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GDNF enhances the synaptic efficacy of dopaminergic neurons in culture

Marie-Josée Bourque and Louis-Eric Trudeau
Départements de Pharmacologie et de Psychiatrie, Centre de Recherche en Sciences Neurologiques, Centre de Recherche Fernand Seguin, Université de Montréal, Canada H3T 1J4

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) is known to promote the survival and differentiation of dopaminergic neurons of the midbrain. GDNF also causes an enhancement of dopamine release by a mechanism which is presently unclear. Using isolated dopaminergic neurons of the rat ventral tegmental area in culture, we have tested the hypothesis that GDNF regulates the establishment and functional properties of synaptic terminals. Previous studies have shown that single dopaminergic neurons in culture can co-release glutamate in addition to dopamine, leading to the generation of a fast excitatory autaptic current via glutamate receptors. Using excitatory autaptic currents as an assay for the activity of synapses established by identified dopaminergic neurons, we found that chronically applied GDNF produced a threefold increase in the amplitude of excitatory autaptic currents. This action was specific for dopaminergic neurons because GDNF had no such effect on ventral tegmental area GABAergic neurons. The enhancement of excitatory autaptic current amplitude caused by GDNF was accompanied by an increase in the frequency of spontaneous miniature excitatory autaptic currents. These observations confirmed a presynaptic locus of change. We identified synaptic terminals by using synapsin-1 immunofluorescence. In single tyrosine hydroxylase-positive neurons, the number of synapsin-positive puncta which represent putative synaptic terminals was found to be approximately doubled in GDNF-treated cells at 5, 10 and 15 days in culture. The number of such morphologically identified terminals in isolated GABAergic neurons was unchanged by GDNF. These results suggest that one mechanism through which GDNF may enhance dopamine release is through promoting the establishment of new functional synaptic terminals.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic peptide part of the cysteine knot protein family that also includes nurturin, persephin and artemin (Saarma & Sariola, 1999). The actions of GDNF are thought to be mediated through binding to the type 1 GDNF family receptor alpha (rGDNFα1), leading to the dimerization and activation of the Ret receptor tyrosine kinase (Jing *et al*., 1996; Treanor *et al*., 1996; Cacalano *et al*., 1998). This could lead to the activation of a complex signal transduction pathway implicating phosphatidylinositol 3-kinase (Pong *et al*., 1998; Soler *et al*., 1999). GDNF has been reported to promote the survival and differentiation of midbrain dopaminergic neurons (Bowenkamp *et al*., 1995; Kearns & Gash, 1995; Tomac *et al*., 1995; Choi-Lundberg *et al*., 1997; Burke *et al*., 1998; Granholm *et al*., 2000), a property that has lead to the suggestion that this peptide could be used as a therapeutic agent for the treatment of pathologies that are associated with a degeneration of dopaminergic neurons, e.g. Parkinson's disease (Beck *et al*., 1995; Bilang-Bleuel *et al*., 1997; Mandel *et al*., 1997).

In addition to preventing the apoptosis and death of dopaminergic neurons, GDNF is known to enhance the ability of these cells to release dopamine. This has been demonstrated in the intact animal as an enhancement of potassium-evoked dopamine release in the striatum using electrochemistry and microdialysis to detect extracellular dopamine (Hebert *et al*., 1996). A similar conclusion has been reached using cultured midbrain neurons (Feng *et al*., 1999). GDNF could cause such an effect through a number of mechanisms. First, the enhanced morphological differentiation of dopaminergic neurons caused by GDNF could be accompanied by the establishment of new synaptic terminals onto target cells. Second, GDNF could cause a facilitation of the ability of pre-existing terminals to release dopamine. Third, GDNF could cause an increase in the excitability of dopaminergic neurons leading to an enhanced responsiveness to synaptic inputs. Finally, an up-regulation of dopamine synthesis or packaging in synaptic vesicles of pre-existing synaptic terminals could occur. At present, solid evidence is available to support only the last of these hypotheses. Indeed, recent amperometry work by Pothos and colleagues has demonstrated that GDNF can cause a significant increase in the quantal size of dopamine-containing small synaptic vesicles in cultured ventral tegmental area (VTA) neurons (Pothos *et al*., 1998). One possibility is that such an increase in quantal size is caused by a stimulation of tyrosine hydroxylase activity (Beck *et al*., 1996), but other mechanisms are possible. GDNF has also been reported to cause an increase in quantal amplitude in cultured *Xenopus* motoneurons where acetylcholine is the neurotransmitter (Liou *et al*., 1997). An increase in quantal size could be one of the major causes of the enhancement of dopamine release by GDNF, but it will be necessary to test more directly the first three hypotheses to determine what is the complete mechanism.

Correspondence: Dr L.-E. Trudeau, Département de Pharmacologie, Faculté de Médecine, Université de Montréal, 2900 Boulevard Edouard-Montpetit, Montréal, Québec, Canada H3T 1J4.

E-mail: louis-eric.trudeau@umontreal.ca

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The present study provides a test of the first of the above-stated hypotheses, i.e. that the enhanced morphological differentiation of dopaminergic neurons caused by GDNF could be accompanied by the establishment of new synaptic terminals onto target cells. We have examined the actions of GDNF on postnatal VTA dopaminergic neurons in primary culture. We have used immunolocalization of synapsin-1 and patch-clamp electrophysiology to determine whether GDNF can act to stimulate the formation of synaptic terminals. Our results show that indeed, GDNF enhances the number of functional synaptic sites in cultured VTA dopaminergic but not GABAergic neurons.

Materials and methods

Microisland cultures

Neuronal cultures were prepared from rat postnatal VTA (first 2 days after birth). The animals were cryoanaesthetized. Midbrain slices were cut manually using the midbrain flexure as a landmark. The VTA was isolated using a custom micropunch and then enzymatically treated with a papain solution for 20 min at 37 °C. After transfer to a trituration solution, tissue blocks were gently triturated by passage through Pasteur pipettes of decreasing diameter until a single-cell suspension was obtained. Intact cells were collected by centrifugation and diluted at a density of ~90 000 cells per millilitre. Cells were plated on glass coverslips which were precoated with 0.15% agarose and sprayed with collagen/poly-D-lysine (each at 0.5 mg/mL) microdroplets using a microatomizer. Cell cultures were incubated at 37 °C in 5% CO_2 atmosphere in Eagle's basal medium with Earl's Salts (Gibco, Burlington, Ontario, Canada) supplemented with penicillin, streptomycin, GlutaMAX-1 (Gibco), Mito-plus serum extender (VWR CanLab, Montreal, Quebec, Canada) and 5% heat-inactivated foetal calf serum (Gibco). Medium was refreshed every 3–4 days by exchanging a third of the medium. In half of the cultures, feeding media were supplemented with GDNF (Alomone Laboratories, Jerusalem, Israel) at 26 pM throughout the culture period. Cultures were treated with a solution of uridine and 5-fluoro-2-deoxyuridine (10 μM) after 3 days in culture to halt glial proliferation. For immunocytochemistry experiments, cells were used 5–-15 days after plating.

Electrophysiology and dye labelling

Electrophysiological experiments were performed in solitary neurons grown in culture for 11–12 days. Patch-clamp recordings in the whole-cell configuration were used to record evoked and spontaneous synaptic activity. The extracellular bathing solution contained (in mM): NaCl, 140; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 2; *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid (HEPES), 10; sucrose, 6; glucose, 10; pH 7.35; osmolarity, 310 mOsm. To record evoked synaptic responses, the patch pipette contained (in mM): potassium gluconate, 140; ethylenediaminetetra-acetate (EGTA), 10; MgATP, 4; GTP, 0.2; HEPES, 10; pH 7.35; osmolarity, 290 mOsm. To record spontaneous miniature synaptic currents, 1 μM tetrodotoxin was added to the extracellular saline and the patch pipette contained (in mM): caesium gluconate, 117.5; NaCl, 10; $MgCl_2$, 4; HEPES, 15; EGTA, 5; ATP (Mg^{2+} salt), 2; guanosine triphosphate (GTP) (Tris salt), 0.2; pH 7.35. Fluorescein dextran (1%, Molecular Probes, Eugene, OR, USA) was added to the recording pipette solution to label cells for post-recording immunostaining for tyrosine hydroxylase. Patch pipettes were prepared with borosilicate glass and had a resistance of ~5 MOhm. Currents were recorded using a PC-505 patch-clamp amplifier (Warner Instruments, Hamden, CT, USA), filtered at 1 kHz and digitized at 2 kHz using Axon Instruments hardware and software (Axon Instruments, Foster City, CA, USA). To evoke autaptic synaptic currents, neurons were stimulated with a brief (2 ms) voltage command pulse to 20 mV from a holding potential of –60 mV and evoked postsynaptic currents were recorded. In some experiments, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) was added to the external medium to block (±)-α-amino-3-hydroxy-5-methylisoxalone-4-propionic acid (AMPA)/kainate receptors which mediate fast excitatory postsynaptic currents in cultured dopaminergic neurons (Sulzer *et al*., 1998; Congar & Trudeau, 1999). In non-dopaminergic neurons, autaptic currents displayed reversal potentials close to –60 mV and were sensitive to 5 μM SR-95531, a $GABA_A$ (γ-aminobutyric acid) receptor antagonist (not shown) (Michel & Trudeau, 2000). The mean amplitude of the evoked autaptic current was calculated from the average of all traces containing a synaptic event (excluding undetectable responses) and the number of synaptic failures was noted. Solutions were exchanged with a gravity-driven local perfusion system. Miniature synaptic currents were analysed using MiniAnalysis 4.0 from Synaptosoft (Leonia, NJ, USA). The frequency and amplitudes of events were measured to generate distribution histograms or cumulative probability plots. Data in the text are expressed as mean ± SEM, unless otherwise stated. Non-parametric tests or two-way ANOVAs were used to evaluate the GDNF treatment effects, as specified. The Kolmogorov–Smirnov test was used to compare cumulative probability distributions.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4, for 30 min. After permeabilization with 0.05% Triton X-100 and blocking in 10% normal goat serum, cells were incubated overnight at 4 °C with primary antibodies against synapsin-1 (Sigma, Oakville, Ontario, Canada), synaptophysin (Sigma), VMAT2 (Chemicon, Temecula, CA, USA), GDNF receptor-alpha (Research Diagnostics, Flanders, NJ, USA) or tyrosine hydroxylase (Sigma). The cells were then incubated with Alexa-488 and/or Alexa-546-conjugated secondary antibodies (Molecular Probes). Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed by epifluorescence microscopy.

Morphological analysis

To investigate the effects of GDNF on the number of synaptic terminals established by VTA dopaminergic neurons, cells were doubled-stained for tyrosine hydroxylase (TH) to identify dopaminergic neurons and synapsin-1 (Syn-1) or synaptophysin to identify putative synaptic terminals. Observations were made with a 40 × objective and rhodamine and fluorescein filter sets. Epifluorescence images were acquired with a black and white video camera (Empix Imaging, Mississauga, Ontario, Canada) or a cooled CCD camera (Orca-II, Hamamatsu, Bridgewater, NJ, USA). Images were acquired with Northern Exposure software (Empix Imaging, Mississauga, Ontario, Canada) or ESee software (Inovision, Raleigh, NC, USA). The digitized images were left untouched except for digital resampling and contrast enhancement. For quantification of the number of Syn-1-positive puncta, digitized photomicrographs were taken from VTA cultures after 5, 10 and 15 days in culture. Boutons were counted automatically using ScionImage software (Scion, Frederick, MD). Results were expressed as number of Syn-1-positive puncta per isolated neuron. Glial cells also usually displayed Syn-1 immunostaining. This latter labelling could easily be differentiated from neuronal labelling because it was not punctate and was concentrated around the nuclear region. These conspicuous large spots were excluded from analyses and from the images shown in

Fig. 7 by automatically rejecting pixel groupings which were larger than 60 pixels. Pixel groupings smaller than 3 pixels were also rejected to avoid counting spots produced by background noise. To determine whether Syn-1 and synaptophysin labelled the same populations of synaptic terminals, we performed double-labelling experiments using antibodies against these two proteins. We found that 92 ± 3% of detected boutons were double-labelled suggesting almost complete overlap ($n = 6$, results not shown).

Results

Effect of GDNF on autaptic currents generated by dopaminergic neurons

Experiments were performed on VTA single-neuron microisland cultures (Furshpan *et al*., 1986; Segal, 1991; Sulzer *et al*., 1998). Neurons were viewed under phase contrast microscopy and patch-clamped for electrophysiological recordings. Patch pipettes contained fluorescein dextran. This permitted intracellular labelling of neurons and, coupled with *post hoc* TH immunocytochemistry, allowed the confirmation of cellular phenotype. We have taken advantage of the ability of dopaminergic neurons to co-release glutamate (Sulzer *et al*., 1998) to monitor the effects of GDNF on synaptic efficacy through the measurement of AMPA receptor-mediated autaptic currents evoked by brief (2 ms) voltage steps to +20 mV.

Figure 1A shows an example of a neuron maintained in microisland culture and intracellularly labelled with fluorescein dextran after 5 min of whole-cell recording. This neuron was subsequently immunostained for TH and thus confirmed to be a dopaminergic neuron (Fig. 1B). All cells recorded were processed for TH immunostaining to ascertain their cellular phenotype. As expected from previous work (Sulzer *et al*., 1998), we confirmed that dopaminergic neurons generally make glutamatergic autapses. In control cultures, excitatory autaptic currents were observed in 79% of TH^+ neurons (23/29). The glutamatergic nature of these evoked synaptic currents was confirmed by their sensitivity to CNQX (10 μM), which produced a complete blockade (Fig. 1C). Dopaminergic (Fig. 2) as well as GABAergic neurons (not shown) in our VTA cultures expressed GDNF receptor-alpha (rGDNFα) as revealed by immunofluorescence staining using a monoclonal antibody.

The effect of GDNF on synaptic transmission from VTA neurons was evaluated by comparing the amplitude of excitatory autaptic currents measured in control cultures and in cultures treated with 26 pM GDNF from their first day in culture. We found that in TH^+ GDNF-treated neurons, the incidence of excitatory autapses was 92% (24/26). Moreover, excitatory autaptic currents from GDNF-treated dopaminergic neurons displayed significantly larger amplitudes than untreated cells (224 ± 58 pA, $n = 23$ versus 703 ± 81 pA, $n = 24$, Mann–Whitney rank sum test, $P < 0.001$, Fig. 3). Because VTA cultures also contain TH^- GABAergic neurons, we tested whether autaptic inhibitory synaptic currents were also enhanced by GDNF. In contrast to dopaminergic neurons, no significant change in GABAergic synaptic current amplitude was detected (150 ± 23 pA, $n = 22$ versus 150 ± 50 pA, $n = 18$, Mann–Whitney rank sum test, $P > 0.05$, Fig. 3). These results suggest that in VTA neurons in culture, GDNF produces a synaptic facilitation that is specific for dopaminergic neurons.

GDNF enhances the frequency of miniature synaptic events

The effect of GDNF on glutamatergic miniature excitatory post-synaptic currents (mEPSCs) in isolated dopaminergic neurons was examined in order to determine whether the enhancement of autaptic current amplitude caused by GDNF was related to a pre- or postsynaptic site of action. We studied the amplitudes and frequency of mEPSCs. Events were measured in the presence of tetrodotoxin (TTX) to ascertain that action potential-mediated synaptic currents were blocked. Dopaminergic neurons cultured for 11–12 days in control conditions displayed low levels of spontaneous synaptic activity (1.53 ± 0.8 Hz, $n = 16$, Fig. 4A and B). GDNF-treated neurons displayed a 3.8-fold higher mEPSC frequency (5.87 ± 0.93 Hz, $n = 18$, Fig. 4A and B). This difference was statistically significant

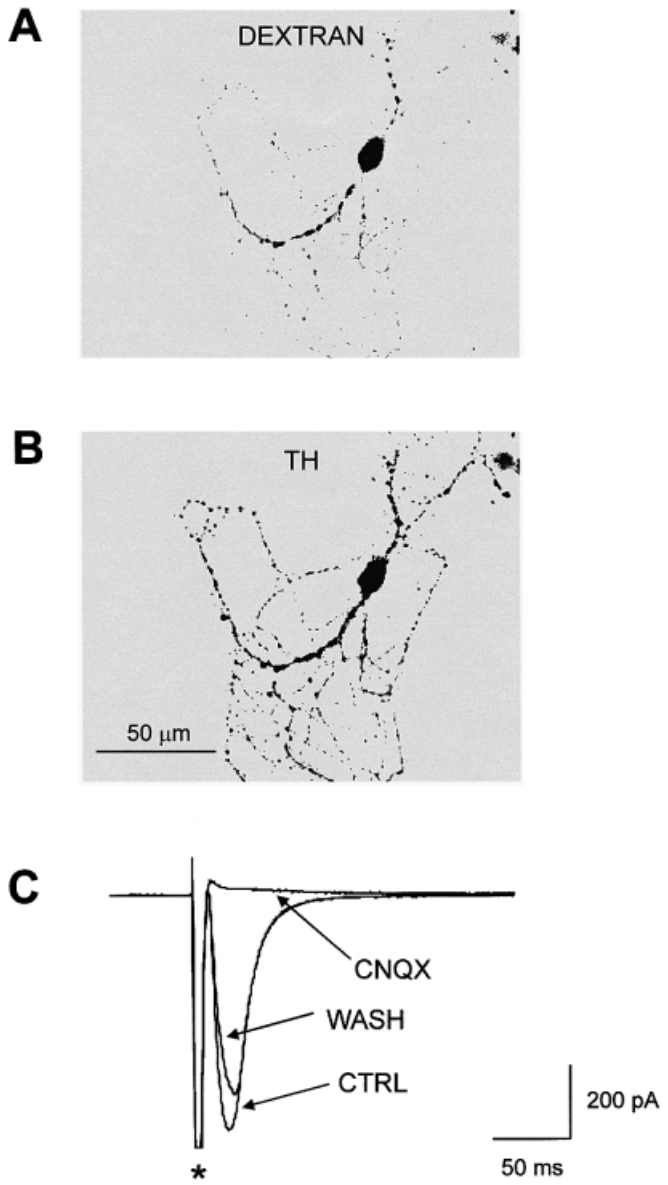

FIG. 1. Morphological and physiological characterization of VTA dopaminergic neurons in microisland culture. (A) Image obtained by epifluorescence showing the characteristic appearance of an isolated neuron growing on a monolayer of glial cells after 12 days in culture. The neuron was filled with fluorescein dextran through a patch pipette during physiological recording (C). (B) Same neuron as in A after immunostaining for tyrosine hydroxylase to confirm the cell's dopaminergic phenotype. (C) Autaptic response recorded from the same voltage-clamped, isolated dopaminergic neuron shown in A and B. Stimulation with a brief depolarizing voltage step (–60 mV to +20 mV for 2 ms) triggered an action current (*) followed by a postsynaptic excitatory autaptic current (CTRL). The response was mediated by AMPA-type glutamate receptors because its was blocked by 10 μM CNQX (CNQX). This block was reversible (WASH). Each trace is an average of 10 sweeps.

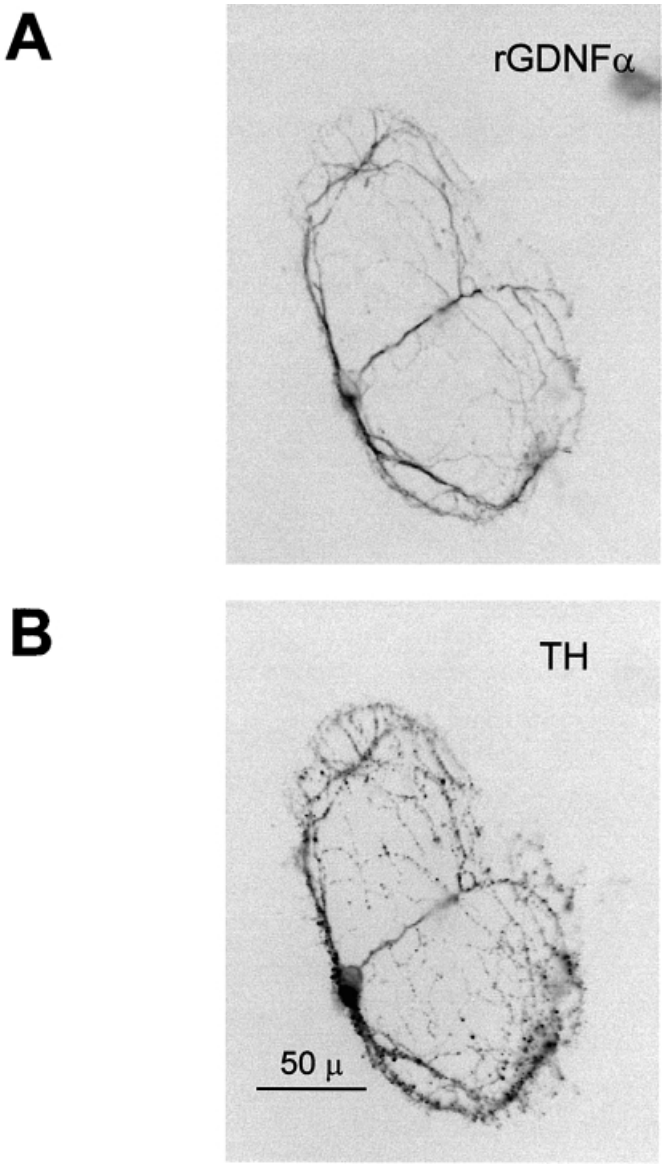

FIG. 2. Localization of GDNF receptors on dopaminergic neurons. (A) Image of a solitary VTA neuron obtained by epifluorescence following immunostaining using a rGDNFα monoclonal antibody. This neuron was subsequently identified as a dopaminergic neuron (B). (B) Same neuron as in A after immunostaining for tyrosine hydroxylase to confirm the cell's dopaminergic phenotype.

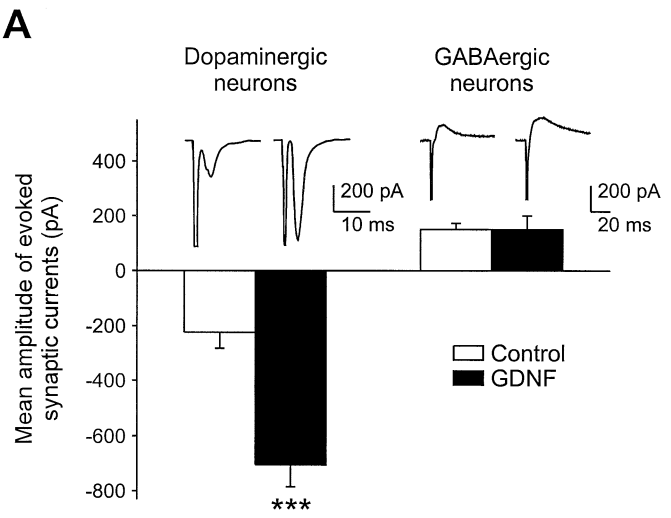

FIG. 3. Summary diagram of autaptic current amplitudes in isolated dopaminergic and GABAergic neurons. Histograms illustrating the mean amplitude of autaptic currents in TH^+ (dopaminergic) and TH^- (GABAergic) neurons. Excitatory autaptic responses recorded in dopaminergic neurons displayed significantly larger amplitudes in GDNF-treated cultures ($n = 24$; CTRL, $n = 23$). In comparison, the mean amplitude of GABAergic autaptic currents was not significantly different between GDNF-treated and control cultures ($n = 18$; CTRL, $n = 22$). Insets show representative recordings of glutamatergic (inward synaptic currents) and GABAergic (outward synaptic currents) autaptic currents in control and GDNF-treated neurons. $***P < 0.001$.

(Mann–Witney rank sum test, $P < 0.001$) and was not accompanied by a change in mEPSC amplitude. Both in control and GDNF-treated cultures, mEPSC amplitude averaged 13 ± 1 pA ($n = 16$ and 18, respectively). Cumulative probability distributions of mEPSC amplitudes were not different between the two groups (Kolmogorov–Smirnov test, $P > 0.05$, Fig. 4A). In parallel to the lack of effect of GDNF on GABAergic autaptic currents, we found that GDNF treatment failed to cause a change in the frequency and amplitude of miniature inhibitory postsynaptic currents (mIPSCs) recorded in TH^- isolated neurons (Fig. 4B). The average frequency of mIPSCs was 1.53 ± 0.4 Hz in control neurons ($n = 10$) and 1.6 ± 0.27 Hz in GDNF-treated neurons ($n = 10$, Mann–Witney rank sum test, $P > 0.05$).

Measurements of the frequency of spontaneous mEPSCs are generally thought to provide a good index of the activity and abundance of functional synaptic terminals. However, they can also be influenced by the resting levels of intracellular calcium in nerve terminals, thus possibly biasing the estimation of the number or activity of active terminals. We thus performed parallel experiments where we compared the rate of mEPSCs between GDNF-treated and untreated cells under conditions where the rate of mEPSCs was stimulated by using ruthenium red (RR), a secretagogue known to act on the presynaptic release machinery and to stimulate the rate of mEPSCs in a Ca^{2+}-independent manner (Trudeau *et al.*, 1996a,b, 1998). As expected, we observed that RR significantly enhanced the frequency of mEPSCs (Fig. 5A). The ability of RR to enhance the frequency of mEPSCs was expressed as a difference score (delta RR), obtained by subtracting the number of mEPSCs recorded in a 15-s episode after RR (40 μM) from before RR. A comparison between the two groups demonstrated that GDNF-treated cells showed a 2.3-fold increase in delta RR as compared to control cells (CTRL, 43 ± 16, $n = 14$; GDNF, 101 ± 23, $n = 14$; Fig. 5A and B; Mann–Witney rank sum test, $P < 0.05$). No such difference was found for RR-evoked GABAergic mIPSCs (Fig. 5B, CTRL, 95 ± 33, $n = 9$; GDNF, 103 ± 27, $n = 9$). These results confirm and extend the data obtained by recording spontaneous mEPSCs. They suggest that GDNF induced either an increase in the efficacy of quantal neurotransmitter release from existing nerve terminals or caused an increase in the number of functional nerve terminals formed by dopaminergic neurons.

GDNF regulates the number of synapsin-1-positive varicosities in isolated dopaminergic neurons

To assess whether the degree of miniature synaptic activity was correlated with an increased number of nerve terminals, we identified putative nerve terminals by immunocytochemical localization of synapsin-1 (Syn-1) or synaptophysin, proteins known to be concentrated in nerve terminals. To first determine whether most Syn-1-positive or synaptophysin-positive nerve terminals established by dopaminergic neurons were likely to be able to release dopamine and not only glutamate, we performed double-labelling experiments

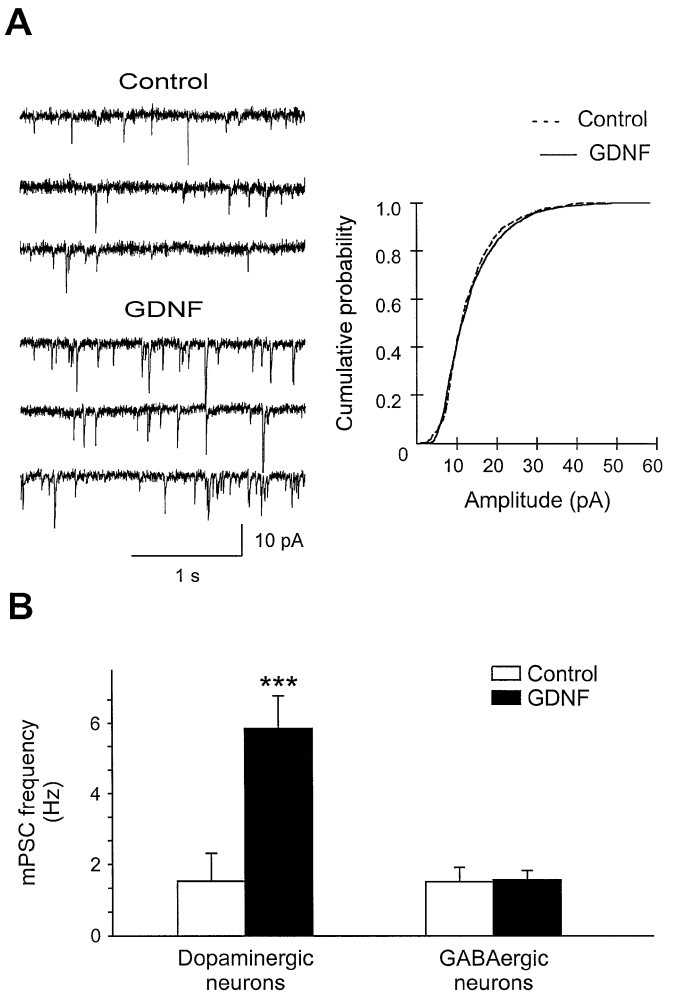

FIG. 4. Effect of GDNF on spontaneous miniature postsynaptic currents (mPSCs) recorded in isolated dopaminergic neurons. (A) Whole-cell recordings of mEPSCs in control and GDNF-treated dopaminergic neurons in the presence of TTX to block action potentials. Control cells displayed a low frequency of spontaneous events (top). The frequency of the spontaneous mEPSCs was markedly higher in GDNF-treated neurons (bottom). Cumulative probability distributions generated from the data of all cells tested also illustrate that the mEPSC amplitudes were not different between the two groups (right). (B) Summary histogram of the mean miniature synaptic event frequency in Hz. The mean frequency was larger after GDNF treatment in TH^+ dopaminergic neurons ($n = 18$), but not in TH^- GABAergic neurons ($n = 10$). $***P < 0.001$.

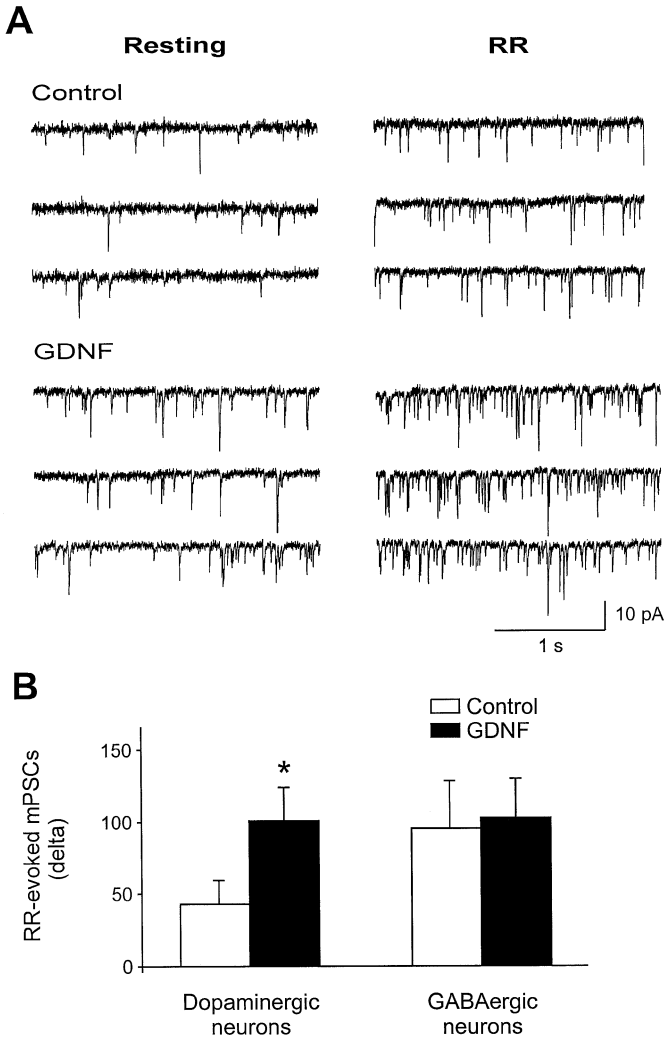

FIG. 5. Effect of GDNF on mPSCs triggered by the Ca^{2+}-independent secretagogue ruthenium red. (A) Whole-cell recordings of mPSCs in isolated dopaminergic neurons. Brief (30 s) perfusion of RR (40 μM) triggered a rise in the frequency of mEPSCs. The number of mEPSCs triggered was calculated by subtracting the number of events during a 15-s period before RR (resting) from that after RR (RR). This calculated difference score (delta) was larger in GDNF-treated neurons than in control neurons. (B) Summary histogram illustrating the frequency of mPSCs triggered by RR measured as delta values. GDNF-treatment caused a significant increase in RR-evoked release in TH^+ neurons ($n = 14$) but not in GABAergic neurons ($n = 9$). $*P < 0.05$.

using antibodies against the vesicular monoamine transporter (VMAT2, Pothos *et al.*, 1998), and synaptophysin. We found that the vast majority of putative nerve terminals were double-labelled. On average, 86 ± 2% of synaptophysin-positive puncta were also VMAT2-positive ($n = 20$, Fig. 6A). In separate experiments we confirmed that as expected, only TH-positive neurons expressed VMAT2 (Fig. 6B), and that synaptophysin and synapsin-1 antibodies labelled the same population of putative nerve terminals ($n = 6$; see Materials and methods; results not shown).

The number of Syn-1-positive puncta found on isolated dopaminergic and GABAergic neurons was quantified after 5, 10 or 15 days in culture in control and GDNF-treated neurons. The number of immunoreactive puncta per neuron was found to increase with time in culture (Fig. 7B). By day 5, most cells had several Syn-1-positive puncta along their somata and dendrites. By day 15, dendrites were densely covered by Syn-1 staining. After day 20, the high density of Syn-1-positive puncta made it difficult to adequately separate individual puncta and thus rendered quantification difficult (not shown). At all ages tested, we found that GDNF clearly increased the number of Syn-1-positive specializations (Fig. 7A). At day 5, the number of puncta was increased twofold in comparison to control neurons (CTRL, 36 ± 4 puncta, $n = 53$; GDNF, 72 ± 6 puncta, $n = 58$). At day 10, the increase was 2.1-fold (CTRL, 72 ± 8 puncta, $n = 29$; GDNF, 152 ± 13 puncta, $n = 29$). At day 15, the increase was 1.7-fold (CTRL, 99 ± 12 puncta, $n = 23$; GDNF, 171 ± 18 puncta, $n = 23$). A two-way ANOVA confirmed an overall significant effect of GDNF treatment ($F = 61.85$, $P < 0.001$) and an overall effect of time in culture ($F = 44.6$, $P < 0.001$). *Post hoc* pair-wise comparisons between individual means confirmed that the effect of GDNF on the number of Syn-1-positive puncta was significant for each tested day in culture (Tukey test, $P < 0.05$). Although TH-negative neurons also showed an increase in the number of Syn-1-positive puncta from 5 to 15 days in

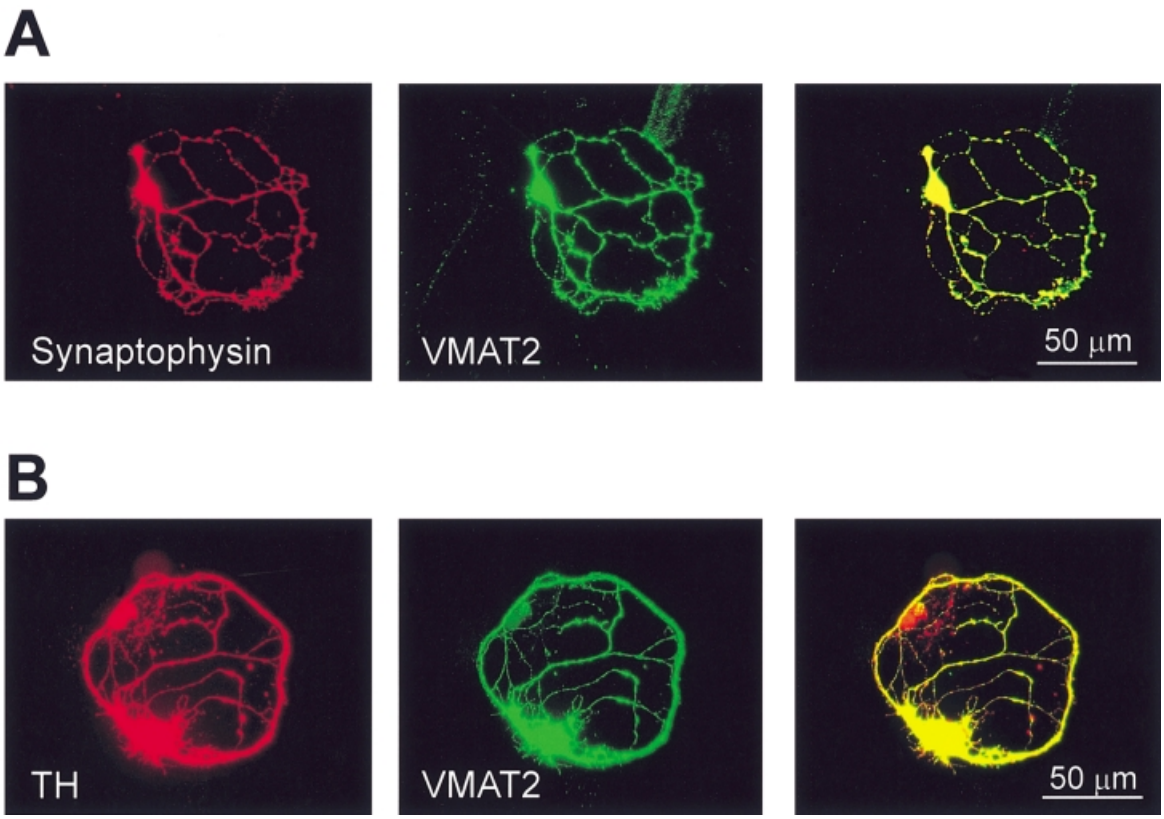

FIG. 6. Localization of the vesicular monoamine transporter in dopaminergic neurons. (A) Images of an isolated dopaminergic neuron immunostained for the synaptic terminal marker synaptophysin (red, left panel) and for the vesicular monoamine transporter (VMAT2, green, middle panel). The right panel is an image overlay illustrating the considerable overlap between the two antigens (yellow). (B) Images of an isolated dopaminergic neuron immunostained for tyrosine hydroxylase (red, left panel) and for the vesicular monoamine transporter (VMAT2, green, middle panel). The right panel is an image overlay illustrating the overlap between the two antigens (yellow).

culture, no significant increase was caused by GDNF. At 5 days, the number of Syn-1-positive puncta was 48 ± 9 ($n = 8$) in control cells and 43 ± 7 ($n = 9$) in GDNF-treated cells. At 10 days, the number of Syn-1-positive puncta was 68 ± 13 ($n = 8$) in control cells and 78 ± 13 ($n = 7$) in GDNF-treated cells. Finally, at 15 days, the number of Syn-1-positive puncta was 112 ± 11 ($n = 7$) in control cells and 122 ± 17 ($n = 6$) in GDNF-treated cells.

Discussion

Glial cell line-derived neurotrophic factor is well known to support the survival and differentiation of dopaminergic neurons. However, the mechanism by which it enhances the synaptic release of neurotransmitter from these neurons is unclear. The present work provides novel information on the mechanism of action of GDNF. We show that in cultured VTA neurons, GDNF acts selectively on dopaminergic neurons to enhance both spontaneous (Fig. 4) and evoked (Figs 3 and 5) quantal release of glutamate. This increase in synaptic transmission is accompanied by an increase in the number of synaptic terminals identified by Syn-1 immunolocalization (Fig. 7). We have found that the enhancement of the number of Syn-1^+ synaptic terminals (~twofold) closely paralleled the enhancement of autaptic EPSC amplitude (~threefold) and RR-evoked mEPSCs (~twofold). Taken together, these results suggest that GDNF stimulated the establishment of fully functional synaptic terminals.

We have observed that the enhancement of the rate of spontaneous mEPSCs by GDNF was slightly larger (~fourfold) than the enhancement of action potential-evoked (~threefold) and RR-evoked (~twofold) release. Although the significance of this observation is still unclear, this may indicate that the newly formed terminals do not yet display a fully mature phenotype at this stage and release more transmitter in an unregulated fashion. Further experiments will be required to test such an idea.

In the present study, we have used glutamate-mediated synaptic events as an assay of the activity of the synaptic terminal established by dopaminergic neurons. This approach was chosen because membrane currents generated by the synaptic activation of dopamine receptors are not detectable in these neurons, as previously reported (Sulzer *et al*., 1998). An alternate approach would have been to use amperometry to directly monitor dopamine release from individual varicosities (Pothos *et al*., 1998). However, this technique would not have allowed us to quantitatively sample the activity of a large number of terminals on any given neuron, something easily performed by recording glutamate receptor-mediated synaptic currents. One limitation of the present study is that we have not verified whether under our experimental conditions, dopamine release was also enhanced by GDNF. This limitation is minimized by the fact that GDNF has already been shown to enhance depolarization-evoked dopamine release in cultured neurons (Feng *et al*., 1999), as measured by using radioactively labelled dopamine. It should also be noted that although our electrophysiological experiments did not directly measure dopamine release, our immunocytochemical experiments (Fig. 7) allowed us to quantify the number of putative synaptic terminals irrespective of the transmitter that is released. We also determined that, at least at the

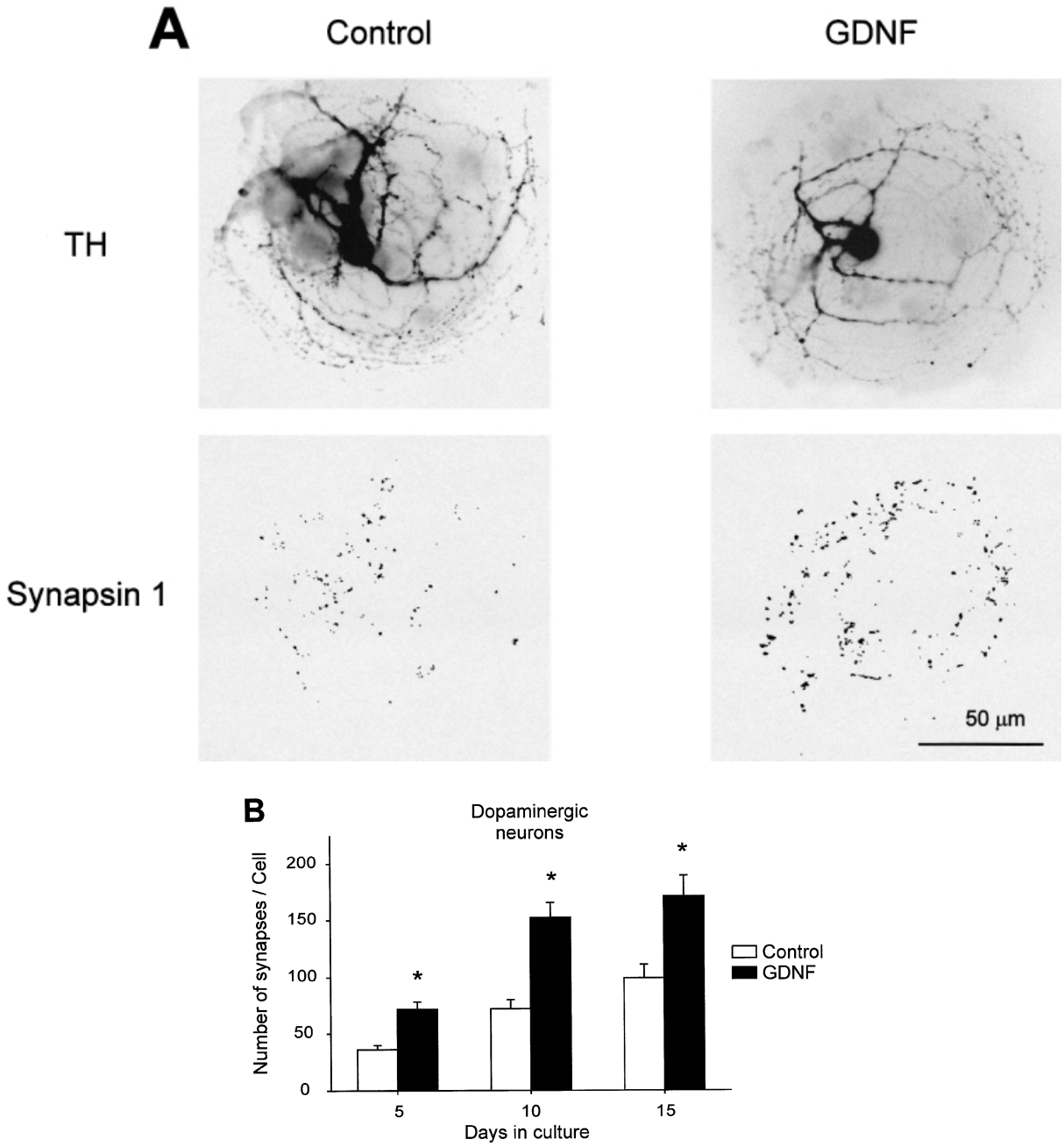

FIG. 7. Time-dependent effects of GDNF on the number of nerve terminals. (A) Images of isolated dopaminergic neurons immunostained for TH (upper set of images). Syn-1 immunostaining of the same neurons was performed to localize sites of putative synaptic terminals (lower set of images). Note that Syn-1 staining was also found on some glial cells (not shown; see Materials and methods). GDNF-treated dopaminergic neurons (right) displayed a larger number of Syn-1^+ puncta than control dopaminergic neurons (left). (B) Summary histogram of the number of Syn-1^+ puncta per neuron in control (open bars) and GDNF-treated (filled bars) isolated dopaminergic neurons. At 5 ($n = 58$), 10 ($n = 29$) and 15 ($n = 23$) days in culture, GDNF significantly increased the number of Syn-1^+ nerve terminals in TH^+ neurons. $^*P < 0.05$.

level of light microscopy, the vast majority of nerve terminals established by dopaminergic neurons under our experimental conditions expressed VMAT2, thereby suggesting that they had the potential to accumulate and release dopamine (Fig. 6). In summary, it is thus very likely that our results reflect an increase in the number of active synaptic terminals formed by dopaminergic neurons, irrespective of whether these release glutamate or dopamine. In this respect, it is interesting to note that previous work has suggested that cultured dopaminergic neurons can form two independent sets of terminals that release either glutamate or dopamine (Sulzer *et al*., 1998). Under our experimental conditions, it seems that most terminals have the capacity to release dopamine (Fig. 6).

Our results suggest that the enhanced neuronal differentiation previously reported to be caused by GDNF is associated with an increase in the number of functional synaptic terminals. This mechanism may contribute to a large extent to the enhancement of dopamine release reported to be caused by GDNF *in vivo* (Hebert *et al*., 1996). Considering the recent observation that GDNF also

causes an increase in the quantal size of vesicular dopamine in cultured neurons (Pothos *et al*., 1998), it is likely that the enhanced dopamine release results from a concomitant increase in the number of functional terminals together with an increase in the quantity of dopamine released from individual terminals. Further work will be required to determine what mechanism predominates *in vivo*.

In the set of experiments reported here, we have not determined the mechanism by which GDNF causes an increase in the number of Syn-1-positive synaptic terminals. Because GDNF receptors are coupled to a tyrosine kinase activity (Jing *et al*., 1996; Treanor *et al*., 1996; Cacalano *et al*., 1998) and can lead to phosphatidylinositol-3-kinase and MAP-kinase activation (Pong *et al*., 1998; Soler *et al*., 1999), it will be important in future experiments to investigate the involvement of these pathways in the facilitation of synapse formation reported here. These pathways could possibly be involved in an up-regulation of the expression of synaptic proteins. It is of interest to note that GDNF has been reported to cause an increase in dopamine release without changing the expression of SNAP-25, syntaxin, Syn-1 or synaptobrevin in cultured embryonic midbrain neurons (Feng *et al*., 1999). However, it is important to note that in these later experiments, the proportion of dopaminergic neurons was very small relative to GABAergic and non-dopaminergic/non-GABAergic neurons, thus complicating the detection of changes that occur specifically in dopaminergic neurons. Besides a direct stimulation of synapse formation, perhaps by stimulating the transcription of synapse-specific genes, an alternate hypothesis to explain the enhancement of synapse number is that GDNF stimulated dendritic development which then indirectly increased synapse number by providing favourable conditions for synaptic growth and development. This could be tested by a thorough quantitative analysis of the effect of GDNF on dendritic development together with an evaluation of the density of synapses on dendrites. A suitable experimental preparation to test this would be low-density primary cultures where individual neuronal processes do not tend to run alongside each other as much as in microisland cultures.

Although the effects of GDNF reported here were restricted to dopaminergic and not to GABAergic neurons, we found that rGDNF-α was expressed in both types of neurons, at least as revealed by immunostaining using a monoclonal antibody against this receptor (Fig. 2 and results not shown). This suggests that the specificity of action of GDNF on VTA neurons must depend on other downstream factors. Previous reports have shown that the expression of rGDNF-α is broad and not restricted to dopaminergic neurons (Trupp *et al*., 1997; Glazner *et al*., 1998).

In summary, our results suggest that GDNF can stimulate the formation of functional synapses in cultured dopaminergic neurons. In light of the considerable efforts that are spent to use this trophic factor in a therapeutic context to help dopaminergic neurotransmission recover, e.g. in the treatment of Parkinson's disease, our observations suggest that GDNF has the potential to not only prevent the degeneration of dopaminergic neurons and to stimulate the growth of neuronal processes, but also to cause an enhancement of dopamine release by increasing synapse number.

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Abbreviations

AMPA, (±)-α-amino-3-hydroxy-5-methylisoxalone-4-propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethylenediaminetetra-acetate; GABA, γ-aminobutyric acid; GDNF, glial cell line-derived neurotrophic factor; GTP, guanosine triphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; mPSC, miniature post-synaptic current; rGDNF-α, glial cell line-derived neurotrophic factor receptor alpha; RR, ruthenium red; Syn-1, synapsin-1; TH, tyrosine hydroxylase; TTX, tetrodotoxin; VMAT2, vesicular monoamine transporter; VTA, ventral tegmental area.

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