Regulation of axonal growth and neuromuscular junction formation by neuronal phosphatase and tensin homologue signaling

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ABSTRACT During the development of the vertebrate neuromuscular junction (NMJ), motor axon tips stop growing after contacting muscle and transform into presynaptic terminals that secrete the neurotransmitter acetylcholine and activate postsynaptic ACh receptors (AChRs) to trigger muscle contraction. The neuron-intrinsic signaling that retards axonal growth to facilitate stable nerve–muscle interaction and synaptogenesis is poorly understood. In this paper, we report a novel function of presynaptic signaling by phosphatase and tensin homologue (PTEN) in mediating a growth-to-synaptogenesis transition in neurons. In Xenopus nerve–muscle cocultures, axonal growth speed was halved after contact with muscle, when compared with before contact, but when cultures were exposed to the PTEN blocker bisperoxo (1,10-phenanthroline) oxovanadate, axons touching muscle grew ~50% faster than their counterparts in control cultures. Suppression of neuronal PTEN expression using morpholinos or the forced expression of catalytically inactive PTEN in neurons also resulted in faster than normal axonal advance after contact with muscle cells. Significantly, interference with PTEN by each of these methods also led to reduced AChR clustering at innervation sites in muscle, indicating that disruption of neuronal PTEN signaling inhibited NMJ assembly. We thus propose that PTEN-dependent slowing of axonal growth enables the establishment of stable nerve–muscle contacts that develop into NMJs.

INTRODUCTION

During embryonic development, motor axons grow to their muscle targets and establish neuromuscular junctions (NMJs). As these incipient synapses mature, two critical changes occur at the nerve–muscle contact sites: acetylcholine receptors (AChRs) are clustered in the postsynaptic muscle membrane, and synaptic vesicles that store and release ACh accumulate within the presynaptic nerve terminal (Sanes and Lichtman, 2001; Madhavan et al., 2005; Hughes et al., 2006; Bruneau et al., 2009). These changes, which ensure reliable synaptic transmission at the NMJ, are set in motion when interaction with a muscle target stalls axonal advance and triggers differentiation. In the past decade, the regulation of axonal elongation and pathfinding has been elucidated in significant detail (Bernhardt, 1999; Stuermer and Bastmeyer, 2000; Wen and Zheng, 2006; Vitriol and Zheng, 2012; Myers et al., 2011), but the molecular signaling that restricts the growth of motor axons after they reach muscle remains poorly understood. This study describes a previously unrecognized function of phosphatase and tensin homologue (PTEN) in controlling axonal growth during neuromuscular synaptogenesis.

Mutations in the gene encoding PTEN lead to many cancers (Snaddon et al., 2001; Kishimoto et al., 2003; He et al., 2007). PTEN is a protein and inositol phospholipid phosphatase that dephosphorylates signaling molecules, such as focal adhesion kinase and the...
adapter Shc, but perhaps the most critical substrate of PTEN is phosphatidylinositol (3,4,5)-triphosphate (PIP3; Gu et al., 1999; Tamura et al., 1999). PTEN removes PIP3's inositol 3-phosphate to produce phosphatidylinositol 4,5-bisphosphate, and by so doing antagonizes the function of phosphatidylinositol 3-kinase (PI3K) that generates PIP3 (Goberdhan et al., 1999; Stocker et al., 2002; Vazquez and Devreotes, 2006; Carnero et al., 2008). Thus PTEN negatively regulates the Akt signaling pathway and inhibits cellular growth (Carnero et al., 2008; Song et al., 2012), and PTEN is now recognized as a potential therapeutic target in tissues that regenerate poorly, such as those of the CNS. Whereas PTEN inhibits neurite outgrowth in cultured PC12 cells treated with nerve growth factor (NGF; Musatov et al., 2004), deletion of PTEN allows axonal regeneration after injury in retinal ganglion cells (RGCs) and corticospinal neurons (Park et al., 2008, 2010; Liu et al., 2010). Moreover, in mice lacking the survival motor neuron protein, PTEN depletion in motor neurons enhances axonal growth (Ning et al., 2010), and inhibition of endogenous PTEN promotes regeneration in peripheral neurons of adult rat (Christie et al., 2010).

Axonal growth is impeded not only by injury but by targets as well. The growth of RGC neurons decreases when they enter the tectum (Harris, 1987; McFarlane et al., 1995; Webber et al., 2003), and the elongation of axons from pontine explants is suppressed when they contact granule cells but not cultured glial cells (Baird et al., 1992). Using Xenopus nerve–muscle cocultures we have shown that spinal neurons extend filopodial processes preferentially toward muscle cells to interact with their synaptic partners (Li et al., 2011). Neurotrophins (NTs), factors that promote neuronal survival and growth (Huang and Reichardt, 2001), suppressed filopodial assembly in neurons and hindered NMJ formation (Peng et al., 2003; Li et al., 2011, 2012), whereas muscle-derived basic fibroblast growth factor (bFGF) induced neuronal filopodia and retarded axonal growth to facilitate NMJ development (Li et al., 2011). Because signaling by PTEN restricts neuronal outgrowth (as discussed above), we tested the potential involvement of PTEN in regulating axonal advance and the establishment of NMJs. We report that in Xenopus nerve–muscle cocultures, axonal growth was enhanced and NMJ assembly was suppressed when PTEN was chemically inhibited, when PTEN expression was reduced in neurons, or when inactive PTEN was introduced into neurons.

RESULTS

PTEN expression in Xenopus spinal neurons

We began this study on PTEN signaling in NMJ formation by using immunoblotting to assess PTEN's expression in Xenopus embryonic nerve and muscle tissues. A PTEN-specific antibody stained a single protein band of 47 kDa in extracts of neural tubes, myotomes, and whole embryos at stages 20–22 (Figure 1A, top panel). In comparison with protein loading shown by anti-tubulin staining (Figure 1A, bottom panel), this suggested that PTEN was well expressed in both Xenopus nerve and muscle tissues. For detection of PTEN's expression specifically in spinal neurons, pure nerve cultures from stage 20–22 embryos were immunolabeled. Anti-PTEN strongly labeled axons and growth cones of these neurons (Figure 1B, C and D); without anti-PTEN, no labeling was seen (Figure 1, D and E). These results suggested that Xenopus spinal neurons expressed PTEN.

Regulation of nerve–muscle interaction by neuronal PTEN signaling

In Xenopus nerve–muscle cocultures, the growth of axons slowed dramatically following contact with muscle cells. This is illustrated by the representative images of nerve–muscle pairs shown in Figure 2, which were captured 30 min apart before (Figure 2, A and B) and after (Figure 2, C and D) axons had contacted muscle. After touching muscle, the axonal growth cone advanced <5 μm in 30 min in this example, which was on average half as fast as before contact (Figure 2G). To test whether PTEN regulates this slowing of axonal growth, we first treated nerve–muscle cocultures with the PTEN-inhibitor bisperoxo (1,10-phenanthroline) oxovanadate (bpV; Schmid et al., 2004; Christie et al., 2010). In the presence of this drug, axons

![Figure 1: Expression of PTEN in embryonic Xenopus spinal neurons.](image-url)
grew ~50% faster after contacting muscle than they did under control conditions (Figure 2, E–G), but the growth of axons not touching muscle did not show a significant change as a result of bpV treatment (Figure 2G). These data suggested that PTEN activity was involved in slowing axonal growth upon muscle contact, and to further explore this premise, we examined the effect of bpV after nerves had touched muscle. Postcontact application of bpV resulted in faster than control axonal advance (Figure 3, A and B), with the mean growth speed ~60% higher after bpV treatment. Thus the target-induced slowing of axonal growth was partially reversed when PTEN was inhibited.

Because PTEN was detected in nerve and muscle tissues (Figure 1A), bath-applied bpV could have affected PTEN in both neurons and muscle cells. To specifically test neuronal PTEN’s involvement in the control of axonal growth, we introduced antisense morpholino oligonucleotides (MOs) into neurons to knockdown PTEN expression. Xenopus embryos at the two-cell stage were injected with PTEN-specific or control MOs, and spinal neurons were cultured from these embryos at stages 20–22. The MOs were tagged with a fluorescein marker and had the same sequences as those designed by others to study PTEN’s function in axonal branching in Xenopus RGCs (Drinjakovic et al., 2010). Neurons from MO-injected embryos were seeded on normal muscle cells, and axonal growth was examined in neurons that fluoresced. Axons with control-MO slowed ~50% after touching muscle cells (Figure 4, A–C), but those with PTEN-MO grew after contact with muscle almost as fast as control axons before contact (Figure 4, D–F and H). Axons with PTEN-MO not contacting muscle cells did not grow significantly faster than control axons (Figure 4H). Demonstrating PTEN-MO’s effectiveness, immunoblotting showed that PTEN protein levels were >50% lower in embryos injected with PTEN-MO than in those injected with control-MO (Figure 4G).

Next, to investigate the importance of PTEN’s catalytic activity in regulating axonal growth, we cultured muscle cells from normal Xenopus embryos with spinal neurons isolated from embryos injected with mRNAs encoding green fluorescent protein (GFP; control) or GFP-tagged wild-type PTEN (GFP-WT-PTEN) or catalytically inactive PTEN (GFP-C124S-PTEN). The C124S mutant has no enzymatic activity, because a critical cysteine in the catalytic domain is replaced by a serine (Maehama and Dixon, 1998; Ono et al., 2001; Steelman et al., 2008). Overexpression of WT- and C124S-PTEN in

**FIGURE 2:** Enhancement of axonal growth by the PTEN-inhibitor bpV. Elongating axons in Xenopus nerve–muscle cocultures were examined by time-lapse recordings. The sample images here show axonal growth over 30 min in control cocultures before contact with muscle (Ctl; A and B) and after (Ctl; C and D), and growth after muscle contact in cocultures treated with a PTEN-inhibitor (bpV, 100 nM; E and F). In both control and bpV-treated cultures, axonal growth slowed after touching muscle, but axons exposed to bpV advanced ~50% faster than control axons. The axon–growth cone position is marked by a white arrowhead at time zero (0’) and by a black arrowhead at 30 min (30’). In 30 min, the control axon advanced 25 μm before target contact (A and B) but <5 μm after contact (C and D). In contrast, addition of bpV caused the axon to grow 23 μm in 30 min after muscle contact (E and F).

(G) Quantification of axonal growth speeds in μm/min using distances axons advanced in control and bpV-treated cultures before and after contact with muscle. Mean ± SEM shown; number of axons examined by time-lapse recording: 10 (Ctl-before contact), 12 (Ctl-after contact), 22 (bpV-before contact), 39 (bpV-after contact). * t test: *, p = 0.01, relative to control axons before contact with muscle; ^, p = 0.03 compared with control axons after muscle contact; n.s., not significant. For the difference between Ctl-before contact and bpV-before contact, p = 0.17.
In cocultures using neurons with exogenous proteins, we again found that control axons expressing GFP slowed significantly after contacting muscle cells and migrated only a short distance in 30 min along the surface of those muscle cells (Figure 5, A–C). Axons over-expressing WT-PTEN (Figure 5, D–F and J) grew even more slowly than GFP axons on the average, while axons with C124S-PTEN seemed to ignore muscle targets and did not slow down upon contacting them (Figure 5, G–I). Quantification of growth speeds (Figure 5J) showed that GFP axons advanced ~50% more slowly after touching muscle cells than before. Expression of excess WT-PTEN in neurons slowed axonal growth in the absence of muscle contact to a level close to that after muscle contact in GFP axons, and interaction with muscle reduced this growth speed further. In contrast, after touching muscle, axons with C124S-PTEN advanced as fast as GFP axons did before muscle contact.

The above results collectively suggested the involvement of PTEN signaling in the slowing of axonal growth during nerve–muscle contact. We next tested whether this function of PTEN affects NMJ formation.

PTEN signaling and NMJ assembly

NMJ development is reliably marked by AChR aggregation at innervation sites in muscle. We quantified nerve-induced AChR clustering in muscle cells after interfering with PTEN signaling by each of the three methods used above: treatment with the PTEN inhibitor bpV, MO-mediated knockdown of neuronal PTEN, and expression of inactive PTEN in neurons. AChRs were labeled with R-BTX in 1-d-old cocultures to determine the percentages of nerve–muscle contacts with AChR clusters.

In control cocultures, AChRs were aggregated at nearly 70% of the nerve–muscle contacts identified (Figure 6, A, B, and E), but in bpV-treated cultures, AChR clusters were found at about half as many innervation sites (Figure 6, C–E). In the presence of bpV, muscle cells retained spontaneously formed AChR aggregates (hot spots, labeled “h.s.” in Figure 5D), which also indicates a suppression of new synapse formation, as discussed elsewhere (Anderson et al., 1977; Li et al., 2011). Similar to bpV treatment, knocking down PTEN expression in neurons made the neurons less synaptogenic: compared with axons with control-MO (Figure 7, A–C), those with PTEN-specific MO (Figure 7, D–F) induced AChR clustering at ~40–45% fewer nerve–muscle contacts (Figure 7G). Last, in cultures prepared using neurons with exogenous proteins, GFP neurons and WT-PTEN neurons triggered AChR aggregation at ~70–75% of the sites at which they contacted muscle (Figure 8, A–D and J). These findings suggest that inhibition of neuronal PTEN signaling, which resulted in faster than normal axonal growth after contact with muscle, also suppressed NMJ formation.

DISCUSSION

In this study, we used molecular and cell biological assays to investigate neuronal PTEN’s involvement in regulating axonal growth and nerve–muscle interaction during NMJ assembly. Our results showed that growing axons slowed after contacting muscle cells in Xenopus nerve–muscle cocultures, but that axons advanced significantly faster after muscle contact when PTEN was inhibited. PTEN was present in Xenopus spinal neurons, and we interfered with its activity in three separate ways: by applying the drug bpV, by using a PTEN-specific MO to lower neuronal PTEN expression, and by expressing catalytically dead PTEN in neurons. Axonal growth was enhanced in all three cases, coupled with a reduction in AChR

heterologous cells respectively lowers and elevates phospho-Akt levels, indicating negative and positive regulation of PI3K/Akt signaling (Wu et al., 1998; Weng et al., 2001; Wan and Helman, 2003).

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FIGURE 3: Effect of bpV on axonal growth after NMJ formation. Spinal neurons were seeded on muscle cultures to allow NMJ formation. After 12–16 h, control or 100 nM bpV-containing medium was added to the cocultures. (A and B) In control cultures, little growth of the axon was seen at nerve–muscle contact sites within 30 min, but in bpV-treated cultures (C and D), axons were observed to resume active growth during the 30-min recording period. (E) Quantification of axonal growth speeds in untreated (Ctl) and bpV-treated cocultures. Mean ± SEM; t test: *, p = 0.05 compared with Ctl. Arrowheads indicate initial and final positions of the terminal ends of axonal shafts (A–D). Number of axons examined by time-lapse recording: 16 (Ctl-after contact), 11 (bpV-after contact).
Neuronal PTEN signaling in NMJ assembly

Although inhibition of PTEN here sped up axonal advance, axons with disrupted PTEN signaling also appeared to slow after touching muscle. PTEN therefore may restrain axonal growth but not abolish an axon’s ability to detect “stop” signals from targets. Specific muscle-derived cues and neuronal signaling cascades that might retard the elongation of axons via PTEN and overcome growth-promoting signals are unknown, but previous studies suggest two intriguing possibilities. Pro-NGF activates the p75 NT receptor (p75NTR) and PTEN downstream from it to offset TrkB stimulation by brain-derived neurotrophic factor (BDNF; Song et al., 2010). PI3K is an effector of TrkB (Patapoutian and Reichardt, 2001; Mullen et al., 2011) and is antagonized by PTEN (Perandones et al., 2004; Carnero et al., 2008), hence PTEN activated in axons by Pro-NGF/p75NTR may counterbalance PI3K signaling downstream from BDNF/TrkB. We recently found that mRNAs for both TrkB and p75NTR are present in Xenopus neural tubes and that expression of excess TrkB and p75NTR in spinal neurons enhanced and suppressed axonal growth, respectively (M. Meng, personal communication; unpublished data).

A second candidate molecule from muscle that may limit the influence of NT/Trk in curbing axonal advance is bFGF. We have shown that bFGF produced by muscle cells induced neuronal clustering at nerve–muscle contacts. We propose that signaling by neuronal PTEN retards axonal growth and thereby facilitates stable nerve–muscle interaction and NMJ development.

Several recent studies have shown that suppression of PTEN signaling promotes axonal regeneration, growth, and survival in adult mouse and rat central and peripheral neurons (Park et al., 2008, 2010; Christie et al., 2010; Liu et al., 2010; Ning et al., 2010). These findings and our results together indicate that PTEN is a critical controller of axonal growth in both embryonic and adult vertebrate neurons. Our current study and previous work (Peng et al., 2003; Li et al., 2011), moreover, support the view that axonal growth and synaptogenic differentiation occur sequentially, with synapse formation following the retardation of axonal advance caused by a growth cone’s contact with muscle. We have shown that NTs, which activate signaling by Trk receptor tyrosine kinases, promote the survival and growth of Xenopus spinal neurons but inhibit nerve-induced AChR clustering (Peng et al., 2003). More recently, we found that exposure to K252a, a Trk blocker, arrested axonal growth and generated an arborized terminal in neurons reminiscent of presynaptic specialization; conversely, tropomyosin-receptor-kinase B (TrkB)-overexpressing neurons grew faster than control neurons and were less capable of inducing AChR clusters in postsynaptic muscle cells (Li et al., 2012). Thus the relative strengths of the opposing NT/Trk and PTEN signals may either sustain axonal elongation or favor stable nerve–muscle interaction and synaptic differentiation.

FIGURE 4: Increase in axonal growth speed following the reduction of PTEN expression in neurons. Spinal neurons were isolated from embryos injected with control MO (Ctl-MO; A–C) or PTEN-specific MO (PTEN-MO; D–F) and cocultured with muscle cells obtained from uninjected embryos. Axons with Ctl-MO and PTEN-MO both slowed after contact with muscle, but after touching muscle, PTEN-MO-axons grew nearly as fast as control axons did before muscle contact. Arrowheads indicate initial and final positions of the terminal ends of axons (A, B, D, and E), and the presence of MOs tagged with fluorescein is shown by the fluorescence of the axons (C and F). (G) Immunoblots showing that PTEN expression was knocked down in Xenopus embryos injected with PTEN-MO but not Ctl-MO (top blot); anti-tubulin staining shows protein loading (bottom blot). (H) Quantification of growth speeds of axons with Ctl-MO and PTEN-MO. Mean ± SEM; t test: *, p = 0.02 compared with Ctl-MO-axons before contact with muscle; ^^, p < 0.005 relative to growth speed of Ctl-MO-axons after muscle contact; number of axons examined by time-lapse recording: 8 (Ctl-MO before contact), 30 (Ctl-MO after contact), 15 (PTEN-MO before contact), 33 (PTEN-MO after contact).
Filopodia that enhance nerve–muscle interaction (Li et al., 2011). Signaling by bFGF via the FGF receptor FGFR1 in neurons slowed axonal growth and favored NMJ establishment (Li et al., 2011), and, notably, this was able to partially rescue NMJ formation in cultures exposed to NTs (Li et al., 2012). Thus the development of stable interactions between nerve and muscle during early stages of contact between these synaptic partners may depend on the balancing of axonal growth promoted by NT/Trk by the growth retardation effect of bFGF/FGFR1 and pro-NGF/p75NTR. Elucidating how these factors control downstream signaling by PTEN and PI3K in neurons may lead to a better understanding of the initiation of neuromuscular synaptogenesis.

In our Xenopus nerve–muscle cocultures, the PTEN-blocker bpV increased axonal growth after muscle contact to a level similar to that achieved by the suppression of neuronal PTEN expression or the introduction of inactive PTEN into neurons. Additionally, NMJ formation was inhibited to nearly the same extent in these cases: exposure of cocultures to bpV and neuron-selective disruption of PTEN reduced nerve-induced AChR clustering by ~40–50%. These results suggest that PTEN regulated NMJ assembly through its control of axonal growth and not by interfering (to a quantifiable level) with postsynaptic aggregation of AChRs, and further, that the synaptogenic capacity of neurons was higher when intact PTEN signaling allowed elongating axons to be slowed upon reaching muscle targets. Intriguingly, in mouse corticospinal neurons in which PTEN depletion promoted regeneration (as noted above), synaptogenesis appeared somewhat defective; this was indicated by a reduction in the density of the presynaptic marker vGlut1 along axons lacking PTEN (Liu et al., 2010).
near targets may help future studies uncover conditions that permit normal synaptogenesis to follow enhanced axonal outgrowth.

**MATERIALS AND METHODS**

**Reagents**

These reagents were purchased from commercial sources: bpV (Calbiochem, San Diego, CA), rhodamine-conjugated α-bungarotoxin (R-BTX; Molecular Probes, Eugene, OR), anti-PTEN goat polyclonal antibody (Imgenex, San Diego, CA); horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA), and TX-100 and enhanced chemiluminescence (ECL) reagent West Pico (Pierce, Rockford, IL). Fluorescein-tagged antisense PTEN-MO and control-MO were from Gene Tools (Philomath, OR).

Xenopus nerve–muscle cocultures

Xenopus spinal neurons and myotomal muscle cells were cultured as previously described (Peng et al., 1991). Briefly, neural tubes and myotomes were dissected from embryos at stages 20-22 and
dissociated in a Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free solution. Dissociated spinal neurons were plated by themselves on glass coverslips (coated with entactin–collagen IV–laminin substrate; Millipore, Billerica, MA) to obtain pure nerve cultures, or seeded on muscle cells plated 4–7 d earlier to generate nerve–muscle cocultures. Cultures were maintained for 18–24 h before use.

Measurement of axonal growth speeds

In nerve–muscle cocultures, the growth of axons approaching muscle cells was monitored before axon tips contacted muscle and also after, if the axons touched the muscle cells they neared. Using time-lapse recordings of 30 min (or more) with 3-min intervals, we measured the distances traveled by the growing tips of axons, using MetaMorph software (Molecular Devices, Sunnyvale, CA) to calculate axonal growth speed.

Expression of exogenous proteins in Xenopus spinal neurons

pcDNA3-GFP-PTEN (Liu et al., 2005) was obtained from Addgene (plasmid 13039; Cambridge, MA), and the GFP-PTEN open reading frame was subcloned into pcRS2+ vector. The resulting pcRS2(+-)

GFP-WT-PTEN was used as a template to generate, by site-directed PCR mutagenesis, pcRS2(+-)GFP-C124S-PTEN that encodes catalytically inactive PTEN. The primers were: 5′-CATGTGCTGCAATTCA

CAGTAAAGCTGAAAGGGC-3′ and 5′-GTCCCTTTCCAGCTT

TACGTGAATTGCTGCAACATG-3′. We used a pcRS2(+-)GFP construct as a control (Madhavan et al., 2006; Li et al., 2011). After pcRS2(+) constructs were linearized, mRNAs encoding GFP, GFP-WT-PTEN, and GFP-C124S-PTEN were synthesized using SP6 polynucleotide with the mMESSAGE mMACHINE kit (Ambion, Grand Island, NY). For expression of exogenous PTEN or for knock down PTEN expression in neurons, mRNAs or MOs were injected into one cell of two-cell–stage Xenopus embryos using a Drummond Nanojet Oocyte Injector (Drummond Scientific, Broomall, PA). Neural tubes were dissected from GFP/fluorescein-positive embryos at stages 20–22 and dissociated spinal neurons were plated by themselves to prepare pure nerve cultures or were seeded on muscle cells isolated from uninjected embryos to generate nerve–muscle cocultures (Qian et al., 2008; Li et al., 2011). Neurons that fluoresced because they carried the MOs or exogenous proteins were examined.

Visualization of PTEN localization and NMJ formation

For labeling PTEN in Xenopus spinal neurons, pure nerve cultures (18-h old) were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 4% sucrose for 30 min and then permeabilized with 0.2% Triton in PBS (10 min). After being blocked with 5% BSA in PBS for 1 h to reduce background staining, neurons were labeled with anti-PTEN and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. To examine NMJ formation, we monitored AChR clustering in nerve–muscle cocultures using labeling with 3 nM R-BTX. In some experiments, cultures prepared using wild-type neurons and muscle cells were maintained overnight in control or bpV-containing medium. In other experiments, neurons were used that had been isolated from Xenopus embryos injected with mRNAs or MOs. Nerve–muscle contacts were randomly chosen under phase-contrast and were then checked for AChR aggregation at innervation sites in muscle, from this, we calculated percentages of nerve–muscle contacts with AChR clusters.

Immunoblotting

Myotomes and neural tubes from Xenopus embryos at stages 20–22 were dissected and dissociated into single cells in Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free Steinberg's solution and then harvested in SDS loading buffer. Extracts of whole embryos at stages 20–22 were prepared using a modified RIPA buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by SDS-PAGE (10% gels) and transferred to polyvinylidene fluoride membranes, which were blocked with 5% milk and then stained with primary and HRP-conjugated secondary antibodies for ECL-based detection.

Microscopy and statistics

Cultures were examined using a Zeiss Axiovert 200M microscope equipped with a Zeiss AxioCamMR camera controlled by AxioVision Pel 4.5 software (Jena, Germany). All experiments were performed three or more times and means ± SEM were calculated; Student’s t tests were carried out with GraphPad Prism (La Jolla, CA) statistical software.

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