis of NMDARs, by dephosphorylating of tyrosine residue 1472 on the NR2B subunit of the NMDAR. Furthermore, prior work showed that reducing STEP by RNA interference in cultured cortical neurons, resulted in the
enrichment of NR1, NR2A and NR2B in the membrane fraction, when examined by immunoblot assays (Braithwaite, Adkisson, et al., 2006).

These previous findings suggested that STEP deficiency could result in higher expression of glutamate receptors within neuronal subsets that normally express high levels of STEP. Neuronal populations that exhibit high levels of STEP include the somatostatin-positive interneurons of the CA1 and the hilus of the hippocampus. The present study compares the expression of NMDARs on hippocampal interneurons obtained from STEP KO vs. STEP WT mice. Immunocytochemical staining for surface NMDA receptor subunits NR1 and NR2B was performed in hippocampal cultures generated from STEP WT and STEP KO postnatal mice.

Materials and Methods:

Animals

STEP WT and STEP KO mice on the C57Bl/6 mice (Charles River Labs) were bred in the Wesleyan University Animal Facility, a pathogen free environment. The KO was originally generated using homologous recombination to remove a portion of the STEP gene, which included the catalytic site. The knockout mice were generated at Pfizer (Venkitaramani, et al., 2009). The WT, KO and heterozygous (HT) genotypes are fertile and visibly indistinguishable. Furthermore, there were no gross anatomical differences between genotypes in Cresyl Violet stained brain sections. Immunoblot analyses showed that no STEP protein is expressed in the KO, while the HT expresses 50% of the STEP protein levels (Venkitaramani, et al., 2009). All experiments involving the use of animals
were in compliance with the guidelines provided by the Wesleyan University Institutional Animal Care and Use Committee and NIH guidelines.

Preparation of primary hippocampal cultures

Postnatal mice (P1) were anesthetized by hypothermia, euthanized, and the brains were removed under sterile conditions. Hippocampal tissue was dissected in sterile Hank’s Balanced Salts Solution (HBSS) without CaCl$_2$ and MgSO$_4$ (Sigma-Aldrich). Tissue was incubated in 0.1% papain for 25 minutes at 37 °C. Papain was replaced with 0.25% trypsin inhibitor and triturated with fire-polished pipettes. Cells were pelleted by centrifugation at 900 rpm, and resuspended in Neurobasal media (Gibco) (containing 2% B27, 0.025mM L-glutamate, 0.5mM L-glutamine, 100u/ml penicillin/ streptomycin, and 5% fetal bovine serum). Cells were plated at a density of 3 x 10$^4$ cells/cm$^2$ on glass coverslips coated with poly-L-lysine hydrobromide (Sigma-Aldrich). The primary neuronal cultures were stored in a humidified incubator (5% CO$_2$) for 21 days at 37 °C in Neurobasal media. Plating media was replaced with serum-free media one day after the initial plating. Half of the media was changed every four days.

Immunofluorescent labeling of pre-synaptic proteins and GABA

Neuronal cultures were gently rinsed in 4% sucrose in phosphate buffered saline (PBS) at 37 °C. and fixed in 4% paraformaldehyde in 0.1 M PO$_4$ (pH 7.4). Samples were treated with 0.1% Triton-X 100 for 10 minutes and blocked with 10% normal goat serum (Vector labs) for 30 minutes. Coverslips were incubated in mouse anti-synaptophysin (Millipore, 1:1000) and rabbit anti GABA (Sigma-Aldrich, 1:500) in the same blocking solution for 1 hour at room temperature. Coverslips were washed with PBS, then
incubated with goat anti mouse Alexa 647 (Molecular Probes, 1:500) and goat anti-rabbit Alexa 488 (Molecular Probes, 1:500) for 1 hour at room temperature. All antibodies were diluted in 10% normal goat serum. Images were photographed under the Zeiss LSM 510 confocal microscope under the 63x objective.

*Immunofluorescent labeling of NR2B and GAD 67*

Living cultures were stained for cell surface expression of NR2B using rabbit anti NR2B (Neuromab) then fixed with 4% paraformaldehyde. Staining was detected using goat anti mouse Alexa 647 (Molecular Probes, 1:500). Samples were treated with 0.1% Triton X for 10 minutes and blocked with 10% normal goat serum (Vector labs) for 30 minutes. Coverslips were incubated in rabbit anti GABA (Sigma-Aldrich, 1:500) for 1 hour at room temperature. Labeling was detected using goat anti rabbit Alexa 488 (Molecular Probes, 1:500). Nuclear counterstaining was performed using Hoechst 33342 (Invitrogen, dilution: 1:1000). Glass coverslips were rinsed and mounted in Prolong AntiFade onto Superfrost Plus microscope slides. Images were obtained on the Zeiss LSM 510 confocal microscope.

*Immunofluorescent labeling of NR1*

Neuronal cultures fixed in 4% paraformaldehyde in 0.1 M PO₄, were stored in antifreeze (30% ethylene glycol, 30% glycerol in PBS) at -20 degrees Celcius until use. For staining, coverslips were warmed to room temperature and rinsed in PBS. Non specific staining was blocked with 0.1% Triton-X 100 and 10% normal goat serum (Vector labs) for 30 minutes. Coverslips were incubated in Rabbit anti NR1 (Santa Cruz, 1:250) and Ms anti GAD67 (Chemcon, 1:500) overnight at 4 °C. Coverslips were washed with PBS,
then incubated with goat anti mouse Alexa 488 (Molecular Probes, 1:500) and goat anti rabbit Alexa 568 (Molecular Probes, 1:500) for 1 hour at room temperature. Images were photographed under the Zeiss LSM 510 confocal microscope with the 63x objective.

**Quantification**

Length measurements of primary dendrites were made using Zeiss LSM Image examiner. Images showing synaptophysin, NR1, or NR2B immunostaining were desaturated and brightness enhancements were made in Adobe Photoshop. The puncta were counted manually along primary dendrites of GABAergic interneurons (Figure 7). Puncta on soma was not included in counts.

**Statistical analysis**

All graphs represent the means ± the standard errors of the means. Statistical differences were analyzed using 1-way Analysis of Variance (ANOVA) with SigmaPlot 11.2 software (Synstat software, San Jose, CA). All data sets passed the tests for normality (Shapiro-Wilk) and the equal variance.
Figure 7. Method for quantification of synaptic punta and NMDA receptor subunit immunoreactivity. A, GABA labeling indicating a GABAergic interneuron and its processes. B, Punctate synaptophysin staining showing sites of synaptic contact on the same hippocampal GABAergic interneuron. C, Measurement of dendritic lengths, using confocal image analysis software. D, Quantification of puncta along a GABA positive dendrite.
Results:

To determine whether STEP deficiency altered neuronal survival in primary neuronal cultures from the hippocampus, cell density was evaluated. There was no significant difference in the density of surviving neurons in the hippocampal cultures after 21 (DIV) \( (p=0.879, n=4 \text{ cultures}) \). Furthermore, there was no statistically significant differences in the density of neurons in all cultures \((p=0.067; n=4 \text{ cultures})\). These data show that the cultures were similar in terms of neuronal density, a variable which if different could markedly effect synaptic density. As shown in Figure 8, immunostaining of GABAergic interneurons allowed for visualization of the soma, dendrites, and occasional dendrite spines. The mouse hippocampal interneurons were typically aspiny, multipolar, and varied in morphology, often exhibiting smooth dendrites with pronounced varicosities along them.
Figure 8. Representative staining of GABAergic interneurons in mouse primary hippocampal cultures at 21 days in vitro (DIV). A,B, GABAergic interneuron with GAD67 immunoreactivity within its soma (green) appears beside a non-GABAergic neuron that is negative for GAD67. The synapses from the GABAergic neuron cover the soma and dendrites of the adjacent neuron. C, GABAergic interneuron with beaded dendrites is shown forming synaptic contacts on a GAD67-negative neuron (D). Scale equals 10 µm
Similar density of primary dendritic synapses

To determine whether STEP deficiency had any effects on synapse formation in hippocampal cell cultures, the density of synapses on the dendrites of interneurons were compared. Antibodies against synaptophysin, a presynaptic protein, labeled discrete 2-3 \( \mu m \) puncta, in areas of synaptic contact (Figure 9). Punctate labeling was present on cell bodies, dendrites, as well as on axons (Figure 10). Counts of synaptic puncta on neuronal primary dendrites showed no statistical difference of the mean density of puncta/100 \( \mu m \) along primary dendritic arbors in the STEP WT (88 puncta \( \pm 13 \)), HT (101 \( \pm 17 \)) or KO (73 \( \pm 12 \)) cultures \( p=0.114 \). (Fig 11).
Figure 9 Confocal image showing high magnification view of synaptophysin-immunoreactive puncta along the primary dendrites of a GABAergic interneuron.

A, Confocal image. B, Intensity values of synaptophysin (blue line) and GABA (green line) immunofluorescent labeling plotted as a function of dendrite length. While the GABA immunofluorescent labeling was more or less continuous along the dendrite, there was considerable variability in the fluorescence emissions along the dendrite, possibly due to a technical artifact of the immunostaining protocol, or some kind of subcellular structure sequestering GAD67 within domains in the dendrites. The synaptophysin labeling was discontinuous, with peaks of fluorescent emissions that were approximately 2-3 µm across, separated by gaps where there was no immunofluorescence. The gaps between synaptic puncta were variable and ranged from 1-5 µm. Scale bar equals 1 µm.
Figure 10 Low-magnification view of a GABAergic interneuron in primary cell cultures of the hippocampus after 21 DIV. Numerous synaptic boutons on the soma and dendrites of STEP WT interneuron are labeled with anti-synaptophysin (red). Cell body and dendrites are labeled with GABA (green). Scale equals 10 μm.
Figure 11. Quantification of synaptophysin-immunoreactive puncta along the primary dendrites of STEP WT, Het and KO GABAergic interneurons. Confocal images of synaptophysin labeling on A, WT, B, Het, C, KO interneurons D. Quantification of the puncta showed no significant difference in the synaptic density between genotypes. N equals number of cells quantified. Scale bar equals 10 \( \mu \text{m} \).
Similar density of surface NR2B containing clusters

Surface levels of NR2B levels were compared in WT and KO cultures, by labeling living cells with antibodies that targeted the extracellular region of the NMDA receptor subunit. NR2B clusters were present along the cell body and the dendrite. NR2B labeling was punctate in appearance (Figure 12a). Examination of the surface expression of NR2B in GABAergic interneurons showed that there was a similar clustering of receptors along the primary dendrites of the two genotypes ($p=0.376$).
**Figure 12. Staining for NMDAR subunit NR2B on STEP wildtype and knockout interneurons in culture.** A, Surface staining for NR2B consisted of discrete puncta as measured by fluorescence intensity in confocal images (B). C, D, Desaturated confocal images of NR2B immunostaining on (C) STEP WT and (D) STEP KO interneurons. E, counts of NR2B puncta from a sample of interneurons show no significant differences in the average number of puncta/100 µm of primary dendrite. N equals number of cells quantified. Scale bar equals 5µm.
**Similar density of surface NR1 clusters**

Surface levels of NR1 containing clusters were compared in WT and KO cultures, by labeling fixed cultures with antibodies that targeted the extracellular region of the NMDA receptor subunit. NR1 labeling was punctate in appearance (Figure 13 A-B) and was located on the cell body, as well as the proximal and distal dendrites. NR1 labeling colocalized with synaptophysin immunoreactivity, indicating that a subset of NMDAR clusters were present at synapses. Furthermore, there were no statistically significant differences in the amount of NR1 clusters /genotype ($p=0.80$) (Fig 13E).
Figure 13. Surface NR1 staining along dendrites of hippocampal interneurons. A, Immunolabeling of NR1 was a series of discrete puncta (red) along primary dendrites. B, Confocal fluorescence emissions for NR1 (red) plotted as a function of distance along the primary dendrites stained for GAD67 (green). A,D, Desaturated confocal images of NR1 staining of STEP WT (C) and STEP KO interneurons (D). E, Quantification showed no statistical difference between NR1/ genotype. Scale bar equals 10 μm.
Figure 14. Colocalization of NR1 and synaptophysin staining. A, Synaptophysin labelling (red) showing distribution of synapses on KO neuron. B, Distribution of NR1 clusters in neuron. C, Overlay of A and B showing colocalization of synaptophysin and NR1. D, E, F, Enlarged view of section showing synaptophysin labelling, NR1 labelling and merged view respectively. Scale bars equal 10 µm.
Discussion

This study tested the hypothesis that a genetic reduction in the expression of STEP would result in higher levels of NR1 and NR2B-containing NMDA receptors on the plasma membranes of hippocampal interneurons. This was based on the established evidence showing that STEP regulates the endocytosis of NMDA receptors by dephosphorylating the NR2B subunit. However, in this experiment no significant difference was found between genotypes.

It was first necessary to determine whether STEP deficiency affected the ability of the cells to form synapses. In this experiment, the genetic reduction of STEP did not significantly affect the ability of synapses to be formed on the dendrites of GABAergic interneurons, as shown by quantification of punctate synaptophysin labeling. The neurons within the STEP WT, HT and KO cultures formed equivalent numbers of synapses. Thus it can be inferred that the GABAergic interneurons observed within these experiments were innervated equivalently among the genotypes.

Punctate, extracellular immunolableling of NMDA subunits NR1 and NR2B were obtained. Since each NMDA receptor contains a pair of NR1, the surface NR1 labeling detected by immunostaining indicates the distribution of all NMDA receptors at the cell surface. NMDA receptors were observed to be distributed along the surfaces of neuronal somas, as well as the dendrites. The number of NR1 containing clusters along dendritic length of 100 µm was (approximately 70%) lower than the quantity of puncta/µm, stained by synaptophysin, indicating that these receptors comprised only a subset of the total cell surface synapses. Furthermore, the number of NR2B containing clusters/100µm was than the number of NR1 containing clusters /100µm of dendrite.
The hypothesis tested in this study was not supported by my data showing that there was no statistical difference in neither NR1 nor NR2B cell surface expression. These results, however, do not provide sufficient evidence to disprove the hypothesis. The results obtained may reflect the limitations of the methodology, in the small sample size, as well as, the resolution of the immunolabelling. Moreover, it is possible that the changes in NMDAR surface expression may not be detectable in cultures functioning at a basal level and further studies would be needed to examine whether significant differences emerge after stimulation, for example with NMDA or (RS)-3,5-dihydroxyphenyl-glicine DHPG. DHPG is a selective metabotropic glutamate receptor agonist which activates mGluR1 and mGlur5 receptors. DHPG induces AMPA receptor internalization in an activity-dependent manner. Furthermore, it is also possible that differences in AMPA receptor expression on the surface of hippocampal interneurons is different in the two genotypes but NMDA receptor expression is not.
Chapter 3

Future directions

The aim of this study was to determine whether a genetic reduction in STEP resulted in a reduction of the surface expression of NMDAR in interneurons. This was examined by the immunolabeling of hippocampal cultures. Contrary to the hypothesis, no significant difference in, neither NR1, nor NR2B cell surface expression was observed between STEP KO and WT genotypes. This result raises several questions concerning the limitations of this study, particularly the resolution of the images.

Fluorescent dyes are useful for labeling subpopulations of interneurons. However, this method provides images of a limited resolution, such that synapses and NMDA receptors may only be resolved on the level of clusters. For example, while labeling resulted in discrete puncta that ranged in diameter from 2-3 µm, actual receptors have diameters on the order of nanometers, so that the actual quantity of receptors is unclear.

A change in the total number of NMDA receptors would not necessarily be coupled with an increase in the number of NMDA clusters. Rather, it is more likely that there would be an increase in the number of receptors within each cluster. In some cases, intensity measurements may be used to distinguish subtle differences that may be beyond the resolving power of the human eye. Nevertheless, intensity measurements coupled with the use of fluorescent dyes, may only distinguish closely juxtaposed or overlapping clusters.

For a greater improvement, rather than organic fluorescent probes, inorganic quantum dots and nano-gold particles may be used (Choquet, 2008). Although quantum dots are bulkier than organic dyes, they are more photoresistant and provide a higher
signal to noise ratio. They are unable to diffuse through the plasma membrane, and as a result are appropriate for surface labeling. They are photoresistant and can be as small as 5nm in size (Choquet, 2008). Lasne and colleagues were able to use such particles to track the motion of AMPA receptors on the surface of living cells in culture (Lasne, et al., 2006). In a similar manner, live imaging studies of living cultures may be done in the future to examine individual receptors on the cell surface of STEP WT and KO interneurons.

Triple labeling immunofluorescence studies may also be undertaken, to simultaneously label GABAergic interneurons, NMDA receptors and synapses. This would allow the quantification of NMDA receptors per synapse on GABAergic interneurons. Furthermore, the quantity of synaptic versus extrasynaptic receptor clusters can be compared between genotypes. In such experiments transgenic mice may also be used, for example, the GAD67-GFP mice used in experiments by Southwell and colleagues (Southwell, Froemke, Alvarez-Buylla, Stryker, & Gandhi, 2010), and generated by Tamamaki and colleagues. In these transgenic animals, the endogenous inhibitory interneurons are fluorescent and were visible in living embryos (E16) (Tamamaki, et al., 2003). Furthermore, triple labeling experiments can be done, specifically by labeling somatostatin-positive interneurons to identify the subset of the GABAergic interneurons enriched in STEP (Choi, et al.)

Previous experiments have suggested that there is indeed a difference in NMDAR surface expression when STEP protein is knocked down in brains. Braithwaite and colleagues used immunoblot assays to demonstrate that the reduction of STEP by RNA interference in cultured cortical neurons resulted in the enrichment of NR1, NR2A and
NR2B in the membrane fraction of immunoblot assays (Braithwaite, Adkisson, et al., 2006). In a similar way, western blot analysis can be done to measure the amount of NMDA proteins in the membranes of neurons form STEP knockout and wildtype neurons mice. This would determine whether there is an overall difference in the surface expression of NMDA in STEP KO and WT mice. However, this approach would not provide information about the types of neurons and whether differences were on a functionally distinct subset of GABAergic interneurons.

In such assays the amount of NMDA receptor protein in surface fractions can be semiquantitively measured. While immunoblot assays are useful in determining the NMDA levels across all neuronal cells, such a technique cannot be used to specifically measure levels within specific cells, particularly the hippocampal interneurons of the hilus. To overcome this obstacle, membrane fractions could be acquired from primary neuronal cultures derived from the medial ganglionic eminence (MGE). This structure is transiently present in the embryonic day 12-14 mouse brain. The MGE is the ventricular zone that gives rise to the somatostatin and parvalbumin subclasses of forebrain GABAergic interneurons (Pleasure, et al., 2000). While cell cultures generated from the MGE would be enriched in interneurons, such an enrichment may lead to a deprivation of excitatory stimulation, leading to an overall decrease in activity dependent processes, but this remains to be tested.

In this study it is possible that neurons were not sufficiently stimulated, so that activity dependent differences between genotypes were not observed. Indeed, previous experiments that examined STEP’s regulation of glutamate receptors were performed above basal levels of cell activity. The Lombroso laboratory, STEP was shown to
mediate AMPA receptor endocytosis after metabotropic receptor activation (Zhang, et al., 2008). In these experiments, the mGLuR activation was achieved by the administration of DHPG, a mGLuR agonist. Chemical and electrical stimulation can be used on cell cultures, to maximize differences in NMDAR levels between STEP KO and wildtype. For example, NMDA, kainic acid or other glutamate receptor agonists could be used to stimulate neurons and promote receptor recycling and STEP activity to allow for the differences to be observed by immunocytochemical studies and immunoblot assays.

Moreover, although dissociated cell cultures are useful for studying the properties of single cells, the in vitro approach does not provide the opportunity to observe the cells as they are configured and connected in the brain. There are many subclasses of GABAergic interneurons, each existing in a particular region of the dentate gyrus, forming connections with distinct cell types at distinct points of along the cell dendrite or body. For example basket cells have been noted to enervate the cell bodies and proximal dendrites of pyramidal cells (Klausberger & Somogyi). Thus hippocampal slices obtained from mice that express GFP within subset of interneurons, allow for the cells to be observed, in as close as their natural environment as possible. However, previous attempts to label NMDA subunits within slices, failed to yield quantifiable result. Punctate labeling was not acquired, possibly due to the dense neuropil. Thus cultured cells were used for these experiments.

Even with these limitations, it is possible that there is an alternative explanation for the prior observations of seizure resistance in STEP KO mice. Even though the interneurons, may not have an enrichment of NMDAR, they might still release more GABA. It would be beneficial to determine the effect of genetic reduction of STEP on
the GABA release of the GABAergic interneurons. This can be done directly by comparing the GABA levels released in the STEP wildtype and knockout brains by microdialysis. Moreover, the GABA release can be indirectly measured by comparing the electrophysiology, of the STEP knockout and wildtype interneurons.

**Conclusion:**

While previous research has suggested a role for STEP in NMDA receptor endocytosis (Braithwaite, Adkisson, et al., 2006), the method used in this current study, by immunolabelling hippocapal cultures, failed to detect an enrichment of NMDAR subunits NR1 and NR2B on the cell surface of the interneurons from STEP KO vs. WT mice. Although a statistical difference in cell surface morphology was not observed, this study does not provide enough evidence to invalidate the hypothesis that a genetic reduction in STEP results in an enrichment of NMDA receptors on the cell surface. By overcoming the limitations of this study, future experiments may yet discern a difference.
References:


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