The Role of Striatal-Enriched Tyrosine Phosphatase in Regulating Excitotoxic Cell Death

by

Daniel Austin
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Abstract

Selective neurodegeneration is a hallmark feature of a variety of diseases, including temporal lobe epilepsy, stroke, ischemia, Alzheimer’s disease, and amyotrophic lateral sclerosis (ALS). Yet the mechanisms that regulate cell death in many of these pathological conditions remain unclear. It is thought that cell survival and cell death signaling pathways become activated during excitotoxic insults, and a particular cell’s response ultimately weighs out in favor of one of the two possible outcomes. A recent study has found that a brain-specific tyrosine phosphatase known as STriatal-Enriched tyrosine Phosphatase (STEP) increases the vulnerability of particular GABAergic interneuron populations to cell death-inducing stimuli (Choi et al., 2007). These results, however, have demanded subsequent work to elucidate components of STEP’s putative role in excitotoxic cell death.

This work is divided between an assessment of STEP’s role in regulating general neuronal cell death and in the previously described GABAergic cell population. Here, I have tested the hypothesis that STEP modulates excitotoxic responses following certain types of insults. Cell death was compared between wildtype (WT) and STEP knockout (KO) primary hippocampal neurons. I demonstrate that cells lacking STEP have an attenuated cell death response to NMDA-mediated insults. I also show that this cell-death response is mediated specifically via this particular glutamate receptor subtype. It is possible, though not conclusive, that STEP’s neurotoxic effects are most noticeable following the activation of extrasynaptic NMDARs. I also collaboratively confirmed a recent finding that STEP is cleaved to its STEP\textsubscript{33} isoform following extrasynaptic NMDAR
stimulation. It remains to be determined whether the cleaved isoform is playing any particular role in excitotoxic cell death, or if it is a byproduct of the aberrant activation of calpain.

To assess the role of STEP in interneuron vulnerability, I next compared cell death in knockout and wildtype somatostatinergic populations. It was previously found that STEP is enriched in this interneuron cell populations \textit{in vivo}. My findings show that enrichment is also present after 12 days of maturation \textit{in vitro}. However, technical challenges in quantifying somatostatinergic cell death made it difficult to determine whether this particular cell population is any less vulnerable in the knockout genotype.

These results provide evidence that inactivating STEP could promote cell survival in hippocampal interneurons following certain excitotoxic insults. Because hilar interneurons regulate the synchronous bursting of granule cells located in the dentate gyrus, therapies that render these neurons resistant to excitotoxic injury could yield novel anticonvulsant treatments.
Introduction

Epilepsy is a diverse set of diseases that share the common affliction of overexcitation in the brain. These periods of overexcitation, termed seizures, can have a debilitating impact, both physical and psychological, on the lives of those who are affected (Acharya et al., 2008; Suurmeijer et al., 2001; Pugh et al., 2005). Research, using mouse models and postmortem analyses of human brain tissue, has revealed common features that emerge in the epileptic brain. The loss of particular neuron populations, termed neurodegeneration, is a hallmark feature of several types of epilepsy, including temporal lobe epilepsy, or TLE (Naegle, 2007). Brain structures, particularly limbic regions like the hippocampus (Figure 1), can undergo cell death caused by multiple seizures. The neurodegeneration that occurs in the hippocampus is not random – particular neuron types are markedly more vulnerable to certain insults.

Sloviter (1987) was one of the earliest studies to demonstrate a reduction in the number of GABAergic interneurons following seizures. Subsequent postmortem studies revealed that TLE patients lose 80% of the somatostatin-immunoreactive interneurons (Robbins et al., 1991). These are a GABA-releasing subset whose name refers to their enrichment in the inhibitory neuropeptide, somatostatin. Analyses of excitotoxic-induced cell death in a kainate rat model of TLE found similar results (Buckmaster and Jongen-Rélo, 1999). While the mechanism remains unclear, work has shown that somatostatin limits the frequency and intensity of discharges associated with epileptiform activity in the hippocampus (Tallent and Siggins, 1999). Figure 1 shows that the majority of these interneurons project inhibitory axons from the hilus to granule cell dendrites located in the outer molecular layer of the dentate
gyrus (Buckmaster, et al., 2002). It is currently hypothesized that a loss of this interneuron population reduces inhibition in limbic brain regions, leading to recurrent seizures in some types of epilepsy.
Figure 1: **The mature rodent hippocampus and its trisynaptic loop.** This limbic structure is located in the temporal lobe (top). A schematic coronal section (bottom) reveals a canonical cellular network, named the trisynaptic loop, which is affected by neurodegeneration. Perforant path axons enter the hippocampus from the entorhinal cortex and terminate on granule cells in the outer molecular layer of the dentate gyrus. Dentate gyrus principal cells project axons (known as mossy fibers) into CA3 and synapse onto Schaffer collateral neurons. A reverberating, excitatory circuit is formed by Schaffer collateral axons that return back to the entorhinal cortex and synapse onto neurons which are part of the perforant path. Within the dentate gyrus, the hilus region (purple area) is enriched in GABAergic interneurons (green cells) that play a central role in gating synchronous bursting of granule neurons in the dentate gyrus. Loss of this inhibition can lead to hyperexcitability in the trisynaptic loop and is thought to contribute to recurrent seizures. Modified from: Purves *et al.*, 1997.
A central question in epilepsy research is whether cell death is the cause of seizures, the product of these periods of over excitation, or a combination of both. Studies have striven to understand how excitotoxic neurodegeneration is regulated, what properties make certain neuron populations more resistant, and the role that aberrant excitation plays in promoting cell death pathways. The principal excitatory neurotransmitter of the CNS, glutamate, is the primary source of feed-forward excitation in the brain. Many researchers have turned to understanding glutamate’s relationship with a diverse set of postsynaptic receptors, as these interactions are a critical foundation for activity-dependent changes in a synapse’s properties. The aberrant release of glutamate and abnormal activation of glutamatergic pathways (due to unusual receptor distributions) are implicated in a variety of neurological disorders such as Fragile X syndrome, ALS and TLE (reviewed in Lau and Zukin, 2007).

**Excitotoxicity triggers survival and death pathways**

During periods of prolonged excitation, the presence of surfeit glutamate situates neurons in an excitotoxic environment that elicits a variety of cellular responses. Novel genes are up-regulated and new protein levels emerge depending on the cell type and properties of the insult, such as its duration and severity (Han and Holtzman, 2000; Hardingham *et al.*, 2001; Medina, 2007). What are the signaling pathways that promote survival in some neurons and death in others? Are the different outcomes due to intrinsic cell differences, such as the expression of
particular kinases and phosphatases – key regulators of cell survival and death signaling pathways?

Researchers have struggled to tease apart the unique features of seizure-resistant cell types. Work has shown that the up-regulation of enzymes which maintain mitochondrial membrane integrity and reduce the formation of reactive oxygen species (ROS) can attenuate the extent of cell death (Mandir et al., 2000; Andrabi et al., 2006). These types of proteins may be broadly regarded as neuroprotective signals, or “pro-survival” enzymes. Proteins that detrimentally influence mitochondrial function or directly down-regulate survival constituents are described as members of “pro-death” signaling pathways. Some have proposed that a cell’s outcome is a tug-of-war between these broad families of proteins, with the balance ultimately tilting in favor of one the two possible outcomes (Xia et al., 1995).

Many studies have found that activation of certain signaling pathways can consistently mediate survival or death. Following excitotoxic insults, activation of prominent cascades, such as the phosphatidylinositol 3-kinase (PI-3K) and extracellular signal-regulated kinase (ERK) pathways attenuates the level of cell death in neurons (Hetman et al., 1999; Almeida et al., 2005; reviewed in Hetman and Xia, 2000). Numerous studies have shown that such pathways up-regulate survival constituents like brain-derived neurotrophic factor (BDNF), cAMP response element-binding (CREB) and ERK. BDNF and ERK help to maintain cellular function through inactivation of apoptosis-inducing constituents; for this reason, increased levels of BDNF and ERK are thought to promote survival. Almeida et al. (2005) found that BDNF treatment increases the amount of Bel-2 present in cultured
hippocampal cells – Bcl-2 promotes mitochondrial membrane integrity by binding to (and blocking the activity of) pro-apoptotic member Bax. Active, phosphorylated ERK (pERK) has also been shown to inactivate the apoptosis-promoting protein Bad, preventing mitochondrial membrane disruption (Jin et al., 2002). Alternatively, upregulation of p38 and c-JUN NH₂-terminal kinase (JNK) pathways induces excitotoxic cell death by increasing levels of Bad, Bax, and decreasing ERK levels (Xia et al., 1995; Ghatan et al., 2000). The cross talk between survival and death pathways has garnered attention because these cascades are dynamically modulated and directly influence each other under excitotoxic conditions.

**Glutamate-induced excitotoxicity is largely mediated through NMDA receptors**

A critical property that determines whether a neuron is injured following presynaptic release of glutamate is the receptor subtypes to which glutamate binds. Utilizing both metabotropic and ionotropic subtypes, glutamate receptors have an array of intracellular mechanisms that are triggered following the binding of neurotransmitter. Glutamate has three ionotropic receptor classes, based on their high-affinity chemical agonist: kainic acid (KA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-d-aspartate (NMDA). NMDA channels are blocked by magnesium ions (Mg²⁺), which are only released after local membrane depolarization (usually via AMPARs), in concert with the binding of glutamate and glycine (Figure 2B). While NMDA receptors (NMDARs) are similar to the other two subtypes in permitting the influx of sodium (Na⁺), these receptors are unique because they are also permeable to calcium (Ca²⁺).
Under normal conditions, Ca$^{2+}$ influx is a critical activator of secondary messengers inside the cell. Calcium influx leads to the activation of Ca$^{2+}$-dependent enzymes that can alter a cell’s physiology. It is well established, for example, that calcium entry (following tetanic stimulations) is one requirement for long-term potentiation (Xia et al., 1996; Kapur et al., 1998). The concentration of intracellular calcium must be carefully regulated – an excessive level of calcium triggers excitotoxic cell death (Coyle, 1983; Choi, 1992). Additionally, work has shown that this glutamate-induced cell death is mediated predominately through NMDA receptors, instead of AMPA or kainate subtypes (Sattler and Tymianski, 2001; Choi, 1992). Because of these features, some researchers have proposed that glutamate-induced excitotoxicity is the result of surfeit calcium entry and aberrant activation of cascades which are normally triggered during LTP induction (see Figure 2).
Neuronal cell death may be due to the exacerbation of processes that are normally required for initiating long-term potentiation (LTP). A. Glutamate release under basal conditions typically promotes sodium influx via AMPA receptors (leftmost panel). LTP can be induced following tetanic stimulation, which leads to glutamate-induced calcium influx via NMDA receptors (middle panel). Molecular cues promote a translationally-dependent increase in the expression of receptors on the dendritic surface, potentiating the synapse (rightmost panel). B, NMDAR activation requires both glutamate and glycine binding. Calcium influx can only occur after depolarization (mediated via AMPARs) and the release of a magnesium ion channel blocker. Following tetanic stimuli, increased glutamate release promotes calcium influx that is associated with downstream LTP processes (middle panel). Aberrant activation of NMDARs during seizures can lead to excess calcium influx, ultimately activating apoptotic pathways that lead to cell death. Modified from: Salter and Kalia, 2004.
The NMDA receptor structure is a dynamic assembly whose regulation is no longer limited to the narrow dimensions of endo- and exocytosis. These channels are mobile, with the ability to move extensively between the synaptic zone and other regions of the dendritic shaft. Modifications can be activity-dependent, as studies have found that extrasynaptic NMDARs move into the synapse-zone following NMDA receptor’s permanent loss of function by the open-channel blocker MK-801 (Tovar and Westbrook, 2002). Additionally, NR2A-containing receptors show a preferential distribution within synaptic sites, compared with NR2B-containing receptors (Tovar and Westbrook, 1999). These findings have improved our understanding of how an NMDAR’s structure, temporal and spatial features can contribute to a cell’s excitotoxic response following an insult.

**Synaptic and extrasynaptic NMDARs function in divergent manners**

NMDARs that occupy regions beyond the synaptic zone are termed extrasynaptic receptors. These populations are thought to seldom bind neurotransmitter, because released neurotransmitter normally disperses into the synaptic space exclusively (and astrocytes quickly remove it from the cleft). It is hypothesized that aberrant firing in a neuronal circuit leads to the release of enough neurotransmitter to saturate synaptic receptors and astrocytes. The result is that excess, lingering glutamate diffuses out of the synapse region and binds to extrasynaptic receptors.

NMDARs in the extrasynaptic region are thought to have distinct, opposing roles to those located in the synaptic zone (reviewed in Hardingham and Bading,
Recent studies have suggested that binding of glutamate to these extrasynaptic NMDARs leads to the activation of pro-death proteins and potent suppression of survival constituents. Exclusively stimulating synaptic receptor populations elicits robust activation of survival-associated cascades involving pERK (Ivanov et al., 2006), BDNF (Vanhouette and Bading, 2003), pCREB (Hardingham et al., 2002), and uncharacterized genes (Medina, 2007). When extrasynaptic receptors are stimulated (either exclusively or concurrently with synaptic populations), expression of these proteins is either unchanged or even down-regulated (Ivanov et al., 2006; Hardingham et al., 2002). Discovering that structurally similar receptors can have vastly contrasting properties, depending on their spatial distribution, has substantial implications in the pursuit of therapeutic interventions for neurodegenerative diseases. These studies have also helped to elucidate why neuroprotective treatments based on broad-spectrum NMDA inhibitors have yielded consistently disappointing results (Lipton, 2004; Lipton, 2006). By selectively blocking extrasynaptic NMDA receptors (based on their location rather than their composition) and sparing those located in the synaptic cleft, one might be able to design novel therapeutic treatments that spare vulnerable brain regions from neurodegeneration.

**STEP is a critical regulator of ERK and NMDA receptors**

Phosphorylation and dephosphorylation serve as critical mechanisms for regulating NMDA receptor expression and conductance properties (for review, see Salter and Kalia, 2004). Active protein kinases phosphorylate tyrosine, threonine and/or serine residues of certain NMDAR subunits. Altering phosphorylation patterns
induces conformational changes that can target the receptor to the plasma membrane (Wang and Salter, 1994). Fyn, a Src-family kinase (SFK), binds to the NR2A subunit and catalyzes the addition of a phosphate group (Figure 3, Figure 2B) when activated in the postsynapse (Tezuka et al., 1999). Thus, activation of Fyn can prolong the presence of NMDA receptors at the synapse surface and alter its response properties to certain stimuli.

Opposing the role of kinases, another protein family functions to strip surface receptors of their phosphate groups. Dephosphorylation by phosphatase proteins induces conformational changes in receptors and can target them for clathrin-mediated endocytosis. Striatal-enriched tyrosine phosphatase (STEP, also known as PTPN5), is a CNS-specific tyrosine phosphatase (Boulanger et al., 1995). It is heterogeneously expressed throughout the brain, with a noted presence in limbic system structures and enrichment in the striatum (hence being named striatal-enriched tyrosine phosphatase). Elements of STEP’s regulation remain unclear, but it is known that its modulation is indirectly dependent on calcium influx. STEP activation relies on the phosphatase calcineurin, whose activity requires elevated levels of intracellular Ca$^{2+}$ (Figure 3).

While STEP activity is reliant on calcium influx, the phosphatase also opposes the expression of NMDARs on the postsynapse surface. This relationship is thought to form a feedback mechanism where active STEP promotes its own down-regulation by reducing surface NMDARs, decreasing calcium influx, and lessening calcineurin activity (Braithwaite et al., 2006). STEP is capable of dephosphorylating the NR2B subunit at Tyr1472, promoting NMDAR endocytosis (Snyder et al., 2005). STEP is
also able to directly dephosphorylate Fyn, inactivating a kinase that promotes NMDA receptor surface expression (reviewed by Braithwaite et al., 2006). Altering the expression of NMDARs changes the dynamics of calcium influx, which is a large determinant of the severity of excitotoxic insults following excessive glutamate release (Figure 3). This is part of the reason that STEP is hypothesized to play a role in regulating excitotoxic cell death.

Figure 3: **STEP contributes to the regulation of pERK and NMDA receptors.** Following its activation by calcineurin, STEP is a potent inhibitor of active (phosphorylated) ERK, reducing the induction of many MAP kinase downstream constituents. Active STEP is also able to directly dephosphorylate NMDARs containing an NR2B subunit, promoting clathrin-mediated endocytosis. STEP indirectly down-regulates expression by inactivating Fyn, a kinase whose activity promotes NMDA receptor insertion at the postsynaptic surface. Modified from: Braithwaite et al., 2006.
STEP has a putative role in modulating neuronal survival that extends well beyond its regulation of NMDAR surface expression. Following its activation by calcineurin, STEP becomes a potent inhibitor of the canonical ERK/mitogen-activated protein (MAP) kinase pathway. This is achieved via its strong affinity to bind, dephosphorylate and inactivate the final kinase in the cascade, ERK (Paul et al., 2002). Phosphorylated (active) ERK translocates into the nucleus and promotes the transcription of a plethora of genes, some of which promote cell survival through a variety of mechanisms (Xia et al., 1995; Xia et al., 1996). Recent unpublished evidence has also implicated STEP in the inhibition of p38, whose downstream targets promote cell death (Xia et al., 1995; Ghatan et al., 2000). These findings put STEP in a central position to regulate multiple cell death signaling pathways simultaneously.

Work in the past two years has reinforced STEP’s role as an important regulator of survival and death cascades. Findings by Choi et al. (2007) have suggested that STEP is a critical determinant of cell death, following status epilepticus, in a subclass of gamma-aminobutyric acid (GABA) inhibitory interneurons. This study revealed that nearly all somatostatinergic interneurons (99%) located in the hilar and CA1 regions of the hippocampus express STEP. This GABAergic subset is one of the earliest cell types to die off following seizure-induced cell death (Buckmaster and Jongen-Rélo, 1999). Following seizures induced by pilocarpine, nearly all (96%) of the hilar neurons dead or dying at six hours after pilocarpine-induced seizures were STEP-immunoreactive (Choi et al., 2007). Additionally, inactivation of STEP in vivo and in vitro improved survivability of hilar
neurons following excitotoxic conditions. Based on these findings, it is thought that STEP-enriched interneurons are vulnerable to cell death because they are incapable of promoting pro-survival signaling cascades (Figure 3). Choi et al.’s results, however, were not acquired in the total absence of STEP, due to the unavailability of knockout mice. Subsequent work is attempting to confirm these findings with knockout mice to better elucidate the conditions which make STEP expression neurotoxic.

While STEP promotes cell death in a mouse model of TLE, work in a rat ischemia model of stroke has yielded contrasting results. Following middle cerebral artery occlusion, STEP mRNA and protein levels are reduced in regions of the brain where marked cell death is measured (Braithwaite et al., 2008). Neuroprotective preconditioning of the brain leads to a global increase in STEP expression. The authors demonstrate that increases in STEP mRNA expression and protein modification are heavily correlated with areas that are spared. Furthermore, ischemic conditions lead to the formation of STEP\textsubscript{33}, a cleaved cytosolic isoform derived from membrane-bound STEP\textsubscript{61}. This study only examined brain regions and did not address particular neuronal subsets, thus it is unknown how these changes in STEP expression affected particular cell types.

So is STEP pro-survival or pro-death? The answer is heavily dependent on the brain region, cell type, and excitotoxic model utilized. A TLE mouse model suggests that STEP is neurotoxic to a GABAergic subset, while a rat ischemia model positions STEP in a neuroprotective role. My work has utilized an \textit{in vitro} model of epilepsy to define the role of STEP during seizure-induced cell death. Here, I have compared the levels of cell death in hippocampal primary cell cultures obtained from isogenic
strains of mice that are wildtype (WT) or STEP-deficient. The results support the hypothesis that neurons containing STEP are more vulnerable to insults involving activation of synaptic and extrasynaptic NMDAR populations. These data support the previous findings of Choi et al. (2007) and suggest that NMDA-mediated activation of STEP may lead to inactivation of the pro-survival ERK/MAP kinase cascade. In doing so, STEP could limit the induction of pro-survival pathway constituents, like BDNF and pCREB. Elucidating the mechanisms underlying why certain cells die in the brain after epilepsy, while others are spared, is one of the ultimate goals of this field of research.
**Materials and Methods**

**Animals**

Wildtype and STEP knockout mice were both on the C57BL background. These populations are isogenic except at the STEP locus. Mice were housed in pathogen-free conditions at Wesleyan University’s Animal Facility. Homozygous matings between wildtype (STEP +/+) or knockout (STEP -/-) mice were utilized to obtain homozygous pups. Parents’ genotypes were confirmed by PCR using STEP and knockout-specific primers. Newborn, postnatal day 0.5 (PND 0.5) pups were used in all primary cell culture experiments. All animals were bred, raised, and euthanized according to the National Institute of Health (NIH) guidelines, and the Wesleyan Animal Care and Use Committee (WACUC) approved of these animal treatment protocols.

**Primary Hippocampal Cell Cultures**

Primary neuronal cultures were derived from hippocampal cells of PND0.5 wildtype and knockout mice. Pups were chilled on ice and decapitated, followed by the sterile dissection of hippocampal tissue from both hemispheres under a microscope. Dissections were done in chilled Hanks’ Balanced Salt Solution lacking magnesium or calcium (HBSS w/o Ca^{2+}/Mg^{2+}; Sigma). Minced tissues were treated with 0.1% papain (Sigma) in Neurobasal media (Gibco) for 25 minutes to dissociate extracellular matrix connections. This reaction was terminated with pre-warmed soybean trypsin inhibitor (Sigma) in HBSS. Cells were gently separated by trituration with three fire-polished pipettes of smaller diameter openings. The cell suspension
was removed from debris, pelleted at room temperature, and then gently resuspended in pre-warmed Neurobasal medium. Cell density was assessed using a hemacytometer and cells were plated at a density of 70,000/cm² on poly-L-lysine-coated (Sigma), culture-grade coverslips (Thermo-Fisher).

Cells were maintained at 37°C with 95% O₂ and 5% CO₂ in a humidified Napco incubator (Winchester, VA). Plating Neurobasal medium, containing 5% fetal bovine serum, 2% B-27 supplement (Gibco), 0.5mM L-glutamine (Invitrogen), 0.025mM L-glutamate (Sigma), and 100 U/mL penicillin/streptomyycin was replaced after one day with medium lacking FBS. Subsequently, half the media was removed every four days and replaced with fresh Neurobasal medium containing B-27, L-glutamine and penicillin/streptomyycin without FBS or glutamate. Media removed on DIV 4 & 8 was saved as conditioned media for use in future experiments.

**Bath NMDA and Glutamate Stimulation**

Neurons were treated after twelve days in vitro (12 DIV) with glutamate receptor agonist compounds. Exogenous glutamate (100 µM; Sigma) or NMDA (50µM; Sigma), dissolved in endotoxin-free, culture-grade dH₂O (Sigma), was added directly to the media and incubated for one hour. Media was replaced with pre-warmed, conditioned Neurobasal media. Cells incubated in glutamate were fixed after eight hours and scored for pyknosis; NMDA-treated cells were fixed and scored twenty-four hours later.
Stimulating Synaptic and Extrasynaptic NMDARs

Stimulation of synaptic and extrasynaptic NMDAR populations was performed using the protocol of Ivanov et al. (2006). Briefly, cells were cultured and plated as described earlier. Twenty-four hours before experiments, neurons were placed into glutamate-free Neurobasal medium. Three hours prior to stimulation, all cells were pretreated with Neurobasal media containing TTX (1µM; EMD Biosciences), CNQX (40µM; Sigma-Aldrich), D-AP5 (100µM; Sigma-Aldrich) and nifedipine (5µM; EMD Biosciences). Immediately before stimulation, cells were washed with media containing TTX, CNQX and nifedipine.

Wells assigned to activation of both synaptic and extrasynaptic NMDARs (“Bath” protocol) were then treated for fifteen minutes with medium containing TTX, CNQX, nifedipine, NMDA (50µM) and glycine (10µM; Sigma-Aldrich). Cells were then washed once with Neurobasal medium and replaced with preconditioned media, and fixed and scored twenty-four hours later.

Wells whose synaptic NMDAR populations were selectively stimulated (“Synaptic” protocol) were treated for fifteen minutes with the GABAergic antagonist biccuculine (10µM), glycine, and nifedipine. Following stimulations, cells were washed and returned to preconditioned medium for twenty-four hours.

Wells with extrasynaptic receptor stimulation (“Extrasynaptic” protocol) were first treated with a cocktail to inactivate synaptic NMDARs: medium containing nifedipine, biccuculine, glycine, and the open-NMDAR channel blocker MK-801 (50µM). Cells were then washed with medium containing TTX, CNQX, nifedipine. Medium containing NMDA (50µM) glycine, TTX, CNQX, and nifedipine was added
for fifteen minutes. Wells were washed with Neurobasal medium and replaced with preconditioned medium for twenty-four hours before fixing and scoring excitotoxic cell death.

**Immunocytochemistry**

Cells were washed twice with pre-warmed phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in 0.1M PO₄ (pH 7.4) at 37°C. After washing with PBS, cultures were incubated for one hour at room temperature (22°C) with blocking buffer containing 0.1% Triton-X100 (Sigma), 10% normal goat serum (NGS), and 1% bovine serum albumin (BSA; Sigma). Cells were incubated overnight at 4°C with the appropriate primary antibodies in blocking buffer. Neuronal specificity was determined using mouse anti-neuronal nuclear marker (NeuN, 1:250; Molecular Probes); STEP-immunoreactive neurons were quantified using mouse anti-STEP (1:1000 Novus Biologicals); somatostatinergic interneurons were detected using rabbit anti-somatostatin (1:500; Peninsula Lab). After washing with PBS, cultures were incubated for one hour with anti-mouse Alexa Fluor 568 and/or anti-rabbit Alexa Fluor 488 secondary antibodies (1:1000; Molecular Probes) in blocking buffer. After subsequent washing with PBS, cell nuclei were stained with Hoechst 33342 dye (Invitrogen). Coverslips were mounted with Vectashield, sealed with clear nail polish and viewed under a Zeiss confocal fluorescence microscope.
**Western Blotting**

Fifteen minutes following the completion of synaptic and extrasynaptic stimulations, cells were washed with PBS and lysed in RIPA buffer with a protease inhibitor cocktail (Sigma). Protein concentration was determined using the BCA method (Pierce Biotechnology). Total cell lysate proteins were run on an SDS/10% polyacrylamide gel and then transferred onto a PVDF membrane (Millipore). Blocking was done in TBST containing 5% milk, followed by overnight staining with primary antibodies against phospho-ERK (Invitrogen), ERK1/2 (Invitrogen), and STEP (Novus Biologicals). The membrane was washed of primary antibodies and probed with HRP-conjugated secondary antibodies (Jackson Immunoresearch) for one hour. Following development with chemiluminescence reagents (Amersham), images were acquired using a Syngene G-Box chemiluminescence system.

**Scoring Excitotoxic Cell Death**

Nuclear pyknosis, a morphological feature of dead or dying cells, was quantified to assess neuronal viability. An equal number of randomly acquired photographs were collected for each treated well. Cells needed to meet the criteria of being NeuN-immunoreactive (NeuN+) before examining their nuclei. Neurons with condensed or fragmented (pyknotic) chromatin were considered dead or dying. All counts were done blind to genotype and treatment, and every round of quantification involved images from both genotypes. After all scoring was completed, decoded data were exported into a Microsoft Excel spreadsheet and the percentage of cell death in each well was calculated as the number of pyknotic neurons out of the total number.
of neurons counted per well. 100 random field photographs were acquired from every NMDA-treated well (average number of total neurons: 1,511); 50 photographs were acquired per glutamate-treated well (average number of total neurons: 738). All bath NMDA experiments were repeated three times in duplicate (n=6 wells) with neuronal cultures obtained from three different breeding pairs. Glutamate data were performed on wells (n=3) obtained from a single set of WT/KO cell cultures. Synaptic (n=2) and extrasynaptic (n=3) results are from two separate WT/KO cell cultures. Statistical significance was determined using a two-tailed Student’s t test. All results are presented as mean ± standard deviation.

**Determining the Percentage of STEP-Immunoreactive Neurons**

To determine the number of STEP-immunoreactive hippocampal neurons following twelve days *in vitro*, cells were cultured, grown, and fixed under normal conditions as described earlier. Wells were stained with either mouse anti-NeuN or mouse anti-STEP antibody. Next, 50 randomly acquired photographs were quantified for the number of STEP-immunoreactive or NeuN-immunoreactive cells. The percentage of cells expressing STEP was calculated by taking the quotient of the mean number of STEP-positive cells and the mean number of NeuN-positive cells in the dish.
Results

Neuronal cell death peaks twenty-four hours after NMDA insults

Prior to comparing NMDA-induced cell death in wildtype and knockout cultures, a time course experiment was conducted to determine the optimal period to wait prior to assessing excitotoxic cell death. Wildtype hippocampal primary cells were grown for 12 days in vitro (DIV) and then exposed to NMDA for 1 hour to provide an excitotoxic insult. Following treatment, cells were incubated in conditioned media for 12, 18, 24, or 48 hours before being fixed with paraformaldehyde. Cells were stained with Hoechst to identify their nuclei and NeuN antibody, which labels mature neurons.

Healthy neurons exhibit a round nuclear morphology along with strong NeuN-immunoreactive staining (Fig. 4A, arrowheads). Following excitotoxic insult, many neurons exhibit condensed (pyknotic) nuclei that are indicative of dead or dying cells (Fig. 4A, arrows). Exposure to dH2O (vehicle) in Neurobasal medium does not induce pyknosis in neurons (Fig. 4A, upper panels). A 1-hour exposure to 50μM NMDA, however, induces apoptosis in most neurons and leads to the formation of pyknotic nuclei (Fig. 4A, lower panels). NeuN immunoreactivity, though slightly fainter following insults, is still detected 24 hours post-insult, allowing for the accurate identification of dead and dying neurons.

The percentage of pyknotic nuclei progressively increased up to 24 hours following NMDA treatment (Figure 4B). Peak cell death (89%) was quantified 24 hours after the insult. The decrease in cell death to 50% after 48 hours is due to the loss of NeuN-immunoreactivity in neurons that have been dead for more than 24
hours (Cevik-Unal et al., 2004). In wells that were fixed before 48 hours, an average of 1,415 cells were counted per coverslip – the 48-hour treatment only contained 976 neurons. 1,372 pyknotic neurons scored 24 hours after an insult – only 485 were counted after 48 hours, presumably due to the inability to score many pyknotic neurons that had lost their NeuN-immunoreactivity. Based on these findings, subsequent comparison of NMDA-treated cells was performed 24 hours post-insult.
Figure 4: Assessing cell death is optimal twenty-four hours after NMDA excitotoxic insults. Cells were administered a 1-hour exposure to Neurobasal medium containing NMDA (50µM), to induce neuronal death, or vehicle (dH2O) as a control. A, Cells exhibit distinct nuclear morphologies following excitotoxic insults. Neurons exposed to vehicle only (upper panels) have healthy, round nuclei (arrowheads) and robust NeuN expression (NeuN panel) 24 hrs later. At this same time point, however, most neurons exposed to NMDA (lower panels) exhibit condensed, fragmented nuclei (arrows) with persisting levels of NeuN immunoreactivity. The rightmost panels highlight neuronal nuclear morphology and NeuN expression; they are magnified from the white-bordered region of the merged panels. The upper panel displays four healthy nuclei (one is marked by an arrowhead), while the lower panel shows two pyknotic nuclei (arrows) and a healthy one (arrowhead). Scale bars: 50µm. B, Peak cell death in wildtype neurons (89%) is measured 24 hrs post-insult. Neuronal cell death increases from 74% (12 hrs post-insult) to 89% (24 hrs post-insult) before NeuN immunoreactivity becomes compromised. Prolonged periods of waiting before assessment (48 hrs post-insult) are not ideal for accurate quantification of cell death, as dead neurons lose their immunoreactivity during the second day. As a result, there are fewer NeuN-positive, pyknotic cells being counted overall (and thus an artificially higher percent survival).
**Neurons lacking STEP are less susceptible to NMDA-induced excitotoxic cell death**

To determine whether STEP is involved in regulating excitotoxic cell death, mature wildtype (WT) and STEP-null (KO) primary hippocampal neurons were exposed to excitotoxic levels of glutamate (100µM) or NMDA (50µM) for 1 hour. 24 hours after the insult, cell death was quantified as previously described. It was first necessary to measure whether KO and WT cells have different levels of basal cell death during *in vitro* development, due to media changes or the addition of vehicle alone (10µL dH₂O). WT and KO neuron populations exhibit comparable levels of basal cell death (4.0±3.3% and 2.3±0.8%, respectively) under non-toxic conditions (Figure 5). Next, activation of the entire glutamate receptor populations was measured to see whether STEP has any influence following glutamate-induced excitotoxicity. After glutamate stimulation, extensive cell death was recorded for both groups (95±1.8% and 97±2.5%, respectively), with no significant difference measured between the populations (n=3). Thus under broad and extreme glutamate receptor activation, no apparent difference between WT and KO groups was measured (see Discussion).

Because STEP activity is dependent on calcium influx and also regulates NMDAR surface expression, I investigated whether NMDA-mediated excitotoxicity was differentially tolerated in the absence of STEP. Wildtype cells exhibited significantly more cell death than knockouts (89.6±3.0% versus 70.8±6.5%, respectively) at twenty-four hours post-insult (n=6, p<0.001). STEP KO cells treated with excitotoxic levels of NMDA survived almost three-fold better following this
treatment regimen. These results suggest that STEP mediates an excitotoxic effect specifically following NMDAR-dependent calcium influx.

Figure 5: **STEP mediates a neurotoxic effect specifically following NMDA-mediated insults.** Wildtype (WT) and STEP knockout (KO) hippocampal neurons were bath treated for 1 hr with vehicle (dH2O only), glutamate or NMDA. No differences in cell death were measured under control conditions or following glutamate-mediated insults. Bath exposure to exogenous NMDA (50μM) for 1 hr resulted in significantly less cell death in STEP KO neurons when compared with wildtype cells (p < 0.001). Survival in the KO cells was nearly three-fold greater (28.4%) than WT cells (10.4%) treated under the same conditions (n=6).
STEP activity is regulated differently following isolated stimulation of synaptic versus extrasynaptic NMDA receptor populations

To further delineate whether STEP is alternatively modulated at synaptic versus extrasynaptic NMDARs, I utilized a protocol that can activate one group of receptors and trigger selective calcium influx (see Materials and Methods). Because of the extensive number of compounds and media changes utilized to inactivate certain receptor types, I first determined whether these treatments alone would induce different levels of cell death in WT and KO populations. Control conditions blocked channels according to the extrasynaptic protocol (Materials and Methods) but did not expose neurons to any NMDA insult (dH2O only). As shown in Figure 6A, the levels of cell death in both WT and KO wells were minimal (5.0±3.2% and 3.1±0.3%, respectively) and not significantly different from each other (n=2). Preferential stimulation of synaptic NMDA receptors did not result in a significant difference in cell death, though the effects were more noticeable in the WT versus KO neurons (Figure 6A). Exposure of extrasynaptic receptor populations to a 15-minute NMDA insult elicited varied responses in WT cells (25.8±25.0%). By contrast, KO cells response to extrasynaptic insults was similar to that following no insult (3.4±0.6%). These data suggest that STEP may exert its neurotoxic effects specifically following extrasynaptic NMDAR activation, though the variation observed in WT cell populations (n=3 wells) prevents any significant conclusions presently.

Confirming earlier findings, a 15-minute bath application of NMDA (50µM) elicited substantial amounts of cell death in the WT population, but only minimal levels in STEP KO neurons (55.2±7.4% vs. 12.9±1.3%, respectively). These findings
are consistent with the results shown in Figure 5, except that the overall levels of cell death are reduced and more disparate between genotypes. Less cell death is expected, given that the exposure time was shorter and thus produced a less severe insult.

To ensure that these stimulations and levels of cell death were consistent with previous studies, I assessed protein expression in wildtype neurons. The profile of pERK following bath, synaptic, and extrasynaptic stimulations has recently been published (Ivanov et al., 2006). Jian Xu of Yale University has found that extrasynaptic NMDAR activation leads to the robust formation of STEP33 (unpublished data). A western blot of pERK and STEP33 shows that these stimulations led to results that are similar to earlier findings (Figure 6B).

Consistent with Ivanov et al. (2006), synaptic stimulation elicits a robust increase in the expression of pERK, while extrasynaptic and bath applications of NMDA (50µM) reveal little difference from basal (Ctl) levels (Figure 6B). Similar to unpublished data, extrasynaptic application of NMDA (50µM) leads to the formation of STEP33, a cytosolic isoform. The appearance of a modest amount of STEP33 was detected following synaptic stimulation, which is unexpected and may be due to some spillover of glutamate from synaptic compartments to extrasynaptic NMDAR sites (Figure 6B). Total ERK 2 (ERK2), whose expression does not change during these stimulations, was used as a loading control.
Figure 6: **STEP may contribute to neuronal cell death predominately following extrasynaptic NMDAR stimulation.**

**A,** STEP knockout (KO) cells trend toward exhibiting less cell death than wildtype (WT) cells following 15 min of extrasynaptic receptor stimulation with NMDA (n=3). STEP KO cells, following extrasynaptic stimulation, have nearly equal levels of cell death as those that are treated with vehicle only. Synaptic stimulation does not show a significant difference between genotypes. Consistent with results in Fig. 5, concurrent stimulation of synaptic and extrasynaptic NMDARs (bath treatment) elicits more robust cell death in WT cell populations (n=2). No data have been statistically analyzed, as experiments have not been repeated three times. **B,** Isolated stimulation of synaptic or extrasynaptic NMDARs alternatively affects STEP\textsubscript{33} formation and active, phosphorylated ERK (pERK). Levels of the neuroprotective protein pERK increase substantially following 15 min of synaptic NMDAR activation, while it is reduced following a 15 min extrasynaptic or bath stimulation. STEP\textsubscript{33}, a cleaved isoform of STEP, appears robustly following extrasynaptic stimulations. Cell culture stimulations and protein samples were done at Wesleyan University in collaboration with Jian Xu, Yale University; Western blot developed by Jian Xu at Yale University.
Mature somatostatinergic interneurons express STEP in vitro

STEP expression is heterogeneous in vitro and in vivo in hippocampal neurons, with robust expression in some and absent in others. Both bath stimulation protocols utilized elicit marked differences in the survival of neurons depending on whether STEP is expressed or not. WT cells can exhibit 42% more cell death than KOs (55% vs. 13%, respectively) following bath-mediated NMDA insults (Figure 6A). Given these results, it is important to ensure that STEP is naturally expressed in a reasonable portion of the general neuron population. If, for example, only 10% of hippocampal neurons express STEP in vitro, it would be challenging to explain how knocking out STEP can rescue up to 42% more neurons from cell death. Only the wildtype neurons that naturally express STEP are expected to show an effect when it is not present in the knockout cultures.

To measure the percentage of cells expressing STEP in mature hippocampal neurons in vitro, an indirect assessment was required. Because NeuN (a mature neuron marker) and STEP primary antibodies are both produced in mice, they cannot be applied to the same wells for direct comparison. Instead, mature (12 DIV) cultures were fixed and stained with either NeuN or STEP. An equal number of randomly acquired (nonselective for certain cell populations) photographs were captured. The total number of STEP-immunoreactive cells was compared with the total number of NeuN-positive neurons. As shown in Table 1, an average of 378±60 NeuN-positive and 335±12 STEP-positive cells were captured (n=2 wells each). Given that a large portion (88.6%) of neurons express STEP in vitro, the previously measured differences in excitotoxic cell death between WT and KO genotypes seem reasonable.
Given that STEP expression is neurotoxic to the general neuron population following bath applied NMDA insults, it was next investigated whether this is also true in a GABAergic subset that expresses STEP. Ninety-nine percent of hippocampal somatostatinergic interneurons express STEP \textit{in vivo} (Choi \textit{et al.}, 2007); it is hypothesized that a similar percentage is also present \textit{in vitro}. In order to examine whether this is also true in primary cell culture, 12 DIV neurons were fixed and stained for somatostatin and STEP. Coverslips were scanned for as many somatostatin-positive cells as could be detected. As Table 1 demonstrates, 163 out of 167 (97.6%) positively identified somatostatinergic neurons express STEP (n=2 wells). These data show that STEP is more highly expressed in the GABAergic subset than in the general neuron population (97.6\% vs. 88.6\%, respectively).

Also highlighted in Figure 7, somatostatin-immunoreactive cells are not the only cells that express STEP – many adjacent neurons also contain STEP (arrow, upper right panel), as previously published for STEP-immunoreactive hippocampal neurons \textit{in vivo} (Choi \textit{et al.}, 2007). These findings lend support to the hypothesis that \textit{in vitro} patterns of STEP expression are comparable to those found \textit{in vivo}. 

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Table 1: **STEP is enriched in somatostatinergic mature hippocampal neurons in vitro.** STEP is expressed in a high proportion (88.6%) of mature hippocampal neurons, based on comparisons of equal, randomly acquired images for NeuN and STEP.

It was previously found that STEP is even more prevalent in certain GABAergic subtypes. To demonstrate that STEP is enriched in somatostatinergic cells, expression was measured in different wells. Out of a total of 167 somatostatinergic cells identified, 163 (97.6%) express STEP (n=2 wells).
Figure 7: **Somatostatin and STEP expression colocalize in vitro.** Subpopulations of STEP-positive neurons are also immunoreactive for somatostatin in mature hippocampal neurons (12 DIV). STEP is expressed in the cell body of 98% of somatostatinergic interneurons (Table 1); somatostatin is found in both the soma and along processes, where it forms puncta (lower left panel). In confirmation with previous *in vivo* findings, STEP expression *in vitro* is not limited to somatostatinergic interneurons; many adjacent non-somatostatinergic neurons also express it (arrow). Scale bars: 50 µm.
Disparate levels of somatostatin are expressed in healthy versus dying interneurons

After confirming that most somatostatinergic cells express STEP, I next investigated whether an absence of STEP rescues these interneurons from NMDA-mediated excitotoxic cell death. STEP KO somatostatinergic populations are expected to survive more robustly when compared with WT somatostatinergic neurons. It is hypothesized that the subset’s vulnerability is directly mediated by the presence of STEP. Since a higher percentage of somatostatinergic cells express STEP than the general neuron population (Table 1), there should be a greater disparity in the levels of WT and KO somatostatinergic cell death, when compared with those found in the general neuron population. If the ratio of cell death were similar to that of Figure 5, it would be difficult to conclude whether STEP contributes more significantly to somatostatin interneuron excitotoxicity than it does to the general neuron population where it is expressed.

In the following experiment, WT and KO cultures were treated with 50µM NMDA for one hour. Assessments of cell death were measured twenty-four hours later by screening a coverslip specifically for somatostatinergic-immunoreactive neurons. 55 somatostatinergic interneurons were captured per treatment condition for subsequent scoring of their nuclei. Unexpected challenges during quantification have been highlighted in Figure 8. There are contrasting levels of somatostatin in dying (Figure 8B) versus healthy (Figure 8C) neurons 24 hours post-insult, possibly due to the termination of neuropeptide synthesis once cell death has begun. Figure 8A, taken at 20x magnification, captures a rare observation – three proximally located
somatostatinergic cells that differ in health. Two of the cells have pyknotic nuclei (Figure 8B, Hoechst panel) and a weak, diffuse pattern of somatostatin (Figure 8B, Som panel). At the same time, a nearby cell is healthy (Figure 8C, Hoechst panel) and prominently expresses somatostatin (Figure 8C, Som panel). Because photographs were acquired based on immunoreactivity (and thus inadvertently on their health), most of the images acquired were found to contain healthy somatostatinergic neurons. Very few expressed robust levels of the neuropeptide and had pyknotic nuclei. As a result of these difficulties, initial quantification yielded unrealistic levels of survival in both WT and KO cell cultures (data not shown). This difficulty and potential solutions are considered further in the Discussion section.
Figure 8: **Expression of somatostatin is highly dependent on the health of cells.** A, 20x-magnified image reveals three somatostatinergic cells in close proximity to each other, two of which are pyknotic (B, red square) and one that is healthy (C, blue square). Scale bar: 100µm. B, upon further magnification of the red-boxed portion, two of the cells have clearly pyknotic nuclei (Hoechst panel) and diffuse expression of somatostatin (Som panel). C, in contrast, reveals a neuron that is healthy and robustly expresses the neuropeptide.
**Discussion**

The tyrosine phosphatase STEP has been shown to regulate important cellular responses following neurotoxic insults. This investigation utilized an *in vitro* model of excitotoxicity to demonstrate that STEP contributes to neuronal cell death after certain insults. These findings are consistent with those first established by Choi *et al.* (2007) and subsequently by Braithwaite *et al.* (2008): STEP expression and regulation influence neuronal viability during periods of excitotoxicity. The current results demonstrate that an absence of STEP is beneficial to survival following bath application of NMDA. STEP’s neurotoxic influence, under the conditions used here, is acting specifically through NMDARs and not other glutamatergic receptor types. STEP may exert its role in hippocampal neurons specifically following the activation of extrasynaptic NMDA receptors. The assessment of somatostatinergic interneuron vulnerability, however, is inconclusive because the methods for quantifying general neuron cell death do not translate into this particular population (see Figure 8). Subsequent work will be required to determine whether this population is particularly vulnerable due to its expression of STEP.

**Evaluating cell death requires an optimized insult and reliable quantification technique**

Eliciting consistent, reliable excitotoxic responses, using either glutamate or NMDA, can be a challenging process. In order to optimize the insult, one can alter the time of exposure, the concentration of the excitotoxic agent, or the amount of time lapsed before assessing the cell death. Previous researchers that examined glutamate-induced cell death have published similar protocols (Almeida *et al.*, 2005; Xia *et al.*,...
1995; Liu et al., 2007). It is important to induce high enough levels of cell death in order to distinguish any significant differences between wildtype and knockout populations. A weak insult only triggers cell death in a small population and may show no difference between genotypes. Exposure to low concentrations of NMDA or glutamate and can even promote survival (Soriano et al., 2006).

The high levels of cell death measured here are attributable to a few critical factors. First, neurons utilized in this study were very mature, obtained from postnatal mice and grown for another 12 days in vitro. More mature neurons express more glutamate receptors on their surface, making them more vulnerable to these insults. Additionally, the cells in this investigation were exposed to excitotoxins for a full hour and were hippocampal, a neuronal type that is more sensitive, in general, than cortical and cerebellar neurons (Ahlemeyer and Baumgart-Vogt, 2005).

Figure 4B shows that the level of cell death peaked 24 hours after NMDA insults. This is based on the number of mature neurons which have pyknotic nuclei, a morphological characteristic of dead or dying cells. Many protocols rely upon alternative techniques for scoring cell death, which measure the prominence of biochemical markers that are associated with dying cells (LDH release and MTT levels, for example). These assays, however, are relatively insensitive and are impractical with cells cultured from P0.5 mice (Carrier et al., 2006). Due to the inherent presence of glia in postnatal cultures, quantification needed to ensure that only neurons were included in the levels of cell death. LDH release assays, for example, cannot distinguish between glia and neurons, as both cell types release LDH during cell death. The usage of NeuN, a mature neuronal marker, in combination with
the quantification of pyknosis, ensured that these results were particular to mature neurons and not inclusive of non-neuronal cell types.

Because dying neurons lose their antigenicity (Cevik-Unal et al., 2004), assessments that rely on neuronal markers must utilize them in a time-sensitive manner. Figure 4B suggests that the fewer cells are dead after 48 hours when compared with 12-24 hours – this is an artifact that is due to the loss of countable pyknotic nuclei because they are no longer NeuN-immunoreactive. This is confirmed by the fact that the overall number of cells quantified in wells treated at 12-24 hours was an average of 1,415, while only 976 were counted after 48 hours. The number of dead or dying neurons was also markedly lower, suggesting that the reduced total count neglects neurons that have died and lost their NeuN-immunoreactivity.

**STEP’s observed neurotoxic effect: Is it specifically modulated via NMDA receptor activation?**

STEP knockout neurons are significantly less vulnerable to bath applied NMDA-induced excitotoxicity, when compared with wildtype cells. This was found using two different protocols for the bath application of NMDA (Figures 5 and 6A). The first provided a longer, more severe insult (1 hour) that yielded a three-fold better survival in the STEP KO cells (p<0.001). The second provided a shorter (15 minute) insult, which elicited a similar profile of cell death in the two genotypes. No differences in cell death were apparent, however, when bath application of exogenous glutamate was measured. While it was a goal to isolate the role of NMDARs, a particular glutamate receptor subtype, there is an issue to consider with the glutamate
stimulation results. These findings did not show any rescue in STEP KO neurons following a severe glutamate insult: the levels of cell death in WT and KO cell populations averaged 95% and 97%, respectively. Treatment with glutamate elicits a broader, more lethal response because it leads to the activation of a more diverse group of receptors and subsequent death mechanisms (Mandir et al., 2000). Similarity between the amount KO and WT cell death could reflect the fact that many of the death pathways elicited under these circumstances would be independent of STEP. Thus STEP’s absence in knockout neurons would be less germane to the levels of cell death quantified. The amount of cell death in both populations is so high, however, that it is not possible to confidently state whether or not there is a “ceiling” effect due to the severity of the insult. Subsequent work will attempt to reduce the total cell death by performing a time series assay, treating cells for 5 minutes to 30 minutes to determine an optimal time of exposure.

Treatment with NMDA is less excitotoxic in neurons lacking STEP, while differences are not measured following severe glutamate insults. What if the levels of glutamate-induced excitotoxic cell death are still equal in WT and KO populations following a less severe insult? It would suggest that STEP modulates a neurotoxic effect particularly following NMDA channel activation. This is reasonable, considering that STEP is activated by the calcium-dependent phosphatase, calcineurin (Paul et al., 2003; Choi et al., 2007). Knocking out STEP, or inhibiting it, could be neuroprotective on many levels, both through altered receptor distributions in the KOs, as well as upregulation of basal levels of pERK. The location of NMDARs in knockout hippocampal cells is still uncertain, but it has been shown that STEP KOs
exhibit an overall increase in NMDAR surface expression (Snyder et al., 2005). If this augmentation were at synaptic sites, the result would increase pERK signaling and could improve survival following excess glutamate release. Both the location and composition of NMDARs may be altered in knockout cells and should be evident by different patterns of calcium influx, when compared with wildtype neurons.

The divergent function of NMDARs has led to reformed concepts to address their differential expression and regulation. It is still unclear what makes these populations so distinct in function – some work has suggested that calcium transients can generate local “microdomains” near the site of entry (Hardingham et al., 2001). It seems plausible that a key difference in NMDAR populations involves the integration and regulation of synaptic populations as part of the postsynaptic density. This massive array of cytoskeletal and regulatory proteins could position and organize NMDARs near other enzymes, like STEP and ERK, in a manner that is absent where extrasynaptic receptors reside. Yet the idea is still largely unproven, as the elucidation of the contrasting role of synaptic and extrasynaptic NMDARs has only emerged in the last decade. The attenuated response of knockout cells to extrasynaptic stimulation implicates STEP as a contributor to some of the pro-death responses previously published (Hardingham et al., 2002; Ivanov et al., 2006; Lee et al., 2005). These results are currently tenuous and will require more trials to reduce the variation in WT levels of cell death.

A cleaved form of STEP emerges following extrasynaptic NMDAR stimulation, contributing to evidence that STEP, like many proteins, is alternatively modulated by synaptic and extrasynaptic receptors. Production of the STEP$_{33}$ isoform,
by the calcium-dependent cysteine protease calpain, has unknown consequences inside the cell (Nguyen et al., 1999). STEP33 is not bound to membrane compartments, allowing it to circulate within cytosolic regions and potentially interact more promiscuously with new targets. Calpain-mediated cleavage of other proteins has been reported during glutamate-induced cell death (Xu, 2007; Wu et al., 2004; Kim et al., 2002). Wu et al. found that calpain cleaves calcineurin, the only established activator of STEP, to a constitutively active form that promotes neurodegeneration. So far, STEP33 has not been shown to regulate any of STEP’s known targets, including pERK, the NR2B subunit, or Fyn (Jian Xu, unpublished data). It is possible that STEP33 is an inert isoform, which results from the dysregulation of calpain following surfeit calcium influx.

It will be important to characterize whether STEP33 contributes to cell death by introducing it into cells and determining its effects under excitotoxic conditions. Additionally, one may consider utilizing STEP mutant constructs that cannot be cleaved by calpain (but are still active). Comparison of the levels of cell death in neurons containing native STEP, versus either STEP33 or mutant constructs, may elucidate whether this cleavage process contributes to excitotoxicity. It is hypothesized that cells containing STEP33 will exhibit more cell death than the native isoforms (STEP61 and STEP46), due to a loss of regulation and aberrant activity in the cytosol. No differences would suggest that STEP33 production does not directly mediate any excitotoxic response, but is instead a byproduct that results after cells lose control of intracellular calcium levels.
Difficulties arise when evaluating somatostatinergic neuron vulnerability

Challenges have made the current quantification method unusable for examining whether somatostatinergic interneurons have different levels of pyknosis in knockout and wildtype populations (Figure 8). This drawback results from identifying somatostatinergic cells based upon their immunoreactivity. Healthy somatostatinergic cells from both WT and KO genotypes synthesize robust levels of the neuropeptide (Figures 7 and 8), while pyknotic cells are barely immunoreactive, potentially due to the cessation of neuropeptide synthesis after 24 hours (Figure 8B). With a limited number of these cells (estimated to be 60-80 per well) found spread out on a coverslip, the ability to perform random field acquisitions, as done with the general neuron assessment, is not practical – it would take several hundred photographs to complete a single coverslip. Quantification through a spectator-selective technique yields an inadvertent bias toward healthy neurons. Alternative mechanisms have been proposed to examine the vulnerability of somatostatinergic cell death. One possible way to offset the faint immunoreactivity of dead or dying neurons is to acquire images at an earlier time point before levels of the neuropeptide are depleted. These neurons are some of the earliest to stain with death markers (Robbins et al., 1991; Buckmaster and Jongen-Rélo, 1999; Choi et al., 2007). Instead of waiting until 24 hours to assess cell death, one may acquire images at 4 or 6 hours, before the production of somatostatin is substantially diminished. It is unknown whether there will be significant levels of pyknosis at this time, however, because nuclear condensation and fragmentation processes require some time to occur.
While methodological challenges have been addressed, it is also felt that a critical component of the Choi et al. (2007) findings should be discussed. Any neurotoxic effect of STEP would require it to be an active enzyme. Currently, the only known activator of STEP is the calcium-sensitive phosphatase calcineurin. An extensive study of calcineurin by Sik et al. (1998) found that it is not expressed in any inhibitory GABAergic cell populations. This investigation found calcineurin protein in principal excitatory cells, but not in interneurons (identified by their expression of GABA). If STEP contributes to somatostatinergic cell death, then how does it become active in these populations? The encountered dilemma suggests that STEP is regulated by an alternative, unidentified mechanism in interneurons. This would be a substantial finding, and would suggest that STEP has distinct regulatory roles in excitatory and inhibitory populations.

In summary, I have shown that STEP deficiency improves neuronal survival following NMDAR-mediated excitotoxic insults. The neurotoxic effects of STEP could be due to the formation of STEP$_{33}$ following calcium influx at extrasynaptic NMDARs. Mature somatostatinergic interneurons express STEP in vitro and may be particularly vulnerable to insults due to their enrichment with STEP. Additional work could further illuminate the current results by characterizing the STEP$_{33}$ isoform and determining whether it is an active protein or byproduct of aberrant calpain activity. Identifying whether there are alternative regulatory mechanisms for STEP in inhibitory versus excitatory neuron populations would also facilitate efforts to dissociate and better contextualize STEP’s role in mediating excitotoxic responses.
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