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Regulation of Neuromuscular Synapse Development by Glial Cell Line-derived Neurotrophic Factor and Neurturin*

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Glial cell line-derived neurotrophic factor (GDNF) is known for its potent effect on neuronal survival, but its role in the development and function of synapses is not well studied. Using *Xenopus* nerve-muscle co-cultures, we show that GDNF and its family member neurturin (NRTN) facilitate the development of the neuromuscular junction (NMJ). Long-term application of GDNF significantly increased the total length of neurites in the motoneurons. GDNF also caused an increase in the number and the size of synaptic vesicle clustering, as demonstrated by synaptobrevin-GFP fluorescent imaging, and FM dye staining. Electrophysiological experiments revealed two effects of GDNF on synaptic transmission at NMJ. First, GDNF markedly increased the frequency of spontaneous transmission and decreased the variability of evoked transmission, suggesting an enhancement of transmitter secretion. Second, GDNF elicited a small increase in the quantal size, without affecting the average rise and decay times of synaptic currents. Imaging analysis showed that the size of acetylcholine receptor clusters at synapses increased in muscle cells overexpressing GDNF. Neurturin had very similar effects as GDNF. These results suggest that GDNF and NRTN are new neuromodulators that regulate the development of the neuromuscular synapse through both pre- and postsynaptic mechanisms.

Studies in the last few years suggest that neurotrophins, originally defined as a family of trophic factors essential for neuronal survival, also regulate synaptic transmission and plasticity (for reviews, see Refs. 1–3). The first evidence for such a new role was the demonstration that brain-derived neurotrophin (BDNF)[1] and neurotrophin-3 (NT3) acutely potentiate synaptic transmission at the *Xenopus* neuromuscular synapse in culture (4). Subsequent experiments from many laboratories have demonstrated regulatory effects of neurotrophins on synapses in a variety of model systems. For example, changes in the level of BDNF in the visual cortex alter the development of ocular dominance synapses (5, 6). Consistent with this, neurotrophins seem to have profound effects on the growth of dendrites of cortical neurons and afferent axons of thalamic neurons (7, 8). In the hippocampus, BDNF acutely facilitates long-term potentiation (9–12). Neurotrophins have also been shown to rapidly regulate synaptic transmission in various cultured neurons (13–16). Mechanistic studies of the role of neurotrophins in synaptic transmission have largely been carried out in the *Xenopus* nerve-muscle co-cultures. Two major effects of neurotrophins have been described on the neuromuscular synapse: acute enhancement of neurotransmitter release (4, 17–22), and long-term regulation of synapse maturation (23–26). Despite of the rapid progress, a number of important issues still await to be addressed. For example, while the acute effects of neurotrophic factors on synaptic transmission have attracted a great deal of interest, much less is known about cellular and molecular mechanisms underlying the long-term synaptotrophic effects. The relationships between the acute and long-term neurotrophic effects remain unclear. Furthermore, the synaptic functions of trophic factors other than neurotrophins were largely unexplored. In this paper, we study the long-term effects of GDNF and its family member neurturin on the development of the neuromuscular synapse, and their potential mechanisms.

GDNF belongs to a newly identified family of neurotrophic factors, which include GDNF, NRTN, artemin, and persephin (27–30). The functions of GDNF ligands are mediated by a two-component receptor complex. One is a common signaling component, the c-Ret receptor tyrosine kinase, and the other a glycosylphosphatidylinositol-anchored protein called GFR-α, which binds ligand with high affinity and determines the specificity (for review, see Refs. 31 and 32). GDNF binds preferentially to GFR-α1, NRTN to GFR-α2, artemin to GFR-α3, and persephin to GFR-α4. At higher concentrations NRTN is also capable of signaling through GFR-α1, and GDNF through GFR-α2 (33–36). The binding of GDNF ligands to GFR-αs leads to recruitment and activation of c-Ret tyrosine kinase activity. One of the major targets of GDNF is the motoneuron in the spinal cord. Several lines of evidence suggest that GDNF attenuates programmed cell death of motoneurons during development (37, 38) and after axotomy in the adult (39). Neurturin has also been shown to regulate motoneuron survival (40–42). *In situ* hybridization experiments demonstrated that GDNF and NRTN are expressed in developing muscle cells (37, 43, 44). GDNF and NRTN are also retrogradely transported by

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[1] The abbreviations used are: BDNF, brain-derived neurotrophin; AChR, acetylcholine receptor; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescence protein; GFR-α, GDNF family receptor α; NMJ, neuromuscular junction; NT3, neurotrophin-3; α-BTX, rhodamine-conjugated α-bungarotoxin; SSCs and ESCs, spontaneous and evoked synaptic currents; PBS, phosphate-buffered saline; DIC, differential interference contrast.

spinal motoneurons (39, 45), GFR-α1, -α2, and c-ret mRNAs and proteins are detected in spinal motoneurons (44, 46–48). Transgenic mice overexpressing GDNF in skeletal muscle cells exhibit hyperinnervation of the neuromuscular junction (NMJ) (49). While these results raise the possibility that GDNF and NRTN produced in the target muscle cells may retrogradely regulate spinal motoneurons, the exact role of these GDNF ligands in the development and/or function of the NMJ remains to be established.

Because of its simplicity and easy accessibility for molecular manipulation at pre- and postsynaptic sites, the NMJ has long been an excellent model system to study synaptic transmission and synapse development (50). One area that the NMJ preparation is particularly useful is to study the development of quantal transmission mechanism. Experiments using *Xenopus* nerve-muscle cultures have described a series of physiological and morphological events associated with the developmental process (51, 52). The physiological events include a gradual increase of the frequency and amplitude of spontaneous synaptic currents (SSCs) (53), and a striking transition from a skew to a bell-shaped distribution of SSC amplitudes (54, 55). Moreover, the amplitudes of impulse-evoked synaptic currents (ESCs) become much larger and more consistent (53–56). Morphologically, synaptic vesicles gradually aggregate to form synaptic varicosities, both pre- and postsynaptic membranes thicken, and basal lamina material appears in the synaptic cleft (51, 52, 57–59). Moreover, ACh receptors (AChR) gradually cluster on the postsynaptic membrane at the NMJ (56). A series of recent studies have demonstrated the long-term enhancement of synaptic efficacy at the neuromuscular synapse by neurotrophins. These effects may involve changes in both the quantal secretion mechanism in the presynaptic site (23–25), and the AChR channel properties in the postsynaptic site (20, 60). In the present study, we have examined the role of the GDNF family of neurotrophic factors in the development of the neuromuscular synapse. We found that long-term treatment of the *Xenopus* nerve-muscle co-culture with GDNF or NRTN significantly promotes axonal growth, and facilitates aggregation of synaptic vesicles in the presynaptic terminals. Furthermore, we show that GDNF and NRTN enhance not only transmitter release, but also AChR clustering. These results define a new role of GDNF and NRTN in quantal synaptic transmission, and provide new insights into how long-term regulation of synapse development can be achieved.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Embryo Injection—Human GDNF, NRTN, or enhanced green fluorescence protein (GFP) (from CLONTECH) cDNA was subcloned into the pSP6TS vector containing the 5′- and 3′-untranslated regions of the *Xenopus* β-globin gene (kindly provided by Yi Rao, Washington University). The cDNA for synaptobrevin-GFP (GFP fused in-frame at the C terminus of synaptobrevin, cloned in pS65T vector) was a gift from Susana Cohen-Cory of UCLA. The plasmids were linearized and extracted by phenol/chloroform. Capped mRNAs for GDNF, NRTN, GFP, and synaptobrevin-GFP were prepared by *in vitro* transcription using the linearized plasmids, RNA polymerase (SP6 or T3), and mMessage mMachine kit (Ambion). Quality of mRNA was determined first by RNA agarose gel, and then by an *in vitro* translation system (TNT-coupled reticulocyte lysate). GDNF or NRTN mRNA, but not GFP-synaptobrevin mRNA, was mixed with GFP mRNA at 1:1 ratio. The mRNAs were injected into one of the blastomeres at the 2–4-cell stage using a Picrospritzer. The final concentration of the mRNAs within an injected blastomere was ~5 ng/μl, and injection volume was ~1.5 nl. After injection, the injected embryos were placed in a 25 °C incubator for 1 day, and neural tube and associated myotomal tissues from stage 20 to 22 embryos were used to prepare nerve-muscle cultures.

Culture Preparation—*Xenopus* nerve-muscle cultures were prepared according to the procedure described previously (55). Briefly, neural tube and associated myotomal tissue of stage 20 to 22 *Xenopus* embryos were dissociated in Ca^{2+}-Mg^{2+}-free saline supplemented with EDTA (58.2 mM NaCl, 0.7 mM KCl, 0.3 mM EDTA, pH 7.4) for 15–20 min. Cells were plated on glass coverslips, and grown in the presence or absence of different factors for 1–3 days at room temperature (20 °C). The culture medium consisted (v/v) of 50% L-15 medium (Sigma), 1% fetal calf serum (Invitrogen), and 49% Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.6). Various neurotrophic factors (human GDNF, NRTN, or transforming growth factor-β1, etc. from PeproTech or Amgen) and/or antibodies (anti-GFR-α1, or anti-GFR-α2, Santa Cruz) were added to the cultures after cells were completely settled (6 h after plating), and kept in the medium until the time of experiments (1 day, 1-day). For longer term experiments (2–3 days), the factors were added every 12–24 h.

GFP-synaptobrevin Imaging—Fluorescent images of GFP-synaptobrevin were acquired by a MicroMax 1300 cool CCD camera (Roper Scientific) mounted on a Nikon Diaphot 300 inverted microscope and analyzed using IPLab software (Scanalytics). Fluorescence images were taken with 1-s exposure time with a ×40, 0.85NA objective. The pseudo color (green) was assigned to fluorescent images and the superimposed DIC and fluorescence images were created by the IPLab software. For quantitative analysis, we first calculated background intensity by averaging the numbers obtained from three non-fluorescent areas along an axon. Next we set the threshold for detecting fluorescent spots to 50% above the background intensity of that cell, and normalized the intensity of the fluorescent spots (50% above the threshold) to the background intensity. Fluorescent spots larger than 2.3 ($1.5^2 = 2.25$) μm^2 were defined as synaptic vesicle clusters. The number, size, and intensity of the fluorescence spots were measured, using the region-of-interest tools in the IPLAB program.

FM Dye Staining—The FM dye labeling was carried out as described (61, 62). Briefly, the fluorescent styryl membrane dye FM 1–43 (Molecular Probes) was loaded into the spinal neurons by incubating the control, GDNF-, or NRTN-treated cultures with high K^+ loading solution containing (KCl, 60 mM; NaCl, 57.6 mM; $CaCl_2$, 3.5 mM; Hepes, 10 mM, pH 7.6; FM 1–43, 2 μM) for 2 min. Cells were then rinsed extensively with Ringer's solution, lightly fixed (2% paraformaldehyde in Ringer's), and rinsed again. The culture coverslips were mounted onto glass slides, and imaged under an upright fluorescence microscope with a standard GFP filter set, and a ×60 oil emersion objective (N.A. 1.5). The images were acquired by the MicroMax camera and analyzed by the IPLab software as described above.

Electrophysiology—Synaptic currents were recorded from innervated muscle cells using whole cell recording techniques at room temperature in culture medium (55). The solution inside the whole cell recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM Hepes buffer (pH 7.2). To elicit evoked synaptic currents, square current pulses (0.5 ms, 0.5–5 volts, 0.05 Hz) were applied with a patch electrode filled with Ringer's solution at neuronal somata under loose seal conditions. All data were collected using a patch clamp amplifier (EPC-7), with a current signal filtered at 3 kHz. The data were stored on a videotape recorder for later playback on a storage oscilloscope (Textronic TDS 420) and a chart recorder (Gould EasyGraf 240), or analysis by a desktop computer. The amplitude, rise, and decay times of SSCs and ESCs were analyzed using the SCAN program (Dagan, Inc.).

Immunocytochemistry—The *Xenopus* cultures were fixed with 2% paraformaldehyde and 0.125% glutaraldehyde (EM Science) for 15 min at room temperature, and washed 3 times with phosphate-buffered saline (PBS). For phospho-Akt (pAkt), the cells were permeabilized with 0.125% Triton X-100 (in PBS) for 5 min. For cell surface protein (GFR-α1, GFR-α2, and c-Ret), the permeabilization step was eliminated. All cultures were incubated with 10% H_2O_2 in PBS overnight at 4 °C to block the endogenous peroxidase activity and rinsed again for 3 times in PBS. The cultures were treated with a blocking solution (50% normal goat serum in PBS) for 3 h in room temperature, and then incubated with the following primary antibodies at 4 °C overnight: c-Ret, GFR-α1, or GFR-α2 (all goat antibodies from Santa Cruz, diluted in PBS by 1:500, or 0.4 μg/ml), and pAkt (rabbit antibody from Promega, diluted at 1:100 in 5% bovine serum albumin in PBS). Pretreatment of the cultures with the peptides used to generate the antibodies against GFR-α1 and GFR-α2 (N-18 and C-20, 4 μg/ml, overnight at room temperature), respectively, prevented the specific stainings by the primary antibodies. Thus, these antibodies were capable of detecting endogenous *Xenopus* GFR-α1 and GFR-α2. After incubation with primary antibodies, the cultures were extensively washed (5 times in PBS), incubated with biotinylated secondary antibody (goat anti-rabbit for GDNF receptors and horse anti-goat for pAkt, 1:1000, all from Vector) in PBS for 30 min, rinsed 5 times again, and reacted with ABC reagent according to manufacturer's instructions (ABC kit, Vector), all done in room tempera-

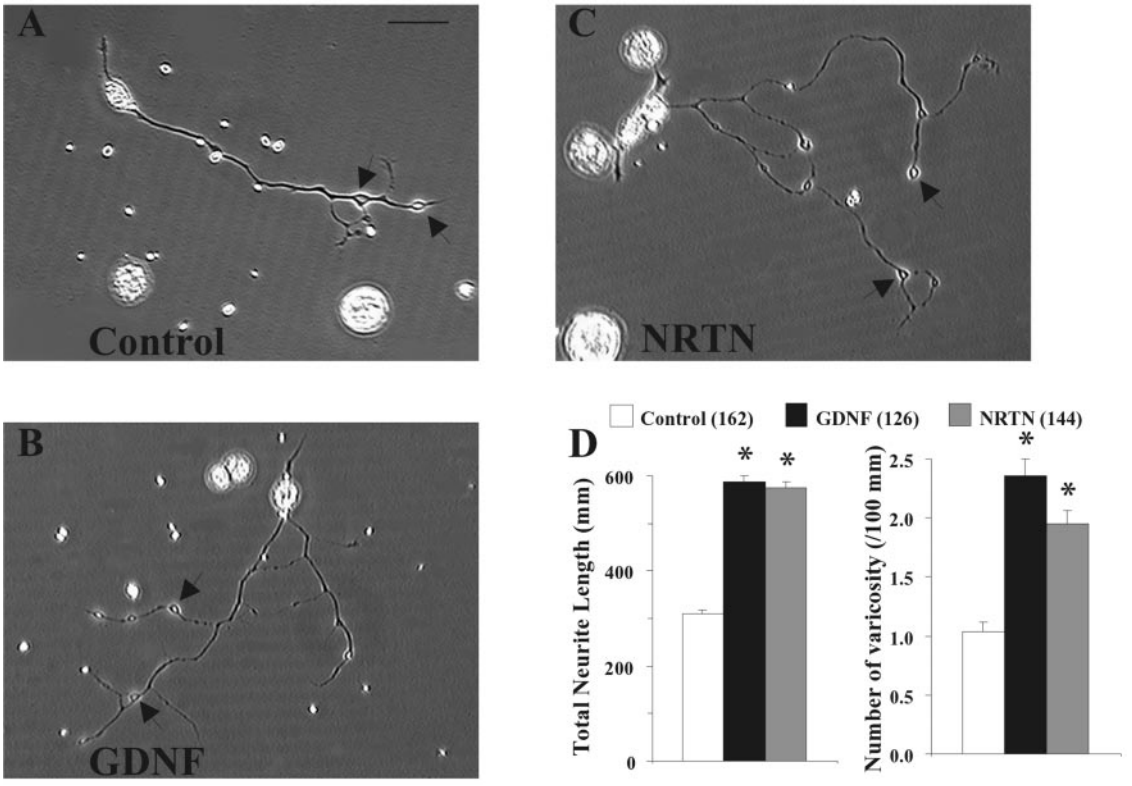

FIG. 1. **Morphological changes in spinal motoneurons induced by long-term treatment with GDNF or NRTN.** GDNF or NRTN was applied to the nerve-muscle co-cultures 6 h after plating, and the cultures were examined by phase-contrast microscopy 1 day later. *A–C*, examples of spinal neurons treated with or without GDNF or NRTN, viewed by a phase microscope. *Scale bar*, 20 μm. *D*, summary of the effects of GDNF or NRTN on total length of axons. *, significantly different from control. Student's *t* test; $p < 0.05$. Unless indicated otherwise, the data in this and all other figures are mean ± S.E. obtained from 1-day-old cultures. The number associated with each column is the number of cells examined.

ture. The cultures were then washed 5 times in PBS, and 2 times in TBS, reacted with diaminobenzidine tetrahydrochloride at low concentration for 1 min, and washed again 2 times in PBS. The cells were dehydrolyzed with alcohol and xyline and the coverslips were mounted onto glass slides with a mounting solution (Fisher). The images of immunocytochemistry were viewed using a DIC microscope with a ×40 objective, captured by the Optronics CCD camera and exported to a desktop computer. At least 20 neurons from several different batches of cultures were examined for each condition, and consistent results were obtained.

ACh Receptor Clustering—AChR clusters were labeled with rhodamine-conjugated α-bungarotoxin (α-BTX), as previously described (63). Briefly, the nerve-muscle cultures (1-day old) were incubated with α-BTX (0.2 μM, Molecular Probes) for 30 min at room temperature. Following labeling, the cultures were rinsed with PBS, and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde (EM Sciences) in PBS for 15 min. The fixed cells then were rinsed with PBS and distilled water for 5 min, respectively, dehydrolyzed, and mounted onto glass slides with a mounting solution (Fisher). Images of AChR clusters on either isolated or innervated myocytes were acquired in the same way described above. The numbers, intensity, and area of the clusters were analyzed using the region-of-interest tools in the IPLAB program.

RESULTS

Morphological Effects on Motoneurons—In an attempt to thoroughly characterize the role of GDNF or NRTN in neuromuscular development, we first examined whether these factors can induce any morphological changes in the presynaptic motoneurons. The nerve-muscle co-cultures were grown in the absence or presence of GDNF or NRTN for 1–3 days. We measured total neurite length (summation of the lengths of all neurites per neuron). Long-term treatment of the cultures with GDNF (1 ng/ml, in this and all other experiments unless indicated otherwise) resulted in a dramatic increase in total neurite length of the motor axons (Fig. 1, *A* and *B*). The effects could be observed as short as 1 day, but longer treatments (2–3 days) elicited more pronounced effects. Quantitation of data from 1-day cultures indicates that treatment with GDNF increased the total neurite length by 83% (Fig. 1*D*). Neurturin had similar effects but required a higher concentration (10 ng/ml, Fig. 1). In these cultures, many neurons exhibited "morphological varicosities" (enlargements larger than 2 times axon diameter) along their axons, resembling synaptic varicosities (Fig. 1). Treatment with GDNF and NRTN also increased the number of these morphological varicosities.

Synaptic varicosities contain clusters of synaptic vesicles as well as other presynaptic elements such as machineries for exocytosis and endocytosis, and therefore are considered as the morphological basis of nerve terminals. To determine whether synaptic vesicles were indeed clustered in these morphological varicosity, we labeled all synaptic vesicles and their precursors using the synaptic vesicle protein synaptobrevin fused with green fluorescence protein at its C terminus (synaptobrevin-GFP). Messenger RNA for the synaptobrevin-GFP fusion protein was injected into one of the *Xenopus* blastomeres at the 2-cell stage. Neurons and muscle cells derived from the injected embryos were plated at low density on glass-bottom culture dishes. Live cells grown in the presence or absence of GDNF or NRTN for 2 days were imaged in an inverted fluorescence microscope. Fig. 2*A* shows an example of a motoneuron exhibiting synaptobrevin-GFP fluorescent spots along their axons. Single vesicles are too small to be resolved by light microscopy. These spots therefore represent clusters of synaptobrevin-GFP containing synaptic vesicles or "pre-assembled terminals" (64). Comparison of differential interference contrast (DIC) and fluorescent images indicated that most of the morphological varicosities along the axons contained the fluorescent spots (Fig. 2*A*), suggesting that they were indeed synaptic varicosities.

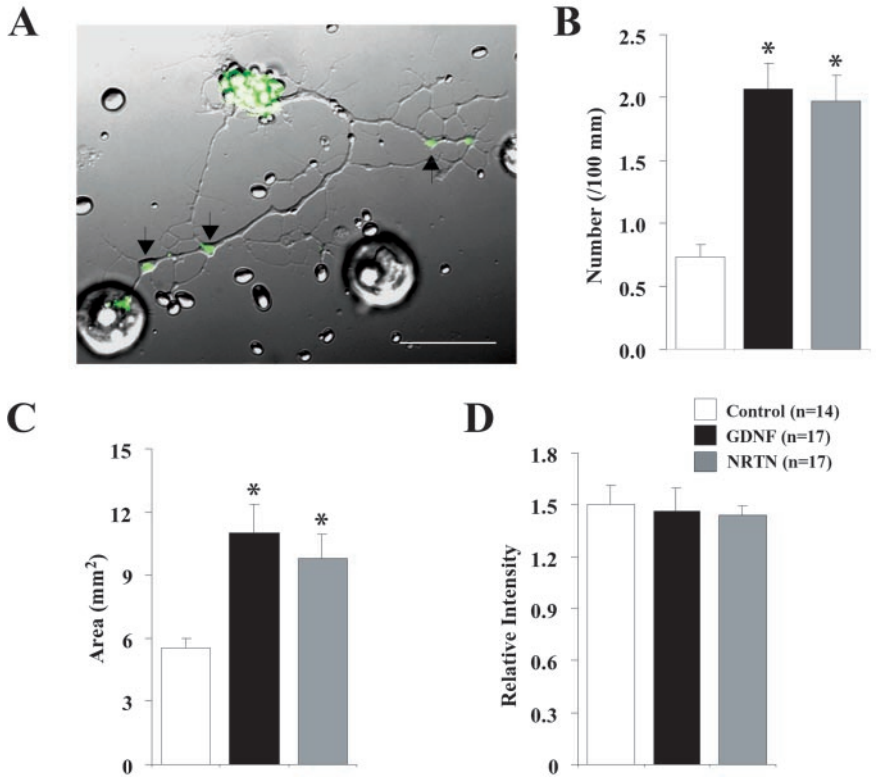

FIG. 2. **Clustering of synaptic vesicles in neurons treated with GDNF or NRTN.** GFP-synaptobrevin mRNA was injected into *Xenopus* embryos at the 2-cell stage, and cultures were prepared using the injected embryos at stage 20–22. The cultures were grown in the presence or absence of GDNF or NRTN for 2 days. Synaptic vesicle clusters were visualized by GFP fluorescence. *A*, superimposed DIC and GFP fluorescence images of a neuron in a NRTN-treated culture. *Scale bar*, 20 μm. *B–D*, quantitative measures of the number, size, and fluorescence intensity of synaptobrevin-GFP clusters, using region-of-interest tool in the IPLAB software.

The average diameter of axons was 1.5 μm and the average diameter of morphological varicosities was around 3.0 μm. To facilitate quantitative analysis, we set the threshold for detecting fluorescent spots to 50% above the background intensity (averaged from 3 non-fluorescent areas along the same axons), and normalized the intensity of the fluorescent spots (50% above the threshold) to the background intensity. Fluorescent spots larger than 2.3 ($1.5^2 = 2.25$) μm^2 were defined as synaptic varicosities. The number, size, and intensity of the fluorescent spots were measured, using the region-of-interest tools in the IPLAB program. Long-term treatment with GDNF or NRTN significantly increased the numbers of the fluorescent spots per unit length of axons (Fig. 2*B*). Moreover, the spots were larger in cultures treated with GDNF or NRTN than those in control cultures. Quantitative analysis showed that GDNF increased the number of the spots by almost 2-fold and the area of the spots by 1-fold, without changing the relative fluorescence intensities (Fig. 2, *C* and *D*). NRTN had similar effects. Since the fluorescence spots were clusters of presynaptic vesicular structures, the increase in the number and area of clusters suggest that GDNF and NRTN facilitate the formation of presynaptic terminals.

Synaptobrevin-GFP labels synaptic vesicles as well as other precursor structures (64). To further investigate whether GDNF or NRTN is truly involved in synaptic vesicle clustering, we used FM dye staining as an alternative method to label synaptic vesicles (61, 62). Depolarization of motoneurons by high K^+ (60 mM) in the presence of FM 1–43 (2 μM) elicits massive vesicle fusion, followed by rapid internalization of FM 1–43 dye which labels all recycling synaptic vesicles. Strong fluorescence spots representing characteristic dye-loaded vesicles were observed along the axons of motoneurons in all cultures (Fig. 3*A*). In neurons treated with GDNF or NRTN, the number and size of the FM dye spots were significantly increased (Fig. 3*B*). Moreover, the fluorescence intensity was also increased by GDNF or NRTN treatment. These results not only suggest an enhancement of synaptic vesicle clustering, but also imply an increase in the number of release sites by GDNF or NRTN treatment.

Acute and Long-term Regulation of Spontaneous Synaptic Activity—To examine the physiological consequences of the presynaptic differentiation induced by long-term treatment with GDNF or NRTN, we recorded synaptic activity at the neuromuscular synapses using whole cell, voltage-clamped recording techniques. In these cultures, synaptic contacts are established within the first day after plating, and synaptic activity undergoes a maturation process that takes 4–5 days (53–56). Fig. 4 shows SSCs recorded from synapses in control, GDNF- and NRTN-treated cultures. The SSCs are induced by spontaneous secretion of individual ACh-containing synaptic vesicles from motor nerve terminals independent of action potentials, since they are not affected in the presence of tetrodotoxin (data not shown). Long-term treatment of GDNF dramatically increased the frequency of SSCs, suggesting an enhancement of transmitter release. The mean frequency of the SSCs recorded from GDNF- and NRTN-treated synapses in 1-day-old cultures were 4.6- and 3.6-fold, respectively, of those in control cultures (Table I). In cultures treated with GDNF or NRTN for 2 days, SSC frequencies were 21.7 ± 3.7 and 18.2 ± 3.2 events/min, respectively, while that in control cultures was 7.0 ± 0.9 events/min.

In addition to their potent effects on SSC frequency, GDNF or NRTN also elicited a small but significant increase in SSC amplitude. Synapses treated with GDNF or NRTN exhibited a significant "right shift" in their cumulative frequency plots of SSC amplitude distribution (Fig. 5*A*). A detailed analysis of SSC time course indicated that treatment with GDNF or NRTN for 1 day increased the average amplitude of SSCs increased by 51.6 and 63.3%, respectively. Similar differences were observed in cultures treated for 2 days (Table I). An increase in SSC amplitude, if accompanied by an increase in SSC decay time, is usually due to an increase in the open time of AChR channels (20, 26, 65). GDNF or NRTN did not affect the SSC decay time in both 1- and 2-day-old cultures (Table I).

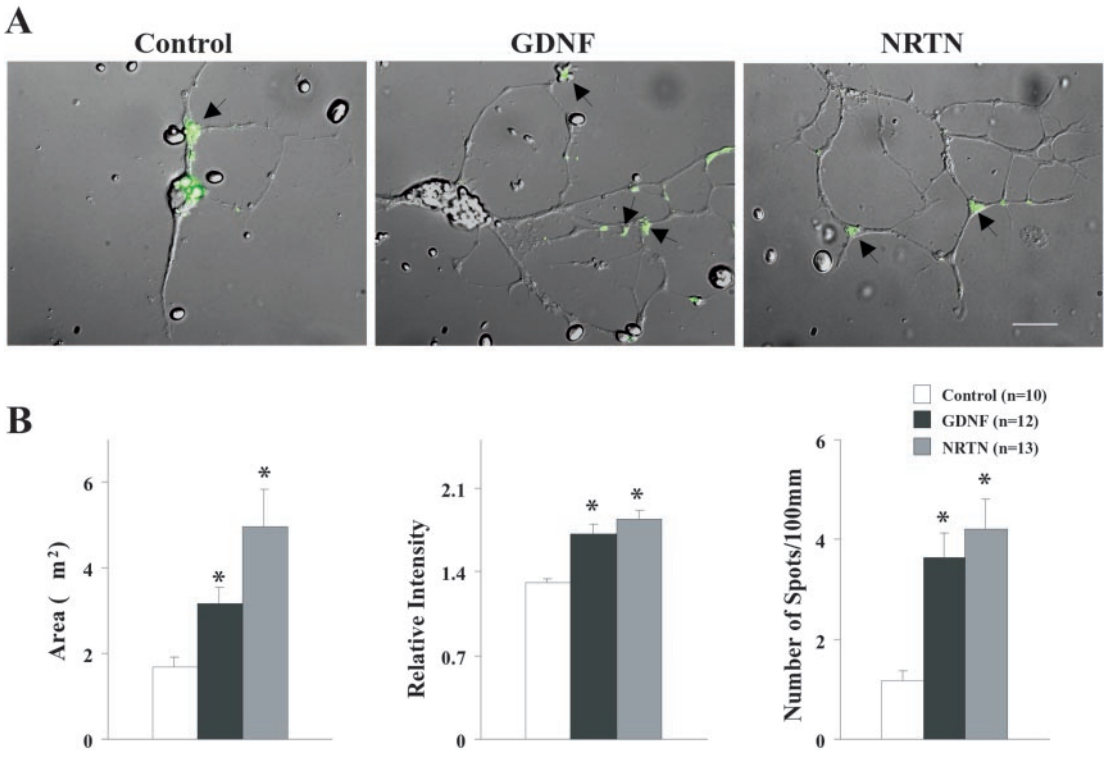

FIG. 3. **FM dye staining of neurons treated with GDNF or NRTN.** FM dye was loaded into spinal neurons by exposure to high K^+ (60 mM) Ringer's solution, followed by extensive wash and light fixing. *A*, superimposed DIC and fluorescence images of FM dye-labeled neurons in control, GDNF- or NRTN-treated conditions. The cell body of the neuron in NRTN-treated culture is outside of the image. *Scale bar*, 10 μm. *B*, quantitative measures of the area, relative fluorescence intensity and number of FM dye-labeled vesicle clusters, using region-of-interest tool in the IPLAB software.

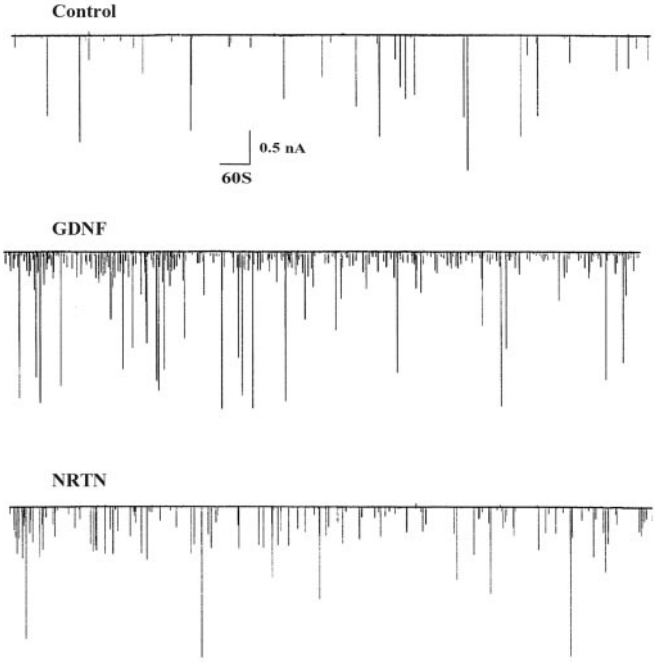

FIG. 4. **Long-term regulation of SSCs by GDNF or NRTN.** GDNF or NRTN was applied to the culture 6 h after plating and kept in the medium for 2 days. SSCs (downward deflections of varying amplitudes) were recorded using whole cell, voltage-clamp recording (Vh = −70 mV, filtered at 150 Hz).

To more accurately measure the SSC decay time, we constructed average SSC waveforms (Fig. 5*B*). Scaling of averaged SSCs in control, GDNF- and NRTN-treated synapses indicate that there was no change in either rise or decay time of SSCs (Fig. 5*B*). These results suggest that the increase in SSC amplitude is not due to an increase in open time of AChR channels.

Neurotrophins have been shown to acutely enhance synaptic transmission at the neuromuscular synapse, in addition to their long-term effects (4, 17, 21). We examined whether GDNF has similar acute action on synaptic transmission. Acute application of GDNF (final concentration 10 ng/ml) to synapses failed to elicit any changes in SSCs (Fig. 6*A*). Quantitative analysis indicated that all parameters of SSCs, including frequency, amplitude, rise, and decay times, remained the same before and after GDNF application (Fig. 6*B*). Application of NRTN (10 ng/ml) also had no effect on SSCs (Fig. 6*B*). Thus, unlike neurotrophins, GDNF and NRTN do not have an acute effect on synaptic transmission at NMJ.

Specificity and Mode of Action—The functions of GDNF family of neurotrophic factors are mediated by a family of GPI-linked receptors called GFR-α, and the c-Ret tyrosine kinase (31, 32). Immunocytochemistry was performed to determine the specific receptors expressed in the *Xenopus* motoneurons. As shown in Fig. 7*A*, GFR-α1, the preferred receptor for GDNF, was detected in almost all the motoneurons by an antibody against human GFR-α1. The staining was specific because it could be blocked by pretreatment with excess amount of peptide antigen (Fig. 7*B*). In contrast, GFR-α2, the preferred receptor for NRTN, was almost undetectable (Fig. 7*C*). Only two out of 20 spinal neurons were stained positively by the anti-GFR-α2 antibody (data not shown). These results are consistent with the recent study showing that majority of motoneurons in the mouse spinal cord express GFR-α1 and not GFR-α2 (44, 47, 48). We also detected the expression of c-Ret in the motoneurons (Fig. 7*D*). Recently, GDNF-induced tyrosine phosphorylation and activation of c-Ret has been linked to the activation of phosphatidylinositol 3-kinase in motoneurons (66). We showed, by immunocytochemistry using an antibody

TABLE I

Effects of GDNF or NRTN on spontaneous synaptic currents (SSCs)

SSCs (approximately 100 SSC events in all cases) recorded before and 10 min after application of GDNF were collected for analysis. Rise time refers to interval between 10 and 90% of the peak amplitude of SSCs. Decay phase of the SSCs is fitted by a single exponential curve and decay time is defined as the time needed for amplitude of SSCs to drop to 1/e of the peak value.

	Rise time	Decay time	Amplitude	Frequency	*n*
	ms		*pA*	*min*	
1 Day					
Control	1.34 ± 0.09	10.97 ± 1.05	349.0 ± 43.3	4.01 ± 0.62	12
GDNF	1.33 ± 0.11	12.22 ± 0.84	416.6 ± 33.7[a]	20.32 ± 2.66[a]	11
Neurturin	1.26 ± 0.07	11.42 ± 0.75	522.9 ± 41.8[a]	13.86 ± 2.87[a]	10
2 Days					
Control	1.11 ± 0.07	7.62 ± 0.57	289.9 ± 42.9	6.95 ± 0.85	13
GDNF	1.18 ± 0.12	9.84 ± 1.05	567.1 ± 85.6[a]	21.73 ± 3.71[a]	11
Neurturin	1.01 ± 0.10	7.52 ± 0.87	392.5 ± 43.1[a]	18.18 ± 3.19[a]	10
NT3	0.85 ± 0.10[a]	10.12 ± 1.07	488.5 ± 119.2[a]	10.51 ± 3.93[a]	6

[a] Significantly different from control (*t* test, $p < 0.05$).

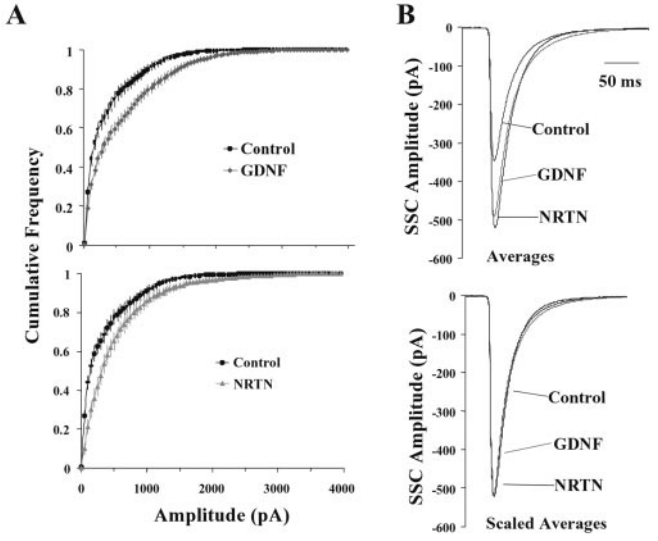

FIG. 5. **Long-term effects of GDNF or NRTN on the amplitude and waveform of SSCs.** *A*, comparison of averaged histograms of SSC amplitude distributions between control and GDNF-treated synapses (*top*), and those between control and NRTN-treated synapses (*bottom*). The data are presented as cumulative frequency (the proportion of total events with amplitudes smaller than a given amplitude). The plots represent averaged amplitude distribution from a number of synapses (control, 13; GDNF, 22; NRTN, 9), each with at least 120 SSC events. The amplitude distributions for GDNF- and NRTN-treated synapses are significantly different from that of control synapses (Kolmogorov-Smirnov test, $p < 0.05$). *B*, the effect of GDNF or NRTN on SSC waveforms. For each synapse, more than 100 SSC events were averaged to obtain a single waveform. Waveforms from synapses recorded in control ($n = 12$), GDNF-treated ($n = 11$), and NRTN-treated ($n = 10$) conditions, respectively, were averaged (*top*). The averaged waveforms for control and GDNF-treated synapses were then scaled to the size of NRTN-treated synapses for better comparison (*bottom*). Note that GDNF and NRTN both increased the amplitude, but not the rise and decay times of SSCs.

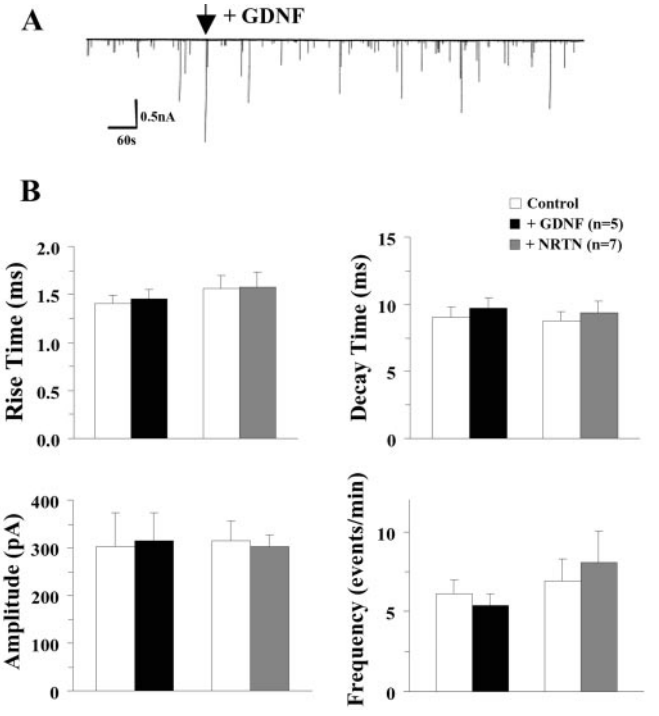

FIG. 6. **Acute effect of GDNF or NRTN on spontaneous synaptic activity.** GDNF or NRTN was applied directly to the medium of 1-day-old cultures. *A*, an example of SSCs recorded from an innervated myocyte before and after GDNF application. *B*, summary of the acute effects of GDNF or NRTN on the properties of SSCs.

against the phosphorylated form of Akt, that application of GDNF rapidly induced the phosphorylation of Akt (Fig. 7, *E* and *F*). These results suggest that human recombinant GDNF is capable of activating c-Ret in *Xenopus* motoneurons.

We then determined dose-response relationships for GDNF/NRTN, using SSC frequency as a functional assay for synaptic efficacy. As shown in Fig. 8*A*, GDNF was able to increase SSC frequency at a concentration as low as 1 ng/ml, or 40 pM. NRTN, on the other hand, requires 5–10 ng/ml to elicit similar effect (Fig. 8*A*). Thus, GDNF appears to be more potent than NRTN in modulating synaptic efficacy. This may be due to the fact that most of the motoneurons expressed GFR-α1 but not GFR-α2 receptors (Fig. 7). Consistent with the above results, the GDNF effects were blocked by preincubation of the cultures with an antibody against GFR-α1, but not by anti-GFR-α2, suggesting that the GDNF actions are mediated primarily by GFR-α1 (Fig. 8*B*). Although GDNF preferentially interacts with GFR-α1 and NRTN with GFR-α2, at higher concentrations the two ligands can cross-talk to the two receptors (33–36). Indeed, the effects of NRTN were also blocked by anti-GFR-α1, but not by anti-GFR-α2 antibodies (Fig. 8*B*). Thus, it is likely that NRTN also signal through GFR-α1 to enhance synaptic transmission at NMJ. Neither transforming growth factor-β1 nor nerve growth factor affected any parameters of SSCs (Fig. 8*C*). NT3 also potentiated SSC amplitude and frequency (see also Ref. 23). However, GDNF and NRTN differed from NT3 in that they had no effect on the rise time of SSCs (Table I).

GDNF or NRTN could be derived from presynaptic motoneurons and act in an autocrine or paracrine manner. Alterna-

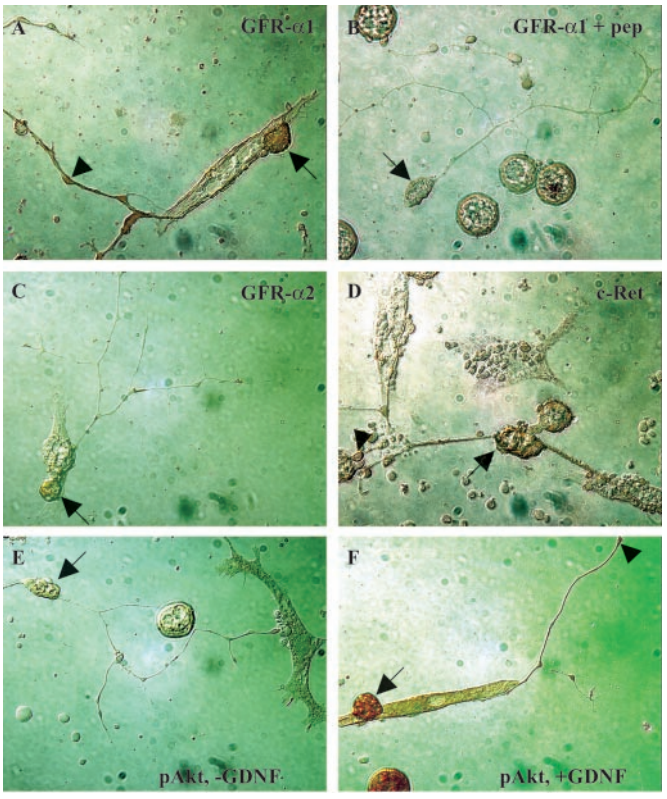

FIG. 7. **Expression and activation of GDNF receptors.** *A–D*, immunocytochemical detection of GDNF receptors. *Xenopus* nerve-muscle were fixed and stained with antibodies against GFR-α1 (*A*), GFR-α1 plus GFR-α1 peptide (*B*), GFR-α2 (*C*), and c-Ret (*D*). *E* and *F*, activation of phosphatidylinositol 3-kinase pathways. The cultures were treated with or without GDNF for 15 min, fixed, and processed for immunocytochemistry using specific antibodies against phospho-Akt 437. *Arrows* point to neuronal cell bodies while arrowheads indicate stainings on neurites and terminals. *M*, muscle cell.

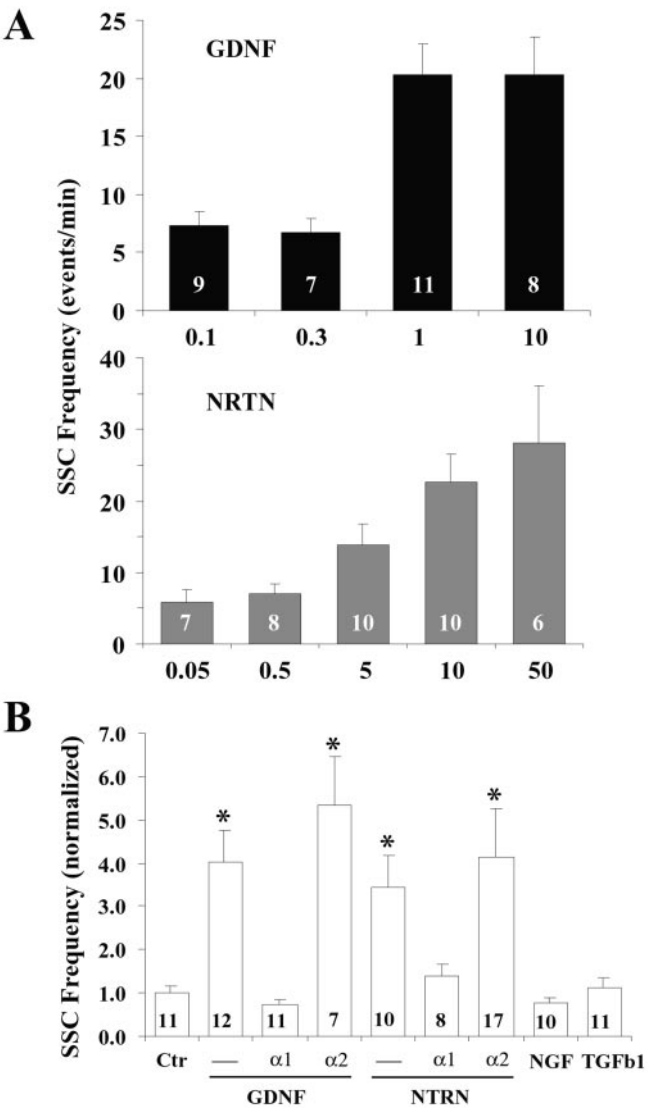

FIG. 8. **Specificity of the long-term effects of GDNF or NRTN on spontaneous synaptic activity.** Various neurotrophic factors were applied to the nerve-muscle cultures 6 h after plating, and the frequencies of SSCs were measured in 1-day-old synapses. *A*, dose-response curves for GDNF and NRTN. The concentrations of GDNF and NRTN are indicated in the *bottom* of the plots (in ng/ml). *B*, effects of various neurotrophic factors and antibodies. GDNF, 1 ng/ml; NRTN, 10 ng/ml; nerve growth factor (*NGF*), 25 ng/ml; transforming growth factor-β1 (*TGF-β1*), 20 ng/ml; anti-GFR-α1 antibody, 50 ng/ml; anti-GFR-α2 antibody, 50 ng/ml. *, groups that are significantly different from others. ANOVA and post-hoc test; $p < 0.05$.

tively, they could be derived from postsynaptic muscle cells and serve as target-derived factors. To determine the mode of action of these factors, we overexpressed GDNF or NRTN either in presynaptic neurons or in postsynaptic muscle cells by injecting its mRNA together with GFP mRNA into one of the blastomeres of 2-cell stage embryos. Nerve-muscle cultures prepared from the injected embryos contained fluorescence negative and positive neurons and muscle cells (Fig. 9*A*). GFP fluorescence has been shown to serve as an excellent indicator of cells expressing the co-injected mRNA (20, 26). Introduction of GDNF mRNA to the postsynaptic muscle cells (M+) markedly enhanced synaptic activity. Both frequency and amplitude of SSCs in M+ synapses were significantly higher than those observed at M− synapses, regardless whether the presynaptic neurons express GDNF or not (Fig. 9*B*). In contrast, overexpressing GDNF in the presynaptic neurons (N+) had no obvious effects on either frequency or amplitude of SSCs (Fig. 9*B*). Similar results were obtained from cultures prepared from NRTN mRNA-injected embryos (Fig. 9*B*), except that NRTN expressed in presynaptic cells also had a small effect on the SSC amplitude (N+/M− synapses). Taken together, these results support the notion that GDNF and NRTN could serve as target-derived factors.

Another important issue was whether overexpressed GDNF or NRTN protein was released at the neuromuscular synapses. To address this issue, we targeted GDNF or NRTN into the postsynaptic muscle cells, using the embryo injection methods described above. We selected synapses in which exogenous GDNF or NRTN was highly expressed in the postsynaptic muscle cells but not in presynaptic neurons, as indicated by the green fluorescence (Fig. 9*C*, top, N−/M+ synapses). If GDNF or NRTN expressed in the postsynaptic muscle cells was released, incubation with the anti-GFR-α1 antibody should prevent the increase in SSC frequency at these synapses. Indeed, the effect of postsynaptic GDNF expression was completely blocked by the antibody (Fig. 9*C, bottom*). Treatment with GFR-α1 also prevented the increase in synaptic efficacy at synapses with postsynaptic expression of NRTN (Fig. 9*C*). These results suggest that exogenous GDNF and NRTN expressed in muscle cells were processed into active form, and released at the neuromuscular synapses. We do not know whether these factors are released when expressed in presynaptic neurons.

Effects on Evoked Transmission—We next examined the effects of GDNF or NRTN on impulse-ESCs elicited by stimulating presynaptic somata of spinal neurons. As expected, long-term treatment with GDNF or NRTN resulted in a significant increase in ESC amplitude (Fig. 10*A*). The average ESC am-

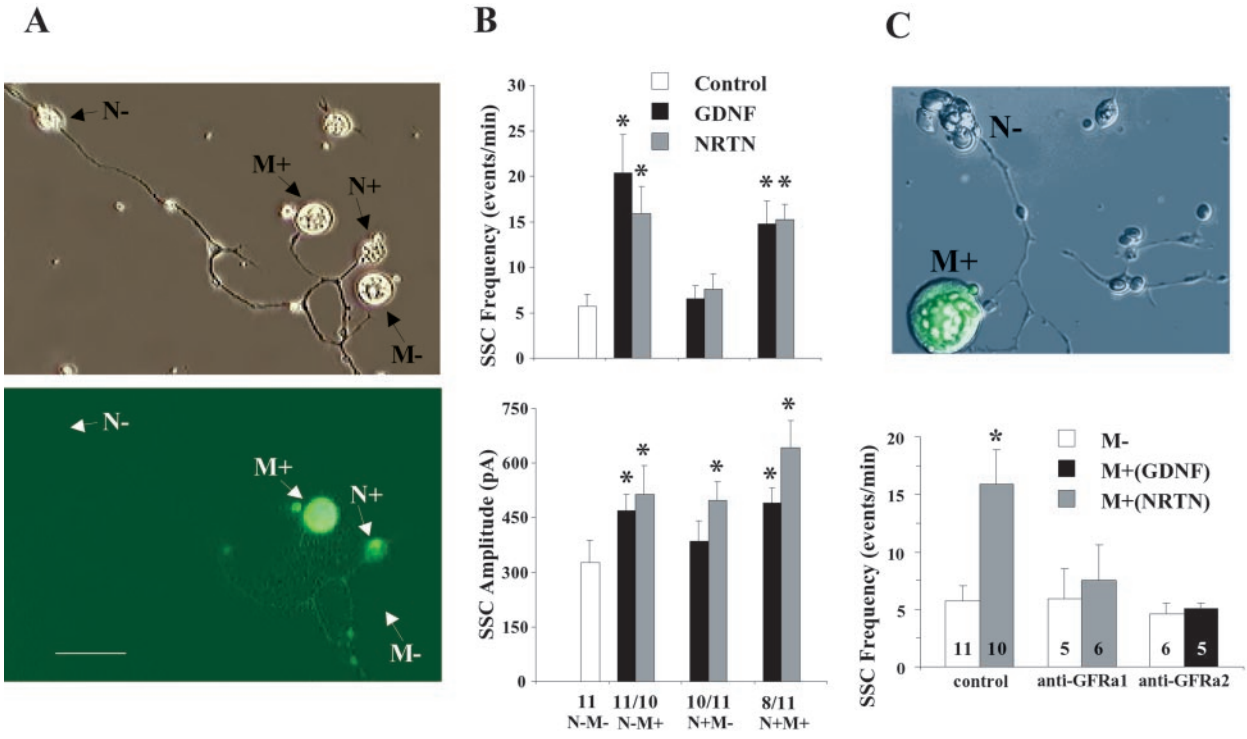

FIG. 9. **Mode of long-term GDNF or NRTN actions.** GDNF (or NRTN) was expressed either in presynaptic neurons in postsynaptic muscle cells through embryo injection. Cells containing exogenous GDNF or NRTN are indicated by the expression of GFP. *A*, phase and fluorescence images of nerve-muscle co-cultures derived from embryos injected with mRNAs for GDNF and GFP. *N+* and *M+* are neurons and muscle cells with GDNF/GFP, respectively. *Scale bar*, 20 μm. *B*, effects of targeted expression of GDNF or NRTN on the frequency of SSCs. Note that SSC frequency is increased only when GDNF or NRTN is overexpressed in the postsynaptic muscle cells (M+), but not in presynaptic neurons (N+). *, significantly different from the N-M− group. Student's *t* test; $p < 0.05$. *C*, release of GDNF at the neuromuscular synapses. Superimposed DIC and fluorescence image (*top*) demonstrates a spinal neuron innervating a myocyte expressing exogenous GDNF, as indicated by GFP fluorescence. Synapses with or without GDNF or NTRN expressed in the postsynaptic myocytes (M+ and M− synapses) were grown in the presence or absence of anti-GFR-α1 (50 ng/ml) antibody for 1 day. Note that postsynaptic expression of GDNF or NRTN enhances synaptic transmission, and anti-GFR-α1 blocks this effect (*bottom*).

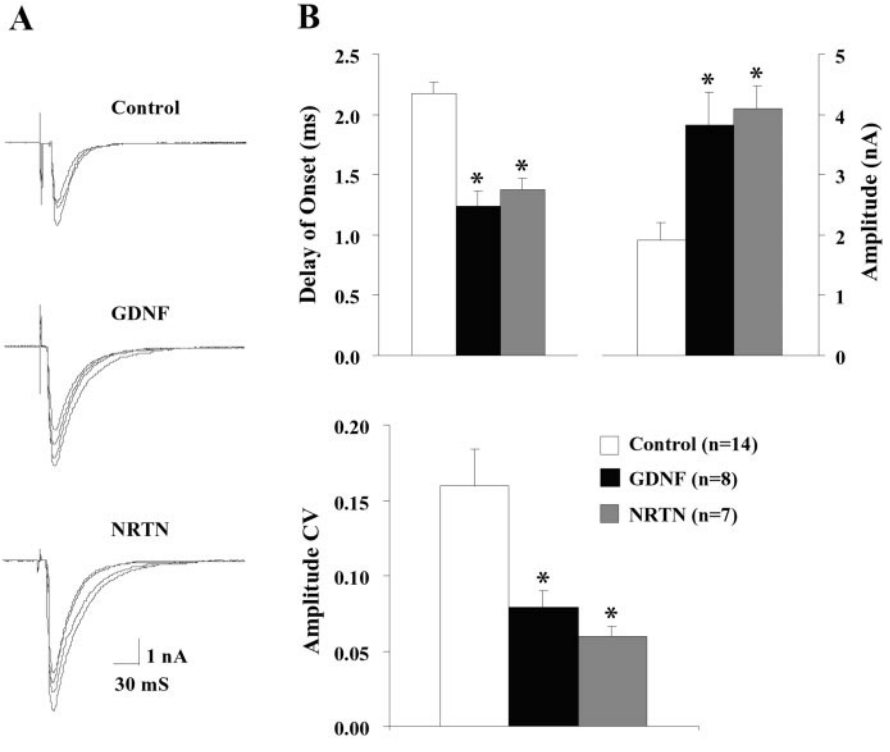

FIG. 10. **Long-term regulation of impulse-evoked synaptic transmission by GDNF or NRTN.** Presynaptic neurons were stimulated at the somata at low frequency (0.05 Hz), and ESCs were recorded at the voltage clamped condition (Vh = −70 mV, filtered at 150 Hz). *A*, examples of ESCs recorded from a control synapse, a GDNF-treated synapse and a NRTN-treated synapse. Several ESCs are aligned at stimulation artifacts and superimposed. *B*, the effects of GDNF and NRTN on ESC amplitude, variability, and delay of onset. Variability is defined as standard deviation divided by the mean of ESC amplitude, and delay of onset is defined as the interval between stimulation artifact and the beginning of synaptic current.

plitude in GDNF- or NRTN-treated synapses was 2.4 and 2.6 times, respectively, of that in control synapses (Fig. 10*B*). Once again, neither the rise time nor the decay time of ESCs was changed after GDNF or NRTN treatment (data not shown). The variability of SSCs contributes to the fluctuation of ESC amplitudes, as reflected by the coefficient of variation ESC

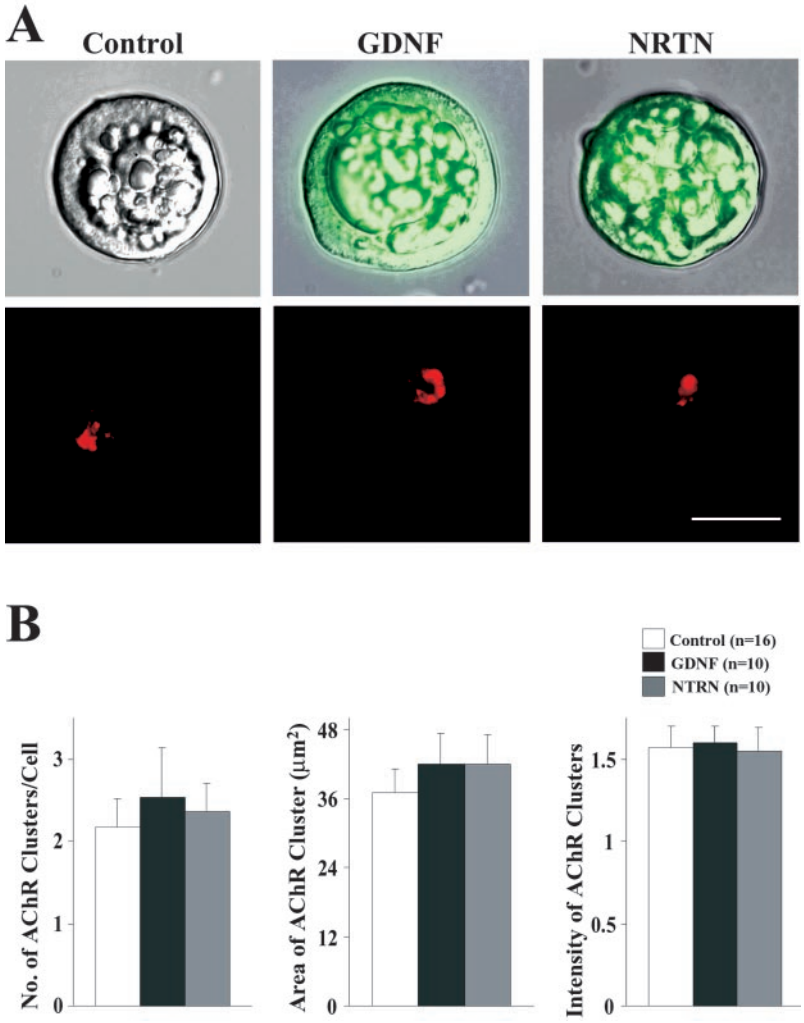

FIG. 11. **Effect of GDNF or NRTN on AChR clustering in isolated myocytes.** GDNF or NRTN was introduced into myocytes by co-injection of its mRNA and GFP mRNA into the 2-cell embryos. The cultures were incubated with rhodamine-labeled α-BTX for 30 min. The muscle cells overexpressing GDNF or NRTN were indicated by the GFP fluorescence (*green*), and AChR clusters were revealed by α-BTX fluorescence (*red*). *A*, *upper panels*: superimposed DIC and GFP fluorescence images of a control myocyte and myocytes expressing GDNF or NRTN. *Lower panels*, AChR clusters revealed as rhodamine fluorescence spots in the same myocytes. *Scale bar*, 8 μm. *B*, quantitative measures of the number, size, and fluorescence intensity of AChR clusters in the isolated myocytes.

amplitude (CV = S.D./mean). CV in normal extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) has often been used to determine the reliability of quantal transmission (20, 23, 26). Treatment with GDNF or NRTN significantly decreased CV of ESCs (Fig. 10*B*). Furthermore, GDNF or NRTN markedly reduced the ESC delay of onset (synaptic delay), the time interval between firing action potential and ESC generation (Fig. 10*B*). All these changes in evoked synaptic activity suggest that GDNF and NRTN promote the development of a more efficient and reliable mechanism for functional synaptic transmission at these neuromuscular synapses.

Postsynaptic Enhancement of ACh Receptor Clustering—The increase in SSC amplitude suggests that in addition to the presynaptic enhancement of transmitter release, GDNF and NRTN have a small postsynaptic effect. Since the decay of synaptic currents, and consequently the open time of AChR channels, was unaffected, the most likely change would be AChR clustering. To test this idea, we prepared the nerve-muscle co-cultures using embryos co-injected with GFP mRNA and GDNF/NRTN mRNA. The muscle cells overexpressing GDNF or NRTN were indicated by the GFP fluorescence. The cultures were incubated with rhodamine-labeled α-bungarotoxin (α-BTX, 0.2 μM) for 30 min at room temperature to visualize clusters of AChR. As shown in Fig. 11*A*, there were very few AChR clusters on the surface of isolated myocytes. On average, there were about 2 clusters per myocyte. Overexpression of GDNF or NRTN had no effect on the number of AChR clusters (Fig. 11*B*). Moreover, imaging analysis indicated that neither the size nor the intensity of AChR clusters was altered by GDNF or NRTN (Fig. 11*B*). Thus, it appears that GDNF or NRTN does not have a direct effect on AChR clustering.

We next examined whether the AChR clusters at the neuromuscular synapses were affected by GDNF or NRTN. We compared the AChR clusters at GDNF+ and GDNF− synapses (Fig. 12*A*). Surprisingly, the area of AChR clusters in GDNF+ synapse was significantly larger, as compared with the GDNF− synapse (Fig. 12*A*). Quantitative analysis showed that the area of AChR clusters in GDNF+ synapses increased by 54.2%, as compared with GDNF− synapses. However, the intensity of AChR clusters was not changed (Fig. 12*B*). Neurturin elicited similar effects at synaptic AChR clusters as GDNF (Fig. 12*B*). Taken together, these results are consistent with the idea that GDNF or NRTN regulates synaptic AChR clustering by acting indirectly on presynaptic terminals, rather than directly on postsynaptic muscle cells.

DISCUSSION

Modulation of the efficacy of synaptic transmission is thought to be the cellular basis for the development and function of the nervous system, as well as for complex behaviors such as learning and memory. Therefore, a great deal of effort has been made to identify secretory factors that regulate structure and function of specific synapses. Recently, the neurotrophin family of proteins has been shown to enhance synaptic transmission in various areas of the central and peripheral nervous systems (1–3). A particularly well studied system is the neuromuscular synapse, where both acute and long-term modulatory effects by BDNF and NT3 have been demonstrated.

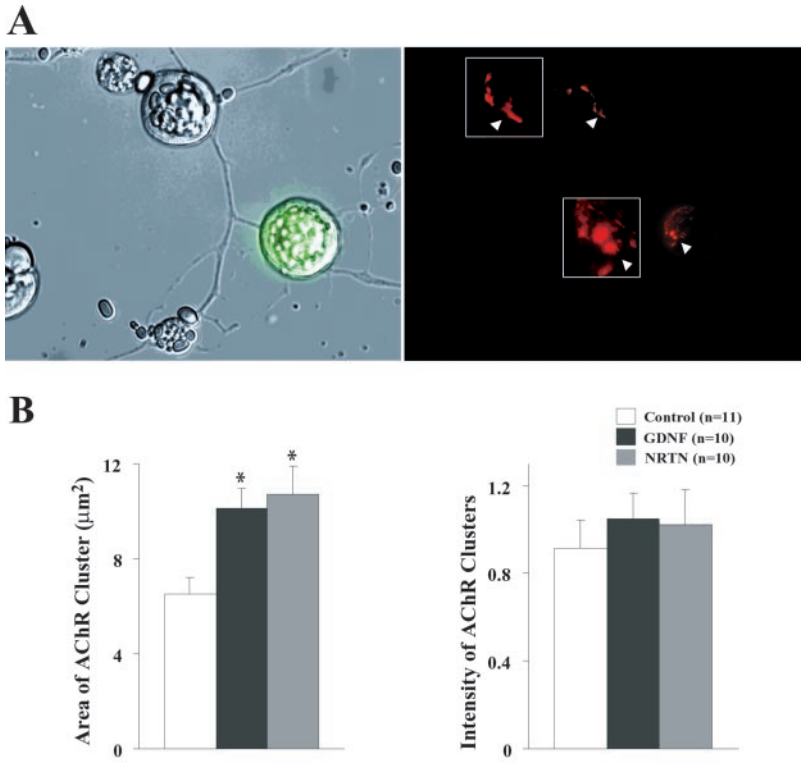

FIG. 12. **Effect of GDNF or NRTN on AChR clustering at the neuromuscular synapse.** Experiments were performed essentially the same way as Fig. 11, except AChR clusters at NMJ were examined. *A*, *left panels*: superimposed DIC and GFP fluorescence images of a single motoneuron innervating two myocytes, one expressing GDNF (*green*) and one without. *Right panels*, AChR clusters revealed as rhodamine fluorescence spots in the same myocytes. *Arrowheads* indicate synaptic AChR clusters. *Insets,* enlarged images at synapse areas. *B*, quantitative measures of the size, and fluorescence intensity of AChR clusters at NMJ.

In the present study, we have examined the long-term effects of the trophic factors from the GDNF family on the development of NMJ. Imaging experiments demonstrated profound morphological changes in the motoneurons and the neuromuscular synapse. The neurites (axons) were markedly lengthened, along with the increase in the number as well as the size of synaptic vesicle clusters. Postsynaptically, GDNF/NRTN enhanced clustering of AChR at the synapses. Consistent with these findings, physiological studies revealed two major effects of GDNF/NRTN on synaptic transmission at the developing neuromuscular synapse: facilitation of transmitter release and potentiation of quantal size. GDNF (and perhaps NRTN) appears to be derived from postsynaptic muscle cells and to act retrogradely. Three pieces of evidence suggest that both GDNF and NRTN signal through GFR-α1 to regulate the neuromuscular synapses: 1) NRTN elicited the same effects as GDNF but required 10 times higher concentrations; 2) most of the *Xenopus* spinal neurons expressed GFR-α1 and only a small population of motoneurons express GFR-α2 (see also Refs. 44, 47, and 48); 3) the effects of both GDNF and NRTN were blocked by antibodies against GFR-α1, but not by those against GFR-α2. These results identify GDNF as a novel neuromodulator that exerts long-term regulatory effects on synaptic transmission, and provide new insights into the developmental regulation of synaptic efficacy.

While both elicit long-term changes in these synapses, GDNF differs from neurotrophins in several aspects. First, GDNF appears to be more potent than neurotrophins. Treatment with GDNF for 1 day at very low concentration (1 ng/ml or 40 pM) results in marked increases in SSC frequency and ESC amplitude, while BDNF or NT3 elicits similar but less dramatic effects at higher concentrations (0.5–2 nM) and requires longer incubation (2–3 days) (23, 25). Second, neurotrophins (20, 23) but not GDNF affect rise or decay time of synaptic currents, whereas GDNF but not neurotrophins reduces synaptic delay. Third, both GDNF and neurotrophins facilitate the formation of synaptic varicosities and regulate AChR clustering (23, 60), but the underlying mechanisms seem to be quite different (see below). Finally, unlike neurotrophins which elicit both acute and long-term effects, GDNF does not acutely regulate synaptic transmission at the *Xenopus* neuromuscular synapses. This is somewhat surprising because opposite results were reported using isolated nerve-muscle preparations from neonatal mouse (67). In that system, acute application of GDNF, but not BDNF, NT3, or a number of other factors, elicits a 2-fold increase in SSC frequency. It should be pointed out that the mouse NMJ preparation does not contain cell bodies of motoneurons, and is prepared at a later developmental stage when elimination of polyneuronal innervation is almost complete. Whether NMJ without motoneuron cell bodies or prepared at a later developmental stage may behave differently in response to neurotrophic factors remains to be investigated.

One of the major effects by GDNF/GFR-α1 pathway is the enhancement of transmitter release. Long-term treatment with GDNF elicited a 4–5-fold increase in frequency of SSCs. Functional transmission, as reflected by impulse-evoked synaptic currents, was also increased. The presynaptic effects of GDNF could be due to an increase either in the probability of transmitter release (Pr), or in the number of release sites. Several pieces of evidence suggest that the increase in Pr explains, at least in part, the marked increase in transmitter release induced by GDNF. First, GDNF affects paired-pulse facilitation, the increase in the amplitude of the second ESC when the synapse is activated by two successive presynaptic stimuli (68). Whereas treatment with GDNF increased the amplitude of the first ESC, the ratio of the amplitudes of second and first was significantly reduced. A decrease in paired-pulse facilitation usually reflects an increase in Pr (69). Second, treatment with GDNF markedly decreased synaptic delay, the time needed for coupling of depolarization/Ca^{2+} influx to transmitter release (Fig. 9). This was probably due to an enhancement of Ca^{2+} influx during evoked transmission. Indeed, our recent Ca^{2+} imaging experiments indicated that Ca^{2+} influx at the presynaptic terminals was dramatically increased at the synapses treated with GDNF (68). Finally, whole cell recording of presynaptic motoneurons demonstrated that GDNF enhances

Ca^{2+} influx by selectively potentiating the N-type Ca^{2+} channels (68). Changes in Ca^{2+} influx directly affects Pr. Thus, an increase in Pr contributes to GDNF-induced enhancement of transmitter release.

The increase in SSC frequency (4–5-fold) was much more pronounced than that in ESC amplitude (2-fold) in GDNF-treated synapses. This result suggests that in addition to the increase in Pr, GDNF treatment may also change the structures of the synapses, leading to an increase in the number of release sites. Consistent with this idea, we found that the number as well as the size of synaptic varicosities (as measured by synaptobrevin-GFP fluorescent spots) was significantly increased in cultures treated with GDNF. Synaptobrevin-GFP fluorescent spots can be divided into two categories based on their mobility: "stationary" spots corresponding to presynaptic boutons capable of releasing transmitters and "transport" spots reflecting pre-assembled "proto-terminals" being transported down the axons (64). It was unclear whether GDNF facilitates the formation of stationary or transport spots. Regardless, the increase in the number and size of synaptic varicosities supports the notion that GDNF promotes the development of presynaptic terminals.

A general increase in the postsynaptic ACh sensitivity could also contribute to an apparent increase in SSC frequency by detecting very small SSC events normally hidden within the recording noise in control condition. The detection limit of our recording was ~20 pA. Thus, a 50% increase of AChR sensitivity would make a 15 pA event visible. Indeed, we found that long-term treatment with GDNF resulted in about 50% increase in the average SSC amplitude. We did not see, however, a preferential increase in the number of SSC events smaller than 50 pA within a fixed time period in GDNF-treated synapses (data not shown). Therefore, the increase in SSC amplitude did not contribute substantially to the increase in SSC frequency. The waveforms of SSCs were not altered, suggesting that GDNF does not change the opening time of AChR channels. When GDNF was overexpressed in innervated muscle cells, the average size of AChR clusters at the developing synapses was increased by about 55%. Interestingly, overexpression of GDNF in isolated muscle cells did not change the size or number of the AChR clusters. This is consistent with the fact that the GDNF receptor c-Ret is not expressed in muscle cells (Fig. 7*D*). Thus, GDNF selectively regulates AChR clustering at the synapses. These results imply that GDNF acts indirectly on presynaptic terminals, possibly by enhancing the release certain factor(s) that are capable of inducing AChR clustering.

Extensive studies in the last two decades have described in detail the sequential events that lead to the development of mature and functional neuromuscular junctions (50). Currently, factors that control or regulate the neuromuscular development and their underlying molecular mechanisms are subjects of intensive investigations. In the present study, we have identified GDNF as a novel, endogenous factor that promotes the maturation of the neuromuscular synapse during early development. We show that the effects of GDNF are quite profound, and are mediated through multiple pre- and postsynaptic mechanisms. The fact that GDNF does not have an acute effect on synaptic transmission also provides a unique opportunity to investigate the molecular mechanisms specific for long-term synaptic effects of neurotrophic factors. Our findings may help understand the possible role of GDNF in synapse development *in vivo*, both at the NMJ and in the brain.

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