Brief Communications

ProBDNF and Mature BDNF as Punishment and Reward Signals for Synapse Elimination at Mouse Neuromuscular Junctions

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During development, mammalian neuromuscular junctions (NMJs) transit from multiple-innervation to single-innervation through axonal competition via unknown molecular mechanisms. Previously, using an *in vitro* model system, we demonstrated that the postsynaptic secretion of pro-brain-derived neurotrophic factor (proBDNF) stabilizes or eliminates presynaptic axon terminals, depending on its proteolytic conversion at synapses. Here, using developing mouse NMJs, we obtained *in vivo* evidence that proBDNF and mature BDNF (mBDNF) play roles in synapse elimination. We observed that exogenous proBDNF promoted synapse elimination, whereas mBDNF infusion substantially delayed synapse elimination. In addition, pharmacological inhibition of the proteolytic conversion of proBDNF to mBDNF accelerated synapse elimination via activation of p75 neurotrophin receptor ($p75^{NTR}$). Furthermore, the inhibition of both $p75^{NTR}$ and sortilin signaling attenuated synapse elimination. We propose a model in which proBDNF and mBDNF serve as potential "punishment" and "reward" signals for inactive and active terminals, respectively, *in vivo*.

Introduction

Activity-dependent synaptic competition plays a critical role in shaping patterns of synaptic connections in the nervous system. At the rodent neuromuscular junction (NMJ), multiple axons compete with one another for the same postsynaptic muscle cell during early postnatal days. The most active terminal gets stabilized, whereas the less active ones withdraw. By the end of the second postnatal week, all axons but one are eliminated, resulting in canonical elimination of polyneuronal innervation (Nguyen and Lichtman, 1996). This synapse elimination is thought to be mediated by an activity-dependent process that involves both "punishment" and "reward" signals from postsynaptic muscle cells (Wyatt and Balice-Gordon, 2003). Despite significant efforts over two decades, the molecular identities of these prospective punishment and/or reward signals remain unknown (Lichtman and Colman, 2000).

Brain-derived neurotrophic factor (BDNF), a neurotrophic factor known to regulate synapse development and plasticity, is secreted from target cells in an activity-dependent manner (Misgeld et al., 2002). BDNF is initially synthesized as a precursor (proBDNF), which is proteolytically processed into mature BDNF (mBDNF) (Lu, 2003). Intriguingly, these two forms of BDNF bind to two different types of cell-surface receptors in motor nerve terminals: proBDNF binds to the pan-neurotrophin receptor p75 ($p75^{NTR}$), whereas mBDNF preferentially binds to the tyrosine receptor kinase B (TrkB). By binding to distinct receptor systems, proBDNF and mBDNF elicit seemingly opposite biological effects at developing neuromuscular synapses (Lu, 2003; Wu et al., 2010; Je et al., 2012). For example, exogenous application of mBDNF triggers synaptic potentiation and maturation of developing NMJs through TrkB, whereas application of

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proBDNF suppresses synaptic transmission and causes axonal retraction by activating presynaptic p75^{NTR}(Yang et al., 2009a). Furthermore, using a triplet culture system that allows gene manipulation in one of two distinctly labeled axons, which innervates a single myocyte, we previously demonstrated that proBDNF serves as a punishment signal that causes axon terminals expressing p75^{NTR} to retract. Neuronal activity converts proBDNF to mBDNF, which serves as a reward signal to stabilize and maintain the axon terminal during synaptic competition (Je et al., 2012).

Although these data strongly support a model in which postsynaptic secretion of a single molecule, proBDNF, regulates synapse elimination and stabilization, it remains unclear whether proBDNF and mBDNF play a role during synapse elimination *in vivo*. Here, we used both pharmacological and genetic manipulations to study the roles of endogenous proBDNF and mBDNF in synapse elimination using mouse *Levator auris longus* (LAL) neuromuscular synapses.

Materials and Methods

Animals. BDNF$^{-/-}$ mice were bred and genotyped as previously described (Ernfors et al., 1994). TrkB knock-in (TrkB^{F616A}) mice, in which endogenous TrkB activity can be inhibited pharmacologically by *in vivo* administration of 1NMPP1, were used (Chen et al., 2005). Neonatal pups of either sex were obtained from C57BL/6 mice and the date of birth was designated postnatal day 0 (P0). p75^{NTR} knock-out mice (p75^{NTR} KO) were purchased from The Jackson Laboratory and were backcrossed onto a C57BL/6 background for more than five generations. All animal procedures conformed to guidelines of the National Institutes of Health.

Drug treatment. Drugs, recombinant proteins, peptides, and antibodies were administered by subcutaneous injection over the right LAL muscle of neonatal mice. Litters were culled to a maximum of 10 pups to minimize variation in growth due to differences in feeding. Factors were diluted to appropriate concentrations in saline containing 0.1% BSA (v/v), and 30–50 μl of was injected twice daily from P1. Doses used for various factors were as follows: IgG (Santa Cruz Biotechnology), 3 μg/ml; affinity-purified anti-p75^{NTR} IgG, 3 μg/ml; purified proBDNF, 0.6 μg/ml; recombinant mBDNF (Regeneron), 0.5 μg/ml; pan metalloproteinase (MMP) inhibitor, 60 μM; general protease inhibitor cocktail, 50 μM; sortilin propeptide (a gift from Dr Peder Madsen, Aarhus University, Aarhus, Denmark) or GST, 10 μM. 1NMPP1 dissolved in saline was administered by intraperitoneal injection to TrkB^{F616A} mice 2–3 times daily from P1 to P7 (5 mM in 20 μl). TrkB^{F616A} mice that received injections of DMSO diluted in the same vehicle solution and wild-type mice that received 1NMPP1, a small molecule of the protein kinase inhibitor protein phosphatase 1 (selective antagonist of TrkB F616), were used as controls.

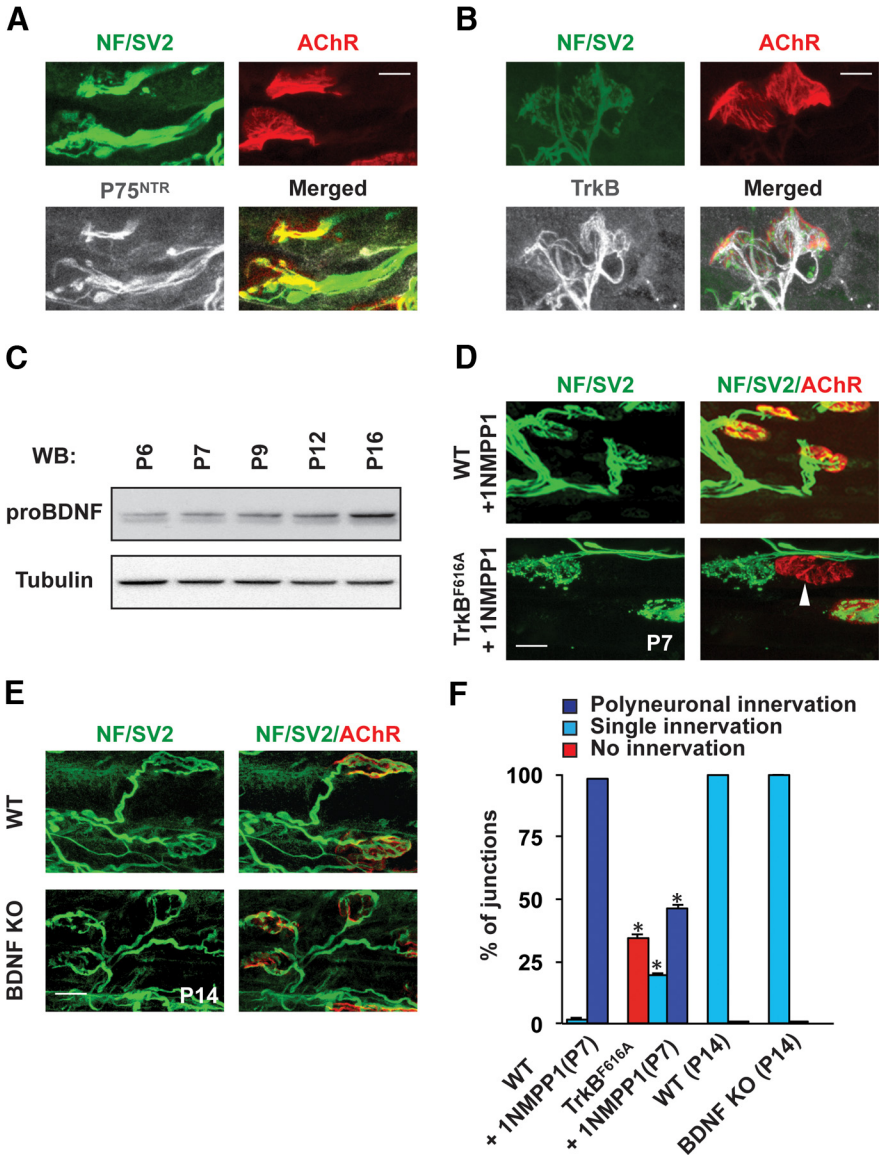

Figure 1. Expression of proBDNF, p75^{NTR} and TrkB at the developing NMJs, and the role of TrkB in synapse elimination. ***A***, Photomicrographs of immunostaining of NMJs showing association of p75^{NTR} immunoreactivity in neonatal axons and nerve terminals. Whole-mount preparations of sternomastoid muscles from P5 mice were stained with specific antibodies against neurofilament (NF) and the synaptic vesicle protein SV2 to label axons (NF/SV2; green), against p75^{NTR} (white), and against postsynaptic acetylcholine receptors (AChRs; red). ***B***, Photomicrographs of immunostaining of NMJs showing association of TrkB immunoreactivity in neonatal axons and nerve terminals. P5 sternomastoid muscles were stained as in ***A***, except that TrkB (white) replaces p75^{NTR}. Scale bars: ***A***, ***B***, ***D***, ***E***; 10 μm. ***C***, Expression of proBDNF in the developing muscle. LAL muscles of neonatal mice were dissected, homogenized, and processed for Western blot using a specific antibody against proBDNF (Yang et al., 2009b). ***D***, Elimination of axon terminals from endplates triggered by the inhibition of TrkB signaling using the TrkB^{F616A} knock-in-1NMPP1 system. To inhibit TrkB signaling in developing NMJs *in vivo*, 1NMPP1 was injected intraperitoneally into TrkB^{F616A} knock-in mice for 5 d (P2–P7). We observed that many endplates, identified by postsynaptic clusters of AChRs, were unoccupied by terminal arbors (white arrowheads). ***E***, Normal synaptic elimination in BDNF KO. In both WT and BDNF KO mice, nearly all of the junctions were singly innervated by P14. ***F***, Quantification of synapse elimination at mouse NMJs. Data represent mean ± SEM. for > 500 endplates from at least 3 mice per group. *Values are significantly different from control, with $p < 0.05$ by Fisher's test.

Immunocytochemistry and morphological analysis. Whole mounts of LAL muscles were processed for immunostaining as previously described (Burns et al., 2007). The following primary antibodies were used: anti-neurofilaments (SMI312, Sternberger Monoclonals); anti-SV2 (SYP, Developmental Studies Hybridoma Bank); α-bungarotoxin (Invitrogen); anti-sortilin (R&D Systems); anti-p75^{NTR} was a gift from Phil Barker

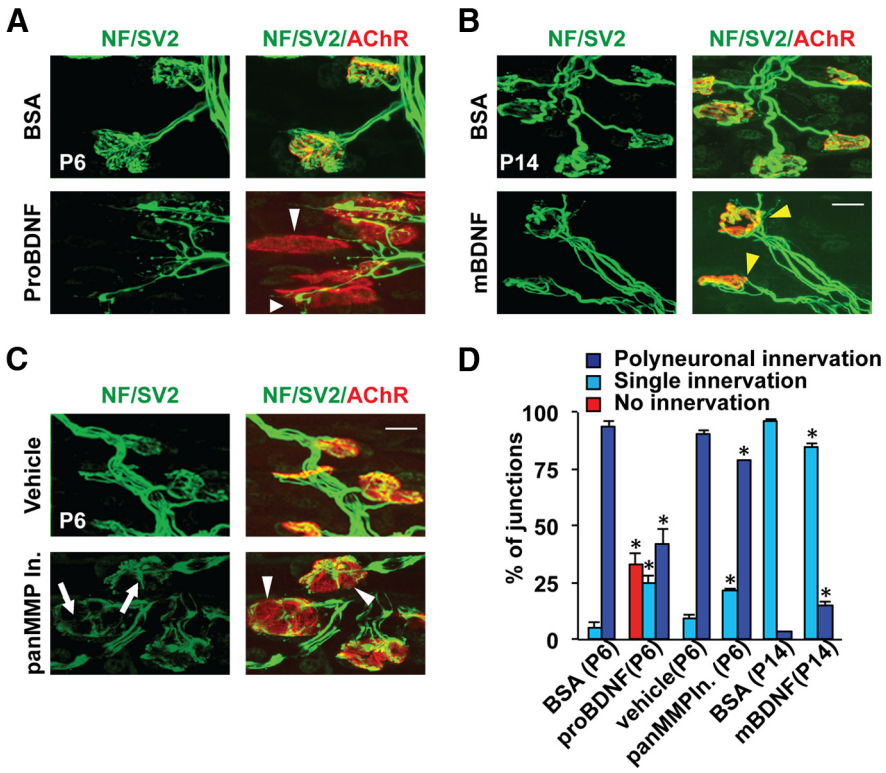

Figure 2. Pharmacological manipulation of synapse elimination. The LAL muscles were immunostained to visualize preterminal and terminal portions of axons, with specific antibodies against NF, the synaptic vesicle protein SV2 (NF/SV2; green) and postsynaptic clusters of AChRs (red). ***A***, Retraction of axon terminals induced by administration of proBDNF. Many endplates show no (white arrowhead) or only rudimentary (white arrows) arbors of nerve terminals. ***B***, Persistence of multiple innervations due to exogenous application of mBDNF. Endplates innervated by multiple axons (yellow arrowheads) were abundant in P14 NMJs treated with mBDNF, compared with NMJs treated with BSA. ***C***, Retraction of terminal arbors induced by treatment of pan-MMP inhibitors. Subcutaneous application of pan-MMP inhibitors (panMMP In) into LAL muscles induced partial elimination of terminal arbors at many endplates (white arrows), as indicated by the presence of AChR-rich patches (arrowheads), that were not juxtaposed with axon termini. ***D***, Quantification of synapse elimination at mouse NMJs following various treatments. *Values differ from control groups (either P6 or P14, treated for the same period with BSA) at $p < 0.05$ by Fisher's test. In, Inhibitors. Scale bar, 10 μm.

(McGill University, Montreal, Canada); the antibody against the cytoplasmic domain of TrkB was from Santa Cruz Biotechnology and the antibody against the extracellular domain of TrkB was kindly provided by Louis Reichardt (University of California, San Francisco, CA). Secondary antibodies included fluorescein-conjugated goat anti-mouse IgG (Roche) and Alexa Fluor 647-conjugated goat anti-rabbit, anti-chick, or anti-mouse IgG (Invitrogen). Images were taken with a Zeiss LSM510 confocal microscope.

Western blot analysis. LAL muscles of neonatal mice (from P5 to P16) were carefully dissected out, homogenized in ice-cold RIPA lysis buffer. We used anti-proBDNF (generated by the Lu Laboratory) and mouse anti-Tubulin (Abcam) as primary antibodies.

Results

Expression of p75^{NTR}, TrkB, and proBDNF at developing mouse NMJs

We tested whether p75^{NTR}, TrkB, and proBDNF are expressed at mouse NMJs during the period of synapse elimination. Previous studies have reported high levels of p75^{NTR} expression in spinal motor neurons, particularly during early development (Yan and Johnson, 1988; Koliatsos et al., 1993; Garcia et al., 2011). We performed whole-mount immunohistochemistry and observed intense p75^{NTR} immunoreactivity associated with motor nerve terminals innervating sternomastoid muscles at P5 (Fig. 1*A*) and P10 (data not shown). In contrast, we observed very little immunoreactivity associated with Schwann cells, indicating that neonatal Schwann cells in association with axons are not expressing p75^{NTR} (data not shown). Next, to determine whether endogenous TrkB protein is expressed in motor nerve terminals at P5 NMJs, we performed whole-mount immunohistochemistry and observed that TrkB immunoreactivity was primarily associated with motor axons and their terminals (Fig. 1*B*).

Although previous studies have demonstrated the expression of BDNF mRNA and protein in developing muscle cells (Funakoshi et al., 1995; Ip et al., 2001), no study has addressed whether proBDNF is expressed in muscles. Using a specific antibody against proBDNF (Yang et al., 2009b), we observed proBDNF expression in muscles during the period of synapse elimination (Fig. 1*C*). These data confirmed the expression of p75^{NTR}, TrkB, and proBDNF at developing mouse NMJs *in vivo*.

Role of TrkB in synapse elimination at mouse NMJs

We next tested whether removal of TrkB signaling, which mediates the mBDNF signal, could accelerate synapse elimination *in vivo*. Because TrkB homozygous knock-out mice die shortly after birth (Klein et al., 1993), we took advantage of TrkB^{F616A} knock-in mice, in which the endogenous TrkB is replaced with a mutated TrkB^{F616A} (Chen et al., 2005). Upon administration of 1NMPP1, which selectively binds to the mutated TrkB receptors, TrkB autophosphorylation and its signaling are blocked in these knock-in mice. Using this chemical-genetic approach, we sought to inhibit TrkB signaling during postnatal NMJ development.

We performed a series of experiments using mouse LAL neuromuscular junction (Garcia et al., 2011). The LAL muscle has been extensively used for pharmacological studies of synapse elimination *in vivo* (Angaut-Petit et al., 1987). At P6, most of the LAL NMJs are polyinnervated, and at P14, nearly all junctions are singly innervated. To test whether removal of TrkB signaling accelerates synapse elimination *in vivo*, we injected both wild-type and TrkB^{F616A} knock-in mice with 1NMPP1 intraperitoneally twice per day from P2 to P7. We found that 1NMPP1 dramatically accelerated synapse elimination at the NMJs of TrkB^{F616A} knock-in mice. At P7, polyneuronal innervation was reduced from 90% in vehicle-treated to <47% in 1NMPP1-treated junctions in the LAL muscles in TrkB^{F616A} mice (Fig. 1*D*; $p < 0.05$, Fisher's test; $n > 700$ NMJs, $N = 3$ mice per condition). Strikingly, the terminal arbors of many NMJs (>34%) appear to have shifted in TrkB^{F616A} mice, leaving some or all postsynaptic AChRs unoccupied (Fig. 1*D*). No sign of sprouting or axonal degeneration was observed in these muscles (Fig. 1*D*). As an additional control, treatment of wild-type LAL muscles with 1NMPP1 had no effect on synaptic stabilization or elimination, suggesting that 1NMPP1 does not elicit toxic side-effects (Fig. 1*D*,*F*). These results highlight the importance of TrkB signaling

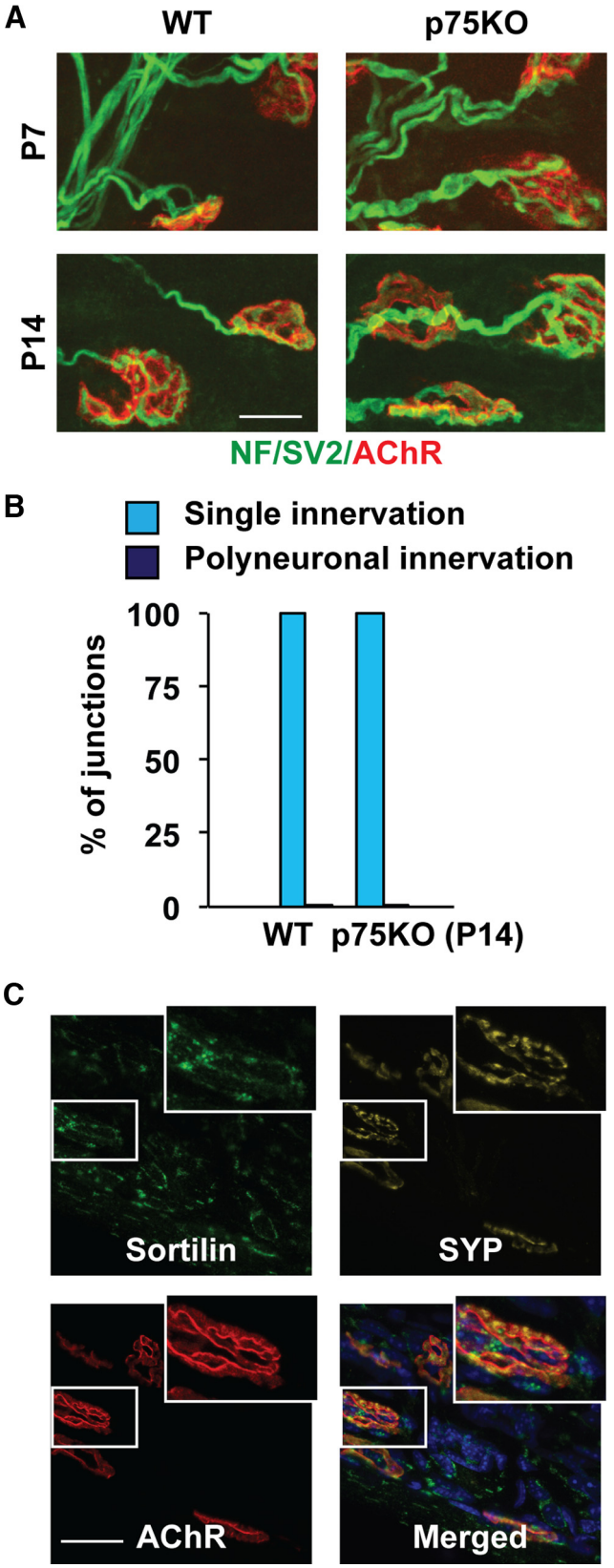

Figure 3. Normal synapse elimination in p75 ^{NTR} knock-out mice and expression of sortilin at NMJs. ***A***, Normal synaptic elimination in p75 ^{NTR} knock-out (p75 ^{NTR} KO) mice. In both WT and p75 ^{NTR} KO mice, almost all junctions were singly innervated by P14. Scale bar: ***A***, ***C***; 20 μm . ***B***, Quantification of synapse elimination in P14 muscles from WT and p75 ^{NTR} KO mice. Data represent mean ± SEM. for >500 endplates from at least 4 mice per group. ***C***, Immunofluorescence staining showing the expression of endogenous sortilin at NMJs. Whole-mount preparations of P5 muscles were immunostained using specific antibodies against sortilin (green), synaptophysin (yellow), and acetylcholine receptors (red). DAPI (blue) was used to label nuclei. The region in the white box is magnified at the top right.

in the stabilization or maintenance of axon arbors during synaptic elimination.

We next tested whether the removal of both the punishment signal (proBDNF) and the reward signal (mBDNF) could affect synapse elimination *in vivo*. To this end, we analyzed NMJs of BDNF knock-out mice (BDNF KO). We observed no changes in synapse elimination at P14 in BDNF KO mice (Fig. 1*E*,*F*; $n > 500$ NMJs, $N = 4$ mice). The lack of phenotype in BDNF KO mice may be due to the removal of both punishment (proBDNF) and reward (mBDNF) signals, suggesting that polyneuronal to mononeuronal synapse elimination occurs by default.

Roles for proBDNF and mBDNF in synapse elimination at mouse NMJs

Next, we performed a series of pharmacological experiments using mouse LAL neuromuscular synapses by infusing recombinant proBDNF and mBDNF (Garcia et al., 2011). At control (BSA-injected) NMJs at P6, AChR-rich postsynaptic sites were completely occupied by multiple axon terminals (Fig. 2*A*). More than 95% of NMJs were multiply innervated (Fig. 2*D*; $n > 500$ NMJs, $N = 4$ mice). In contrast, the proBDNF-treated muscles exhibited dramatic retraction of axon terminals from postsynaptic sites (Fig. 2*A*). Most NMJs were either innervated by a single axon or were even devoid of any axon (Fig. 2*D*; 34% NMJs with no innervation, 26% with single innervation, $n = 800$, $N = 4$ mice). Following the proBDNF treatment, postsynaptic AChR clusters appeared more elongated and larger, compared with controls. These AChR clusters were observed at the junctions on thin muscle fibers that lack innervation, suggesting that the phenomenon is likely to be secondary to denervation or muscle fiber atrophy-associated remodeling of AChR clusters (Fig. 2*A*) (Misgeld et al., 2002). Conversely, in P14 LAL muscles treated with mBDNF, ~23% of the NMJs remained innervated by multiple axons (Fig. 2*B*, $n > 500$ NMJs, $N = 4$ mice). In comparison, nearly all (98%) NMJs were singly innervated by P14 in the BSA-treated muscles (Fig. 2*B*,*D*; $p < 0.05$, Fisher's test, $n > 650$ NMJs, $N = 4$ mice). Together, exogenous proBDNF promoted the elimination of nerve terminals, whereas mBDNF led to persistence of multiple innervations at many NMJs.

Previously, we reported that metalloproteases mediate conversion of proBDNF to mBDNF at developing neuromuscular synapses (Yang et al., 2009a). Therefore, we tested whether the inhibition of proteases that convert proBDNF to mBDNF results in accumulation of endogenous proBDNF at the NMJs *in vivo*, leading to accelerated elimination of axon terminals. Intriguingly, we observed the significant reduction of polyneuronal innervation at P6 NMJs when LAL muscles were treated with pan MMP inhibitors for 6 d (P1–P6) (Fig. 2*C*,*D*). Approximately 22% of NMJs were singly innervated in pan MMP-treated muscles, compared with 2% in vehicle-treated muscles (Fig. 2*D*; $p < 0.05$, Fisher's test, $n > 800$ NMJs, $N = 4$ mice).

Role of p75 ^{NTR} in synapse elimination at mouse NMJs *in vivo*

If p75 ^{NTR} mediates the punishment signal, ablation of p75 ^{NTR} might allow retention of polyneuronal innervations at NMJs later in development. To our surprise, p75 ^{NTR} knock-out (p75 ^{NTR} KO) mice showed normal synapse elimination at P14 (Fig. 3*A*,*B*; $n > 500$ NMJs, $N = 4$ mice). More detailed analysis of the developmental time course revealed that the patterns of polyneuronal

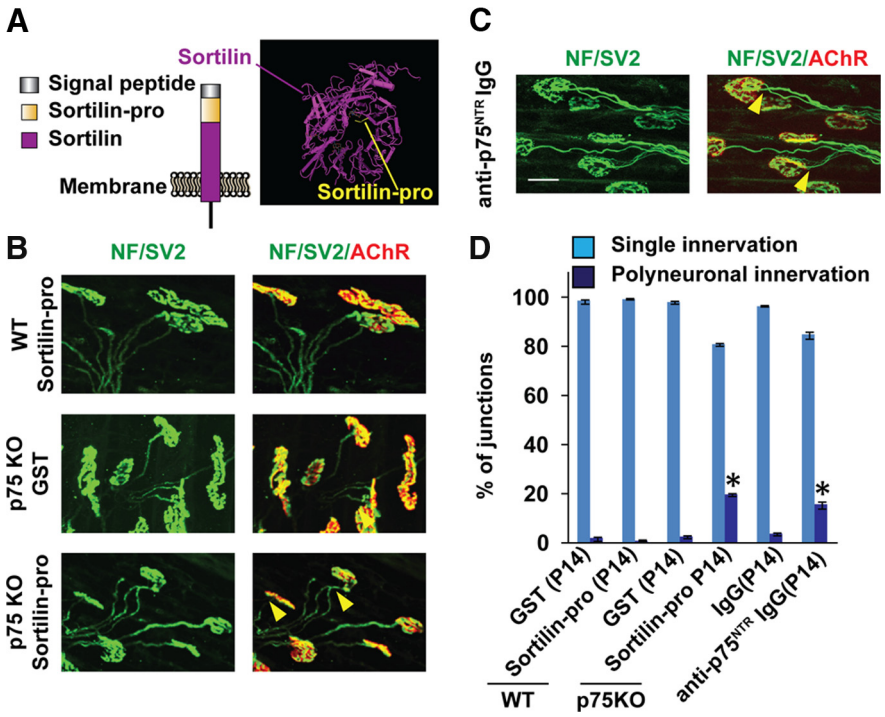

Figure 4. Synapse elimination mediated by endogenous proBDNF via both sortilin and p75NTR. ***A***, The 3D structure of sortilin. Top, Schematic diagram of sortilin. Sortilin-pro, Sortilin-propeptides. Bottom, 3D representation of sortilin (purple) and putative binding sites of sortilin propeptides (yellow). The 3D image was constructed using PDB (PDBID: 3F6K; http://www.rcsb.org/pdb/explore/explore.do?pdbId=3F6K) (Quistgaard et al., 2009). ***B***, Substantial polyinnervation in P14 NMJ of p75^{NTR} KO mice injected with sortilin propeptides. Note that synaptic elimination proceeds normally in WT mice injected with sortilin propeptides and also in p75^{NTR} KO mice injected with GST peptides, which served as a negative control. Yellow arrowheads indicate endplates innervated by multiple axon terminals. Scale bar: ***B***, ***C***; 20 μm. ***C***, Persistence of polyneuronal innervation upon exogenous application of antibodies against p75^{NTR} (anti-p75^{NTR} IgG). LAL muscles were treated with anti-p75^{NTR} IgG twice daily for 14 d (P1–P14). Endplates innervated by multiple axons (yellow arrowheads) increased in the anti-p75^{NTR} IgG-treated muscles, but not increased muscles treated with the control IgG. ***D***, Quantification of synapse elimination at mouse NMJs following various treatments in ***B*** and ***C***. *Values differ from control groups (WT, IgG-treat or GST-treated, $p < 0.05$, ANOVA).

innervation in p75^{NTR} KO mice were analogous to those in wild-type littermate controls at P7, and P14 (Fig. 3*B*). The lack of phenotype at NMJs of p75^{NTR} KO mice led us to hypothesize that proBDNF-mediated synaptic retraction requires simultaneous activation of p75^{NTR} and a complementary receptor sortilin, a coreceptor that binds to pro-neurotrophins (Nykjaer et al., 2004; Teng et al., 2005; Jansen et al., 2007). Consistent with this hypothesis, we observed that sortilin is expressed in both motor neurons and postsynaptic LAL muscles (Fig. 3*C*).

To test whether blockade of sortilin signaling in p75^{NTR} KO mice prevents synapse elimination, LAL muscles from either p75^{NTR} KO mice or their WT littermates were subcutaneously injected with recombinant GST-tagged sortilin propeptides (Fig. 4*A*) from P1 to P14. Sortilin propeptides are known to inhibit binding of sortilin ligands including neurotensin and proBDNF, but not proNGF (Munck Petersen et al., 1999; Quistgaard et al., 2009). At P14, AChR-rich postsynaptic sites were completely innervated by a single axon in two control groups (98%, p75^{NTR} KO mice injected with GST alone, $n > 300$ NMJs, $N = 2$ mice, 99%, WT mice injected with sortilin propeptide, $n > 600$ NMJs, $N = 4$ mice) (Fig. 4*B*,*D*). Intriguingly, many NMJs were innervated by multiple axons in P14 muscles from p75^{NTR} KO mice injected with sortilin propeptides (19%, $n > 1000$ NMJs, $N = 6$ mice; $p < 0.05$, ANOVA), indicating that inhibition of both p75^{NTR} and sortilin attenuated synapse elimination *in vivo* (Fig. 4*B*,*D*).

The effectiveness of postnatal injection of sortilin propeptide suggests the importance of inhibiting the punishment signal during the first two postnatal weeks, when synaptic competition occurs. To test this idea, we locally injected an affinity purified anti-p75^{NTR} antibody to the LAL muscle of wild-type animals twice daily from P1 to P14. Because p75^{NTR} and sortilin cooperate to promote proneurotrophin binding (Nykjaer et al., 2004), it is likely that the anti-p75^{NTR} antibody blocks the proBDNF-sortilin interaction. Remarkably, anti-p75^{NTR} antibody treatment resulted in many NMJs of the LAL muscles retaining polyneuronal innervation at P14 (Fig. 4*C*,*D*; ranging from 11% to 22% NMJs, $n = 825$ NMJs, $N = 8$ mice). In comparison, <3% of NMJs were innervated by multiple axons at P14 in the LAL muscles treated with control IgG ($p < 0.05$, Fisher's test, $n = 425$ NMJs, $N = 4$ mice). Together, blockade of p75^{NTR} signaling during the period of synapse elimination attenuates synapse elimination at developing mouse NMJs.

Discussion

There are two hypotheses for activity-dependent synaptic elimination: the "synaptotoxin" hypothesis proposes that the postsynaptic cell secretes a destabilizing factor that remove presynaptic terminals, and the "synaptotrophin" hypothesis proposes that axons compete against one another for a trophic factor derived from the postsynaptic cell (Snider and Lichtman, 1996). The *in vivo* results presented in this study, together with the *in vitro* data published earlier (Yang et al., 2009a; Je et al., 2012), suggest a new model in which a single molecule, BDNF, can be either the punishment or the "reward signal", depending on proteolytic conversion. Thus, proBDNF from postsynaptic muscle cells serves as a "punishment signal" that induces retraction of nerve terminals through p75^{NTR}. In parallel, neuronal activity drives secretion and/or activation of metalloproteases to convert proBDNF to mBDNF, a reward signal for which all terminals compete.

Although our "gain-of-function" pharmacological studies demonstrated that the conversion of the punishment signal (proBDNF) to the reward signal (mBDNF) is critical for synapse elimination, results from the "loss-of-function" experiments using knock-out mice were not so straightforward. For example, BDNF KO NMJs showed normal synapse elimination (Fig. 1*E*,*F*). This result is not completely unexpected, because genetic compensation is quite common in neurotrophin knock-out mice (Conover et al., 1995; Liu et al., 1995). Furthermore, deletion of the *p75^{NTR}* gene did not attenuate synapse elimination. This may due to that the punishment signal (proBDNF) at the p75$^{NTR-/-}$ NMJ may be mediated or compensated for by other proBDNF receptors such as sortilin. In support for this hypothesis, the injection of a sortilin antagonist to the LAL muscle in the p75$^{NTR-/-}$ significantly increased polyinnervation (Fig. 4*A*),

whereas blockade of sortilin signal alone in the wild-type muscle did not affect synapse elimination (Fig. 4*A*,*B*).

The other possibility to explain the lack of phenotype in $p75^{NTR-/-}$ NMJ is that the effect of inhibiting $p75^{NTR}$ is only relevant during the period of synapse elimination. In this model, deletion of the $p75^{NTR}$ gene in early embryos may eliminate the need for competition, leading to a default single innervation at the postnatal NMJ. Notably, similar "default" mechanisms have been reported. For example, although agrin is required for AChR clustering later in development, the AChR clusters form normally by default in early embryos of agrin knock-out mice (Lin et al., 2001). Additionally, in the visual cortex, complete inhibition of neuronal activity early in development does not prevent the formation of ocular dominance columns, although postnatal manipulation of visual activity drastically alters ocular dominance (Crowley and Katz, 1999, 2000). In this study, we show that infusion of the $p75^{NTR}$ blocking antibody to wild-type NMJs during the process of synapse competition markedly attenuated synapse elimination (Fig. 4*A*,*B*). Similarly, inhibition of TrkB signaling by daily injection of 1NMPP1 to $TrkB^{F616A}$ knock-in mice accelerated synapse elimination (Fig. 1*D*).

Together, our study supports a model that single innervation of a NMJ may be a default mechanism during early development, and the punishment (proBDNF)-reward (mBDNF) system acts postnatally to ensure the precision of the motorneuron-muscle connections *in vivo*.

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