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Xiya Zhu April 09, 2013

Selective Stabilization of Synaptic Inputs onto Motoneurons by Postsynaptic trkB Receptors

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Abstract

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Following peripheral nerve injury, synaptic terminals withdraw from the cell bodies of motoneurons in the spinal cord in a process known as synaptic stripping. Stripping is thought to be the result of a decrease in the release of brain derived neurotrophic factor (BDNF) from injured motoneurons. This decrease in binding of motoneuron BDNF to trkB receptors on synaptic terminals results in their withdrawal, implying that this retrograde signaling maintains synaptic contacts. However, trkB receptors are plentiful on BOTH motoneurons and on synapses onto them. We hypothesize that the motoneuron trkB receptors play a role in the maintenance of synaptic inputs onto motoneurons. In male and female mice in which the gene for trkB was knocked out selectively in a subset of motoneurons, the proportion of the motoneuron somata contacted by excitatory (VGLUT1+, VGLUT2+) and inhibitory (GAD67+, glycine+) synaptic inputs was measured. Results from wild type (WT) and trkB knockout (KO) cells were compared. Simply eliminating expression of the trkB receptor in motoneurons resulted in a significant reduction in VGLUT2+, GAD67+, and glycine+ synaptic contacts but had no effect on the magnitude of excitatory VGLUT1+ synaptic inputs. The magnitude in the reduction in synaptic inputs was nearly twice as great for inputs immunoreactive for GAD67 as for other inputs. The reduction in synaptic coverage by VGLUT2 and glycine was much greater in males than in females, whereas no sex difference was found in reduction of GAD67+ contacts. Thus, expression of trkB in motoneurons plays an integral role in the maintenance of inhibitory synapses and this role is different in males and females. Motoneuron BDNF, acting in an autocrine/ paracrine manner through these postsynaptic receptors, could have a self amplifying effect to stabilize synaptic terminals.

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Introduction

Peripheral nerve injuries are common. There are several hundred thousand new injuries in the US every year. Despite the fact that axons in injured peripheral nerves can regenerate and reinnervate their targets, functional recovery is very poor and often results in some sort of permanent disability or discomfort (Brushart, 1998; Frostick et al., 1998). The reasons most often given for poor functional recovery include slow axon regeneration times (Fu and Gordon, 1995, 1997; Gordon, 2009), reinnervation of the inappropriate targets by the regenerating motor axons (Evans et al., 1991; de Ruiter et al., 2008), and changes in circuitry of the central nervous system (CNS). Among these CNS changes is synaptic stripping, the withdrawal of synaptic inputs from the cell bodies of axotomized motoneurons following peripheral injury (Blinzinger and Kreutzberg, 1968). Both excitatory and inhibitory synapses are withdrawn, beginning 3-5 days following injury (Lindå H, 1992). Over time, many of the withdrawn terminals are restored, regardless of whether axon regeneration is successful (Alvarez et al, 2010). However, the withdrawal of some synaptic terminals from motoneurons, especially those originating from primary afferent neurons is permanent, (Alvarez et al, 2010). Therefore, synaptic stripping could be an important contributor to the poor functional recovery seen in patients with peripheral nerve injury.

Synaptic stripping has been found to occur on brainstem motoneurons after transection of the facial nerve (Blinzinger and Kreutzberg, 1968), which do not contain sensory axons, suggesting that the withdrawal of terminals might be due to changes in the properties of the axotomized motoneuron. Specifically, the stripping of terminals has been postulated to be the result of decreased availability of retrograde neurotrophic

molecules from the axotomized motoneurons (Titmus and Faber, 1990). Neurotrophins are a family of four proteins essential to the survival, development, and function of neurons. They include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). The most widely studied of the neurotrophins is BDNF. It is secreted from the somata and proximal dendrites of motoneurons (Thoenen, 1995) and could help stabilize synaptic terminals through retrograde signaling, by binding to its receptor, tropomyosin related kinase B (trkB), on presynaptic terminals (Davis-Lopez de Carrizosa et al., 2009a). Injury to their axons results in very short increase in motoneuron BDNF synthesis followed by a marked and prolonged decrease (Gordon, 2009). It has been hypothesized that such a decrease in neurotrophic support leads to synaptic stripping (Titmus and Faber, 1990). Consistent with this theory, in intact mice, there is a 50% loss of synaptic coverage onto the motoneuron somata if BDNF was knocked out conditionally in motoneurons (Krakowiak et al., 2010). Synaptic stripping was not observed when BDNF was applied continuously to the proximal stump of a cut nerve (Davis-Lopez de Carrizosa et al., 2009a,b).

According to the hypothesis described above, BDNF acts as a retrograde signal by release from motoneurons and binding to its trkB receptor on synaptic terminals making contact with motoneurons. However, in addition to their location on synaptic terminals, trkB receptors also are found abundantly on the post-synaptic motoneurons. Thus BDNF secreted from motoneurons could act both as a direct retrograde signal to stabilize synaptic terminals and by binding to trkB receptors on the same or surrounding motoneurons. The importance of this autocrine or paracrine signaling pathway in

synaptic stabilization is unknown. One goal of this project was to investigate the role of this signaling pathway on synaptic stability using mice in which the trkB receptor is knocked out selectively in motoneurons and not in the synaptic terminals onto the motoneurons. BDNF has been shown to act as an autocrine factor for maintaining neuronal survival during target-independent stages of development (Davies and Wright 1995). Additionally, BDNF serves as a self-amplifying autocrine factor in promoting axon formation in embryonic hippocampal neurons (Cheng et al., 2011). Thus motoneuron BDNF, acting in an autocrine/ paracrine manner through trkB postsynaptic receptors, could have a self amplifying effect to stabilize synaptic terminals. I hypothesize that the elimination of motoneuron trkB receptors will result in a withdrawal of synaptic terminals.

Testosterone is known to be an important regulator of both BDNF and trkB expression in neurons (Osborne et al., 2007; Verhovshek et al., 2010). Estrogen has also been shown to regulate the expression of BDNF mRNA and protein in the rat hippocampus (Solum et al., 2002). Given these hormonal influences on BDNF and trkB expression, one might hypothesize that the effects of peripheral nerve injury-induced synaptic stripping might be different in male and female mice. A second goal of this project was to investigate sex differences in the role of trkB receptors in synaptic maintenance. Selective elimination of trkB expression in motoneurons may lead to a differential amount of synaptic withdrawal in males compared to females.

Motoneurons receive synaptic inputs from different sources and synapses from different sources have different properties. Some inputs to motoneurons are excitatory and some are inhibitory. Some are from primary sensory axons in peripheral nerves

and others are from neurons within the CNS. In the cerebellum, hippocampus, and cortex, BDNF-trkB signaling has been associated with the specific development of inhibitory synaptic inputs (Rutherford et al. 1997; Marty et al. 2000; Seil and Drake-Baumann 2000). Thus, a third goal of this study was to investigate the composition of synaptic inputs to motoneuron from different sources in motoneuron-specific trkB knockout mice. Two types of excitatory synapses and two types of inhibitory synapse were studied in female and male mice.

Methods

Animals:

The mice used in the following experiments were adult females and males (>2 months old) weighing 21 to 26 grams. These mice were SLICK::trkB$^{f/f}$ mice bred using the *Cre/lox* system. Because mice made null for trkB at the time of conception do not survive (Klein et al., 1993), conditional gene knockout was performed. Mice with loxP sites flanking the coding region of the trkB gene (trkB$^{f/f}$) were bred with mice expressing a tamoxifen-inducible Cre and Yellow Fluorescent Protein (YFP) under the control of the *thy-1* promoter, a construct that directs expression to a subset of motoneurons and a small number of other neurons. These latter mice are known as SLICK (single-neuron labeling with inducible Cre-mediated knock-out) (Young et al., 2008). In one group of the resulting SLICK::trkB$^{f/f}$ mice, Cre recombinase was activated by two bouts of treatments of three days of oral treatment with the synthetic estrogen, tamoxifen, separated by two weeks. This treatment regimen has been shown to result in neuron-specific expression of Cre (Wilhelm et al, 2012). Once Cre is expressed, elimination of the trkB gene in motoneurons marked by the presence of YFP follows. Motoneurons not expressing YFP (or Cre) are assumed to be trkB+. Nearly all of the afferent axons forming synapses onto motoneurons do not express YFP and also are assumed to be trkB+. A second group of SLICK::trkB$^{f/f}$ mice was not treated with tamoxifen and acted as a control.

Retrograde Labeling:

In both experimental and control mice, the spinal motoneuron cell bodies were marked either through retrograde labeling (2 female mice), or by immunoreactivity to

NeuN (6 male mice), a neuronal nuclear antigen present in cytosol of all neurons. In retrograde labeling experiments, the gastrocnemius and soleus muscles of isoflurane anesthetized mice were injected with 1μL each of the beta subunit of cholera toxin B conjugated to Alexafluor 594 (1mg/1mL) using a 35G injection needle. Injections were made bilaterally. Three days after muscle injections, the mice were euthanized with a lethal dose of pentobarbital (150mg/kg) and perfused with saline and paraformaldehyde fixative. The spinal cord segments L3 through L5 were harvested and cryoprotected in 20% sucrose overnight. The spinal cords were cut transversely into 20 μm thick sections using a cryostat and mounted on Superfrost Plus slides.

Immunohistochemistry:

To visualize the synaptic terminals onto motoneurons, immunofluorescence staining was conducted by reacting separate sets of sections with antibodies either to GAD67, VGLUT1, VGLUT2, or VGAT. Glutamate decarboxylase 67 (GAD67) is an enzyme in the synthetic pathway for the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). The vesicular GABA transporter (VGAT) functions in the loading of synaptic vesicles containing both GABA and the inhibitory amino acid transmitter, glycine (McIntire et al., 1997). All GAD67+ terminals also contain VGAT. Terminals that contain VGAT but not GAD67 contain only glycine (Chaudhry et al., 1998). There are three vesicular glutamate transporters which function in the loading of synaptic vesicles containing the excitatory transmitter, glutamate. The overwhelming majority of terminals in the spinal cord containing the VGLUT1 isoform are from primary afferent neurons (Hughes et al., 2004). Synaptic terminals containing vesicular

glutamate transporter 2 (VGLUT2) are excitatory synaptic inputs from interneurons (Todd et al. 2003).

Sections were first incubated for 1 hour at room temperature in buffer containing 0.1M phosphate-buffered saline (PBS) with 0.4% Triton X (PBS-T) and 10% normal goat serum (NGS). Following pre-incubation, the tissues were incubated in different primary antibodies overnight in a humid chamber at 4°C. The primary antibodies used were either mouse monoclonal anti-GAD67 (1:200), rabbit polyclonal anti-VGLUT1 (1:2000), rabbit polyclonal anti-VGLUT2 (1:200), or mouse monoclonal anti-VGAT (1:200). The GAD67 and VGAT antibody binding was followed by incubation with a goat anti-mouse secondary antibody conjugated to Alexafluor 647 (1:200). The binding of VGLUT1 and VGLUT2 antibodies were detected by goat anti-rabbit secondary antibody conjugated to Alexafluor 647 (1:200). Retrograde labeling of the motoneurons was enhanced with rabbit anti-cholera toxin B (1:200) followed by a goat anti-rabbit secondary antibody conjugated to Alexafluor 546 (1:200). Motoneurons without retrograde labeling were reacted with either mouse or rabbit anti-NeuN (1:200), followed by goat anti mouse or goat anti rabbit secondary antibody conjugated to Alexafluor 546. Washes were performed with 0.1M PBS. All slides were cover slipped with Entellan.

Image Analysis:

Images of thin (<1μm) optical slices of spinal cord sections were obtained using a Zeiss LSM510 confocal microscope at a magnification of 63x. Cell bodies of trkB knockout (KO) motoneurons innervating hind limb muscles were identified by the presence of both YFP (green) and NeuN or cholera toxin B (red). Cell bodies of

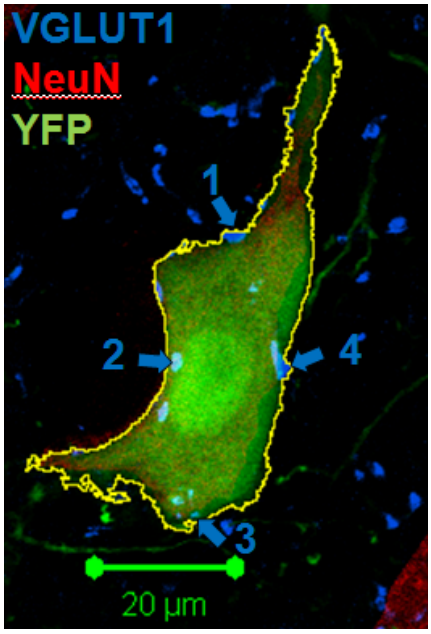
motoneurons containing trkB receptors, no YFP, and the presence of NeuN or cholera toxin B were considered wild type (WT). Images of 10 KO motoneuron cell bodies and 10 WT cells were obtained from each side of the spinal cord for GAD67, VGAT, VGLUT1, and VGLUT2 visualized with far red (647nm) optics. In the wild type control group (SLICK::trkB$^{f/f}$ mice not treated with tamoxifen), 20 WT cells were selected from each side of the spinal cord for study. From these images, the coverage of the motoneurons by different types of synaptic inputs was measured using the computer program FIJI. A region of interest (ROI) was created around the perimeter of each labeled motoneuron somata and very most proximal dendrites in the red channel of the RGB images, using the thresholding function of the software. A plot profile, indicating the intensity of fluorescence beneath ($\pm$1 micrometer) this outline was then generated from the blue channel of the RGB image, the component obtained using far red optics. Mean fluorescence intensity in the blue channel within the area bounded by the ROI was determined and used to determine an intensity threshold of the fluorescence intensity within the cell. Profile plot values greater than this threshold were assumed to indicate contact of the motoneuron soma by immunoreactive synaptic terminals (Figure1). The proportion of the entire cell perimeter so contacted was found for each cell and expressed as percent synaptic coverage.

Statistics:

Percent coverage in WT and KO cells in male and female tamoxifen-treated SLICK::trkB$^{f/f}$ mice and in mice not treated with tamoxifen was subjected to a one way analysis of variance (ANOVA). Data from each of the five synaptic types were

compared separately. In all cases the omnibus test of the ANOVA was significant (Table 3), so post-hoc paired testing (Tukey's Honest Significant Differences, HSD) was conducted. Probabilities of <0.05 were considered significant.

A.



B.

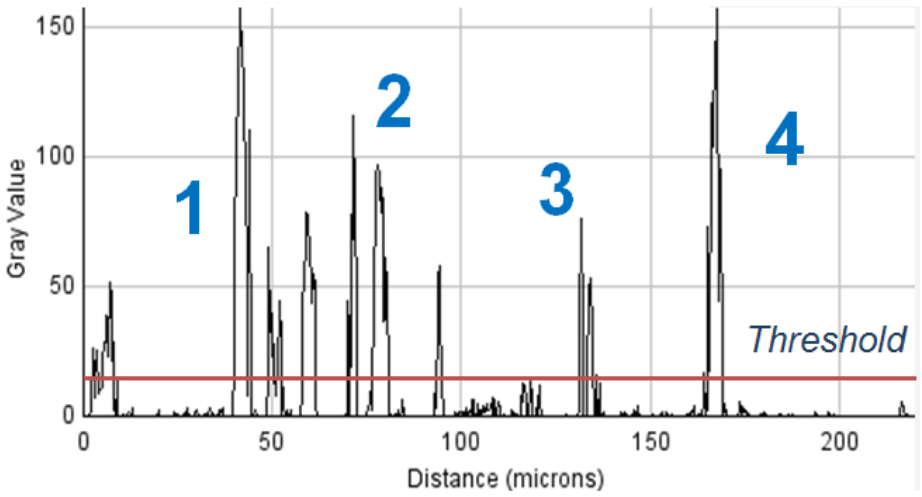


Figure 1. (A) Single knockout (KO) motoneuron with immunopositive VGLUT1 synaptic terminals. Numbers correspond to numbers on profile plot to indicate presence of synaptic terminals. Yellow outline is the region of interest (ROI) (B) Profile plot: Numbers indicate location of synaptic inputs as they intersect with ROI. Red bar denotes threshold level.

Results

The contacts made by synaptic terminals from different sources were studied using immunofluorescence analysis of identified motoneurons that either contain the trkB receptor or in which the receptor was knocked out. Images of examples of such wild type (WT) and trkB knockout (KO) motoneurons and contacts on them made by different synaptic terminals are shown in Figure 2.

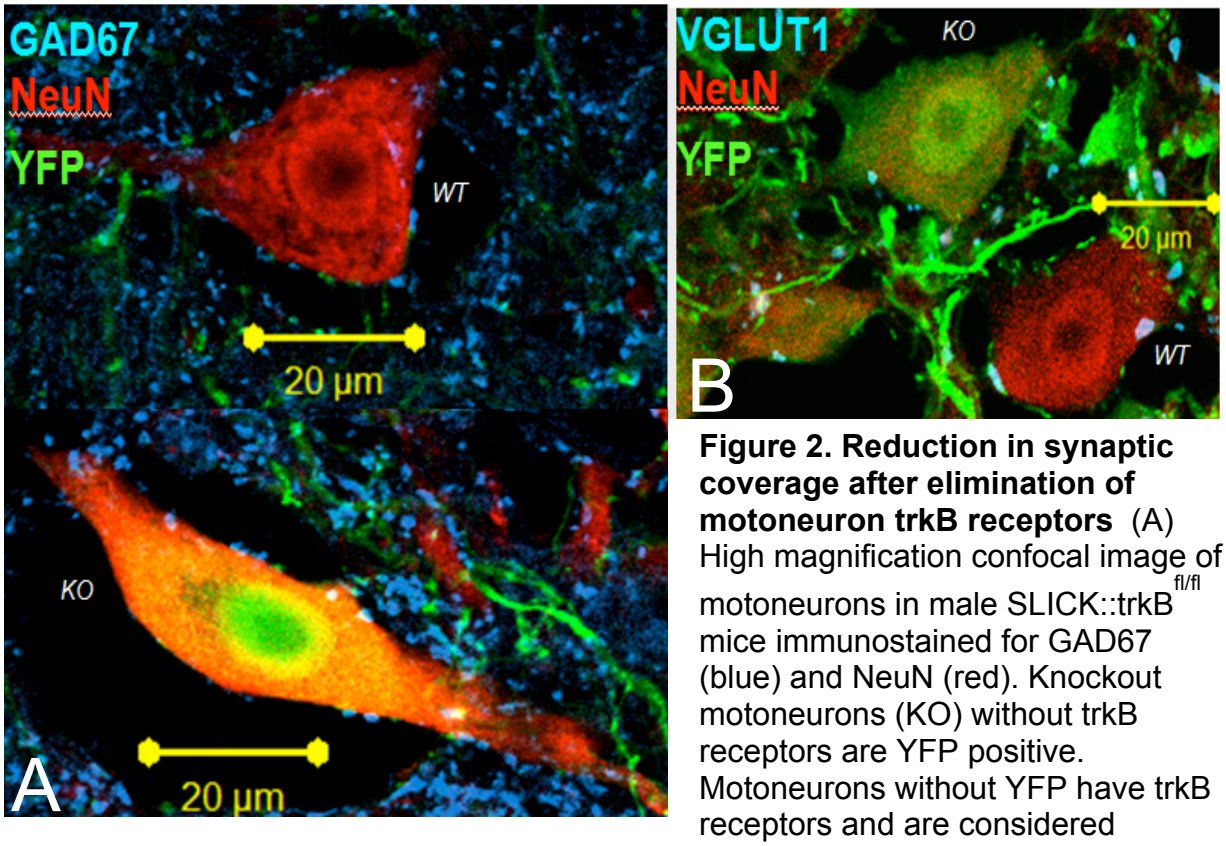


Figure 2. Reduction in synaptic coverage after elimination of motoneuron trkB receptors (A) High magnification confocal image of motoneurons in male SLICK::trkB$^{fl/fl}$ mice immunostained for GAD67 (blue) and NeuN (red). Knockout motoneurons (KO) without trkB receptors are YFP positive. Motoneurons without YFP have trkB receptors and are considered wildtype (WT). (B) KO and WT motoneurons immunostained for VGLUT1 (blue) and NeuN.

VGLUT1+ synapses in trkB-KO mice

The cumulative distribution of percent synaptic coverages by excitatory terminals containing VGLUT1 on the somata and proximal dendrites of all labeled motoneurons in male and female SLICK::trkB$^{f/f}$ mice is shown in Figure 3A. In males, the distributions for WT and KO cells are virtually identical, and this similarity is reflected in the mean coverages of different animals (mean =11.69%±0.09% SEM in WT cells, 11.54%±0.10% SEM in KO cells, HSD, p=0.99). In females (Fig 3A: right), the distribution of VGLUT1 synaptic coverages from KO cells is shifted to the left of that for WT cells, suggesting that a reduction of synaptic coverage was found in the absence of neuronal trkB. However, differences in mean percent coverage (WT: 11.86%±0.43% SEM, KO: 10.52%±0.45% SEM) were not statistically significant (HSD, p=0.0502). The percent coverage by VGLUT1+ terminals in WT cells from tamoxifen treated male mice (mean=11.69%±0.09% SEM) was similar to that found in WT mice (male SLICK::trkB$^{f/f}$ mice not treated with tamoxifen) (11.84%±0.26% SEM, HSD, p=0.99), demonstrating that tamoxifen treatment had no effect on synaptic coverage. The mean percent synaptic coverage (±SEM) by VGLUT1+ terminals between male and female mice is compared in Figure 4. No significant difference in VGLUT1 coverage was observed between male and female WT motoneurons (HSD, p=0.995), nor between male and female KO motoneurons (HSD, p=0.102).

VGLUT2+ synapses in trkB-KO mice

The cumulative distribution of percent synaptic coverages by immunopositive excitatory VGLUT2+ inputs originating from interneurons, from data from all

motoneurons in tamoxifen treated SLICK::trkB$^{f/f}$ male and female mice is shown in Figure 3B. In males, the distribution of synaptic coverages by VGLUT2 from KO cells is shifted to the left of that for WT cells, and this reduction in synaptic coverage in KO cells is significant (KO: mean=17.64%±0.22% SEM, WT: mean=20.91%±0.20% SEM, HSD, $p<0.0002$) . Similarly, in females (Figure 3B: right), the distribution of synaptic coverages for KO cells is shifted to the left of that for WT cells, reflecting a significant reduction (KO: mean=20.79%±0.53% SEM, WT: mean=24.36%±0.03% SEM, HSD, $p<0.0007$). The mean percent coverage (±SEM) by VGLUT2+ inputs is significantly different between male and female KO motoneurons (HSD, $p<0.0004$), as shown in Figure 4. However, there is no significant difference in VGLUT2 synaptic coverage between male and female WT motoneurons (HSD, $p=0.12$).

GAD67+ synapses in trkB-KO mice

The cumulative distribution of percent synaptic coverages by inhibitory inputs containing GAD67, as derived from all motoneurons in male and female mice, is shown in Figure 3C. In males (Fig. 3C: left), the shift in the distribution of synaptic coverages for KO cells to the left of that for WT cells reflects a significant reduction in GAD67 coverage after motoneuron trkB elimination (KO: mean=11.16%±0.37% SEM, WT: mean=16.04%±0.25% SEM, HSD, $p<0.0002$). The shift in synaptic coverage distribution to the left for KO cells is further observed in females, with the reduction in mean synaptic coverage from WT to KO also being significant (KO: mean=10.85%±0.15% SEM, WT: mean=15.49%±0.08% SEM, HSD, $p<0.0002$) (Fig. 3C: right). No sex difference in GAD67 synaptic coverage was found between male and

female KO motoneurons (HSD, p=0.88), or between male and female WT motoneurons (HSD, p=0.65) (Figure 4).

Glycine+ synapses in trkB-KO mice

The cumulative distribution of percent synaptic coverages by inhibitory VGAT+ inputs in all motoneurons from male and female mice is shown in Figure 3D. In both males and females, the distribution for KO cells is shifted to the left of that for WT cells, suggesting a decrease in synaptic coverage by terminals containing VGAT. In males, the mean percent synaptic coverage decreased from 36.16%±0.18% SEM in WT cells to 28.68%±0.09% SEM in KO cells. In females, the mean percent synaptic coverage decreased from 36.87%±0.46% SEM in WT cells to 31.00%±0.40% SEM in KO cells. Because terminals that contain VGAT contain both GAD67 and glycine, the mean percent coverage by GAD67 was subtracted from the mean percent coverage by VGAT to calculate the percent synaptic coverage by only glycine+ inputs. These data are represented in Figure 4. A significant reduction in coverage by glycine+ inputs (HSD, $p<0.0006$) is noted between male KO (mean=17.53%±0.41% SEM) and WT (mean=20.13%±0.24% SEM) motoneurons. In contrast, coverage by glycine+ terminals in female KO motoneurons (mean=20.16%±0.51% SEM) is not significantly different compared to the measurement found in female WT motoneurons (mean=21.35%±0.41% SEM; HSD, p=0.24). Additionally, there is a significant sex difference in coverage by inputs containing glycine only between KO motoneurons (HSD, $p<0.0027$), but not between WT motoneurons (HSD, p=0.22).

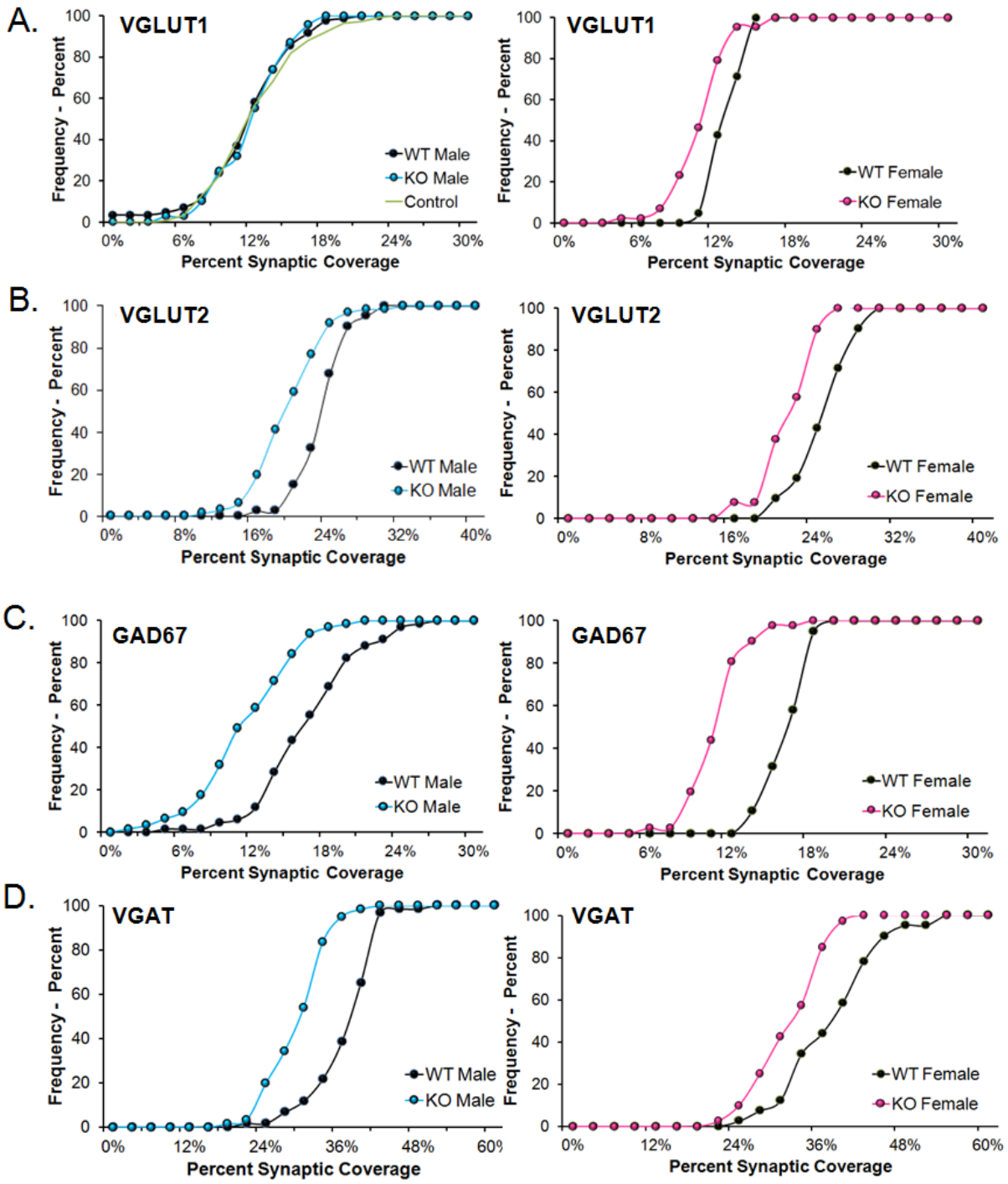


Fig. 3. Cumulative frequency plot of percent coverage by synaptic terminals onto the soma and proximal dendrites of labeled motoneurons in tamoxifen treated SLICK::trkB$^{f/f}$ male and female mice. WT refers to wildtype motoneurons without YFP that still retain trkB receptors. KO refers to knockout motoneurons labeled with YFP that do not have trkB receptors. Data from all the mice in each group were pooled. (A) Percent coverage by excitatory VGLUT1+ inputs. Control refers to motoneurons from male SLICK::trkB$^{f/f}$ mice not treated with tamoxifen. No significant difference in percent synaptic coverage found between WT Male and Control. (B) Percent coverage by excitatory VGLUT2+ inputs. (C) Percent coverage by inbitory GAD67+ inputs. (A) Percent coverage by inhibitory VGAT+ inputs.

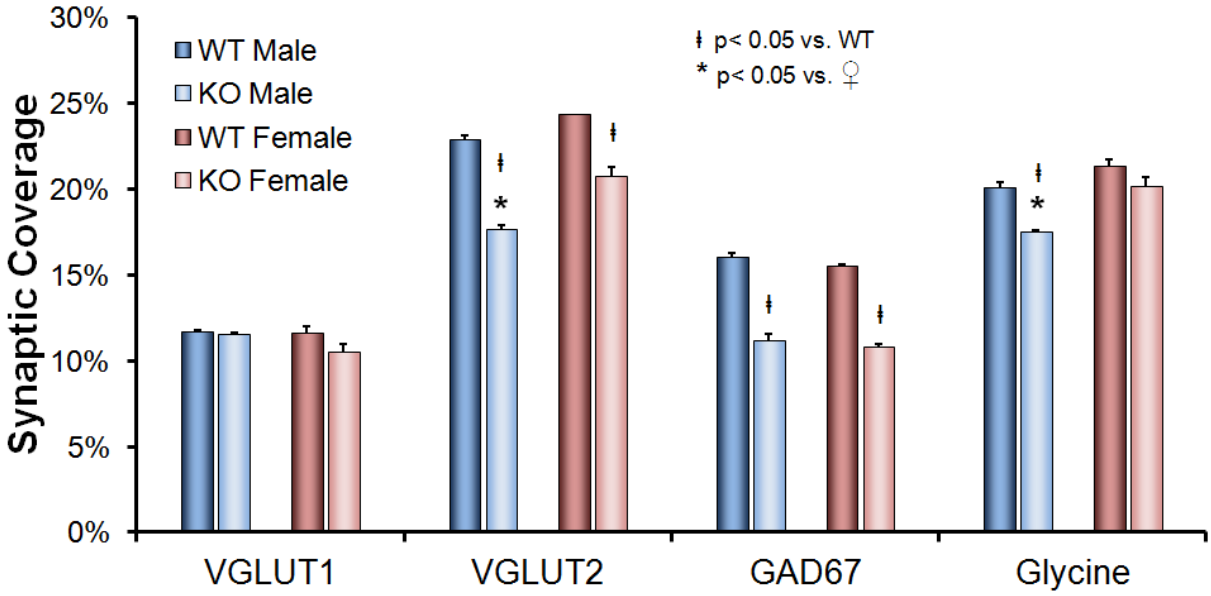


Fig. 4. Synaptic terminals are withdrawn in motoneuron-specific trkB KO mice. Mean percent synaptic coverage (±SEM) by synaptic terminals containing VGLUT1, VGLUT2, GAD67, or glycine in male and female tamoxifen treated SLICK::trkB$^{f/f}$ mice. This value is calculated by averaging data from individual mouse. Percent synaptic coverage by inputs containing glycine equals to percent coverage by VGAT minus percent coverage by GAD67. WT refers to wildtype motoneurons without YFP that still retain trkB receptors. KO refers to knockout motoneurons labeled with YFP that do not have trkB receptors. Significant sex differences in synaptic coverage are noted. No significant reduction found in VGLUT1.

Difference in percent synaptic coverage by inhibitory vs. excitatory terminals

The proportional reduction in mean percent synaptic coverage (±SEM) from WT to KO motoneurons by synaptic terminals containing VGLUT1, VGLUT2, GAD67, or glycine in male and female mice is shown in Figure 5. Data analysis was conducted using Fischer's LSD (Least Significant Difference). Probabilities of <0.05 were considered significant. In both males and females, the magnitude of reduction in GAD67 coverage is significantly greater than the reduction in coverage by VGLUT1 (LSD, Male: p<2.1E-07; Female: p<1.37E-06), VGLUT2 (LSD, Male: p<0.02; Female: p<8.38E-05), and glycine (LSD, Male: p<5.88E-07; Female: p<4.21E-08). When calculating the percent difference in synaptic reduction by pooling the data from both sexes, the magnitude of reduction by inhibitory contacts containing GAD67 and glycine

(40.22%) is almost twice the magnitude of reduction by excitatory contacts containing VGLUT1 and VGLUT2 (23.45%). These results point to a preferential depletion of inhibitory synapses in the absence of motoneuron trkB. Significantly less reduction in VGLUT1+ contacts is observed in males than in females (Males: -1.23%±1.48% SEM; Females: -9.70%±3.84% SEM; LSD, $p<0.01$). However, the decrease in coverage by VGLUT2 (Males: -23.00%±0.33% SEM; Females: -14.66%±2.12% SEM; LSD, $p<0.02$) and glycine (Males: -12.94%±1.62% SEM; Females: -5.38%±3.94% SEM; LSD, $p<0.02$) is significantly more in males than in females. There is no difference between males and females in the amount of decrease in GAD67+ inputs (Males: -30.45%±2.03% SEM; Females: -5.38%±3.94% SEM; LSD, $p=0.91$). A sex difference is therefore present in the withdrawal of certain types of synapses when trkB is selectively eliminated from postsynaptic motoneurons.

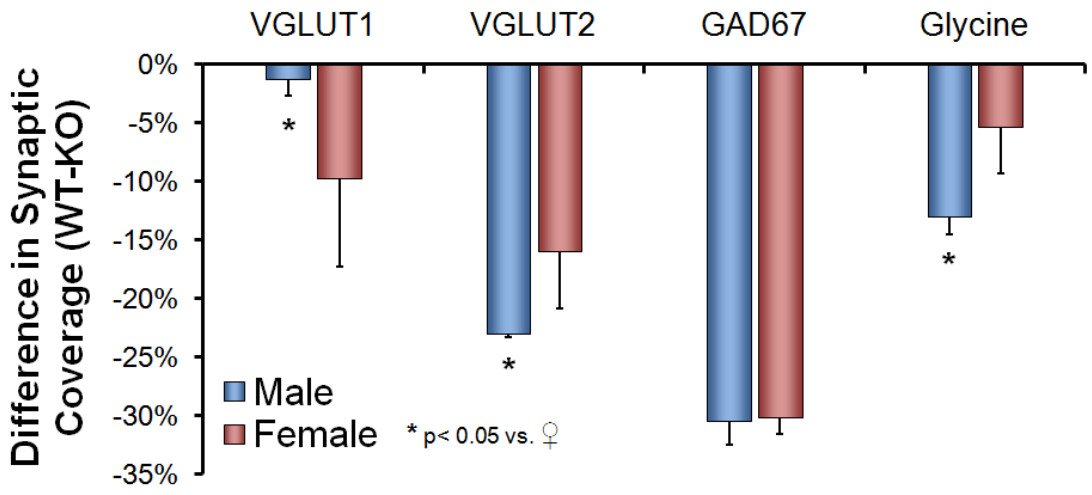


Fig. 5. Difference in mean percent synaptic coverage (±SEM) from WT to KO motoneurons by synaptic terminals containing VGLUT1, VGLUT2, GAD67, or Glycine in male and female tamoxifen treated SLICK::trkB$^{f/f}$ mice. WT refers to wildtype motoneurons without YFP that still retain trkB receptors. KO refers to knockout motoneurons labeled with YFP that do not have trkB receptors. Significant sex difference in reduction is noted for VGLUT1, VGLUT2, and glycine. No sex difference found in GAD67 reduction.

Discussion

After peripheral nerve injury, functional recovery in patients is often very poor. I have speculated that changes in circuitry of the central nervous system (CNS) following injury in the periphery could contribute to this poor recovery. One of these changes is the withdrawal of synaptic terminals from the somata and proximal dendrites of the motoneurons, in a process known as synaptic stripping (Blinzinger and Kreutzberg, 1968; Oliveira et al., 2008). This withdrawal process has been proposed to be due to a lack of neurotrophic support from the injured motoneurons (Titmus and Faber, 1990). In support of this hypothesis, in injured motoneurons treated with neurotrophins such as BDNF and NT-3, the effects of axotomy-induced synaptic stripping are reversed (Davis-Lopez de Carrizosa et al., 2009a,b). Secreted BDNF from motoneuron soma and proximal dendrites is thought to maintain synaptic terminals onto motoneurons through retrograde signaling by binding to its receptor trkB on presynaptic terminals (Davis-Lopez de Carrizosa et al., 2009a). Many trkB receptors are also located on the postsynaptic motoneurons themselves, but how these receptors are involved in stabilizing synapses is unclear. Investigating the role of motoneuron trkB receptors in synaptic maintenance was the main goal of this study.

The main finding of this study is that when trkB receptors are eliminated from motoneurons, a significant withdrawal of synaptic terminals is found. Contacts containing VGLUT2, GAD67, and glycine were reduced significantly on the somata and proximal-most dendrites of motoneurons lacking the trkB receptors, relative to those found on WT motoneurons in tamoxifen treated SLICK::trkB$^{f/f}$ mice. These results suggest that postsynaptic motoneuron trkB receptors play an integral role in the active maintenance of synaptic contacts onto motoneurons. The binding of BDNF to those

trkB receptors on motoneurons can signal changes in downstream gene expression of molecules involved in synaptic maintenance. One possible change is an increase in production of BDNF, which can stabilize synapses through retrograde signaling. Another possibility is that BDNF-trkB binding leads to an increase in production of synapse cell adhesion molecules. A reduced expression of cell adhesion molecules such as Netrin G-2 ligand has been speculated to be involved in synaptic stripping in motoneurons (Berg et al., 2010). The elimination of motoneuron trkB receptors may have resulted in a decreased availability of BDNF or cell adhesion molecules, causing synaptic withdrawal to occur.

Although my findings indicate that signaling through the binding of BDNF to motoneuron trkB is necessary in maintaining synaptic contacts onto motoneuron soma and proximal dendrites, the source of the BDNF is unknown. BDNF is present and secreted not only by the postsynaptic motoneuron but also presynaptic terminals. I therefore propose two possible mechanisms of synaptic stabilization involving the BDNF binding to motoneuron trkB, either from the postsynaptic motoneuron or from presynaptic terminals. Secretion of BDNF by presynaptic terminals could bind to postsynaptic trkB receptors on motoneurons (an anterograde signal), which then stimulates the production of a retrograde signal back to presynaptic terminal to stabilize synapses. To determine the plausibility of this mechanism, one could selectively eliminate presynaptic BDNF from different afferent neurons containing GAD67, glycine, or VGLUT2. If synaptic stripping resulted, then the hypothesis that presynaptic BDNF functions in maintaining synaptic contacts by binding to postsynaptic motoneuron trkB receptors would be supported. A second possible mechanism is that secretion of

motoneuron BDNF results in its binding trkB receptors on the same or neighboring motoneuron in an autocrine and/or paracrine manner and this binding induces the production of more BDNF in the motoneuron leading to a self-amplification effect. This additional BDNF can then be secreted from the soma and proximal dendrites and retrogradely bind to presynaptic trkB receptors to stabilize synapses (Figure 6). To evaluate this mechanism, one could measure the change in BDNF levels in postsynaptic motoneurons in trkB KO mice. If BDNF levels decrease in the absence of motoneuron trkB receptors, then the reason is likely due to the loss of the BDNF-trkB self-amplification pathway. Current literature supports the function of BDNF as a self-amplifying autocrine factor that elevates cytoplasmic cAMP and protein kinase A activity, which triggers further secretion of BDNF and membrane insertion of trkB (Cheng et al., 2011). The second mechanism that we proposed is more compatible with this finding. Therefore, we believe that synaptic terminals are actively maintained onto motoneurons by the binding of BDNF to trkB on motoneurons in an autocrine/paracrine pathway leading to the amplification of BDNF, which then stabilizes terminals through retrograde signaling.

In particular, I found evidence for a preferential withdrawal of inhibitory synaptic contacts following the elimination of motoneuron trkB. Comparing the amount of reduction by different types of synapses, the synaptic coverage by inhibitory terminals containing GAD67 had the greatest reduction from WT to KO motoneurons compared to all other types of synapses. In contrast, a significant change in coverage was not found at all by excitatory terminals containing VGLUT1. The reduction seen in excitatory synapses was mostly accounted for by the withdrawal of VGLUT2+ inputs. However,

the magnitude of reduction in coverage by GAD67+ inputs was almost twice of that by VGLUT2+ inputs. Taken together, these results provide strong evidence that postsynaptic motoneuron trkB plays a more prominent role in the maintenance inhibitory synapses than in excitatory ones. This finding is consistent with previous literature showing that BDNF-trkB signaling is associated with the specific development of inhibitory synaptic inputs in certain regions of the brain (Rutherford et al. 1997; Marty et al. 2000; Seil and Drake-Baumann 2000). Motoneurons are contacted by synaptic inputs from a heterogeneous source of neurons. The maintenance of synaptic contacts by some neurons, such as primary afferent neurons containing VGLUT1 or a subset of interneurons containing VGLUT2, might not involve signaling through postsynaptic trkB receptors. Instead, stabilization of synapses containing VGLUT1 may require signaling through the binding of NT-3 to its receptor tropomyosin related kinase C (trkC), not BDNF and trkB. To further investigate this, an experiment can be conducted to see if selectively knocking out BDNF in motoneurons leads to a similar pattern of reduction of the different types of inputs.

Interestingly, I also find a sex difference in synaptic coverage in the absence of postsynaptic trkB. The reductions in contacts containing VGLUT2 and glycine in trkB KO motoneurons were significantly greater in male than in female mice (Figure 5). This indicates that the role of motoneuron trkB receptor in synaptic stabilization is different in males and females. Reasons for this sex difference are unknown, but could be due to androgen signaling, a known regulator of BDNF expression (Verhovshek et al, 2010). For example, in motoneurons of the rat lumbar spinal cord, androgens may alter the expression of BDNF mRNA, impairing the targeting of BDNF proteins to dendrites to

regulate synaptic signaling (Ottem et al., 2010). If increases in androgen receptor signaling can promote neuronal BDNF expression (Wood et al., 2012), leading to greater synaptic stabilization, it is a paradox that we see a greater reduction in synaptic coverage in male mice. However, this finding in males might be explained that in the absence of motoneuron trkB, more BDNF is available to bind instead to the low-affinity common neurotrophin receptor p75, which is known to initiate cell death (Casaccia-Bonnefil et al., 1999). To further elucidate the influences of sex hormones on synaptic composition, trkB KO male mice can be treated with the androgen receptor blocker flutamide and compared to trkB KO females to see if reductions in synaptic coverage are similar.

Investigating the role of motoneuron trkB receptors gives a more complete understanding of how synapses are actively maintained in contact with motoneurons. Knowing this mechanism will be important in helping determine methods to prevent synaptic stripping from occurring and to aide patient recovery after peripheral nerve injury.

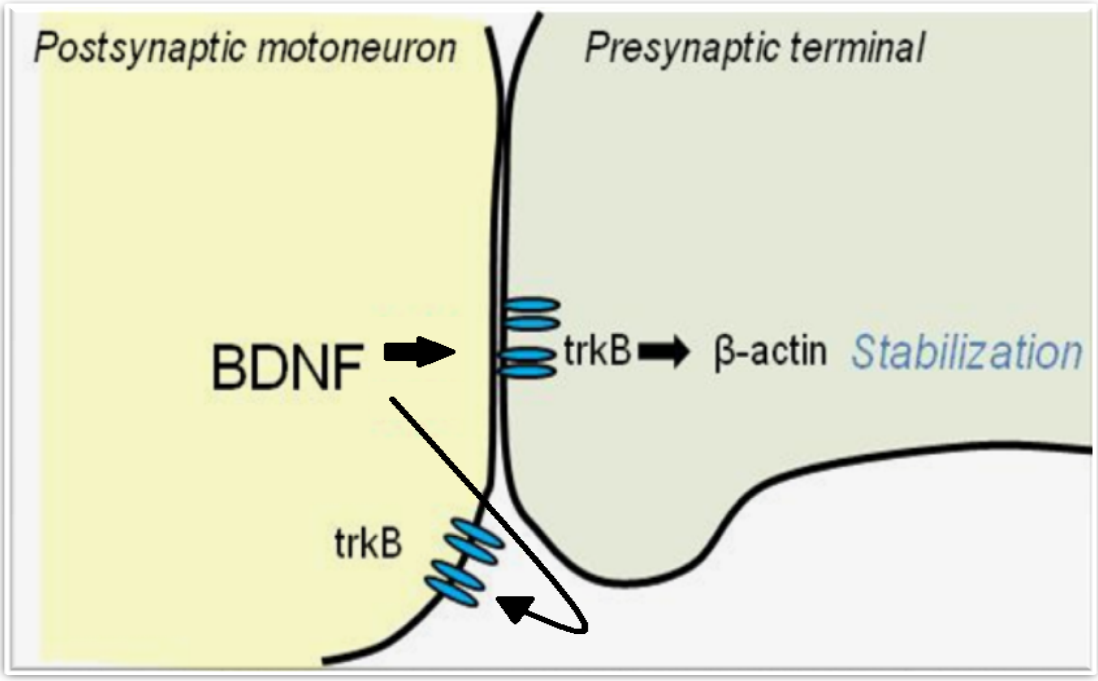


Figure 6. BDNF-trkB self amplification pathway: Binding of BDNF to postsynaptic motoneuron triggers production of additional BDNF, which is subsequently secreted and binds to presynaptic trkB receptors to stabilize the synaptic terminal.

Table 1. Groups of mice used in the study

Gender	Number of mice	Tamoxifen treatment
Female	N=2	Yes
Male	N=3	Yes
Male	N=3	No

Table 2. Antibodies used in immunohistochemistry staining

	Antibody	Concentration	Source
1°	Rabbit polyclonal anti-VGLUT1	1:2000	Synaptic Systems
	Rabbit polyclonal anti-VGLUT2	1:200	Synaptic Systems
	Mouse monoclonal anti-GAD67	1:200	Millipore
	Rabbit polyclonal anti-VGAT	1:200	Synaptic Systems
	Mouse monoclonal anti-NeuN	1:200	Millipore
	Rabbit polyclonal anti-NeuN	1:200	MIllipore
2°	Goat anti-Rabbit Alexafluor 647	1:200	Invitrogen
	Goat anti-Mouse Alexafluor 647	1:200	Invitrogen
	Goat anti-Rabbit Alexafluor 546	1:200	Invitrogen
	Goat anti-Mouse Alexafluor 546	1:200	Invitrogen

Table 3. Results of one way analysis of variance

Synapse Type	df Effect	df Error	F	p
VGLUT1	4	20	4.265672	0.011733
VGLUT2	3	15	5.465676	0.009673
GAD67	3	15	86.2607	1.09689E-09
Glycine	3	16	18.55682	0.000018

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