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Neurotrophins Stimulate the Release of Dopamine from Rat Mesencephalic Neurons via Trk and p75^{Lntr} Receptors*

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We analyzed the short term effect of neurotrophins on mesencephalic neuronal cultures of embryonic (E14) rats with respect to which receptors mediate the actions. Brain-derived neurotrophic factor (BDNF) or neurotrophin-3 enhanced within minutes in a dose-dependent manner (2, 20, 100 ng/ml for 5 min) depolarization-induced (KCl, 30 mM 5 min) and basal dopamine release, but nerve growth factor (NGF) was only effective at high doses (100 ng/ml). The effect of BDNF, but not of NGF, was blocked by K252a or K252b. BDNF, but not NGF, phosphorylated trkB receptors. The NGF-induced, but not the BDNF-induced effect upon the release of dopamine was blocked by anti-p75 antibody MC192. C_2-ceramide, an analogue of ceramide, the second messenger of the sphingomyelin pathway, and sphingomyelinase itself induced a release of dopamine comparable with the effect of NGF. NGF, but not BDNF, increased ceramide production. In addition, simultaneous treatment with BDNF and NGF led to a partial prevention of the NGF-stimulated, p75^{Lntr}-mediated effect.

We conclude that BDNF stimulates the release of dopamine by activation of the trkB receptor, whereas NGF affects the release via the p75^{Lntr} receptor inducing the sphingomyelin pathway.

Neurotrophins mostly have been discussed with respect to their function in influencing survival and differentiation of certain populations of neurons during development. In transgenic mice, inactivation of neurotrophins and their receptors mainly results in an impairment of the peripheral nervous system, whereas in the central nervous system only minor effects have been observed (for review see Ref. 1). However, there is growing evidence that neurotrophins in the central nervous system might be involved in activity-dependent changes of neuronal structures and functions. In contrast to the periphery, neurotrophins in the central nervous system are synthesized in neurons, and their expression (for review see Ref. 2) and release (3, 4) are regulated by neuronal activity. The release occurs within 1 min after depolarization mainly at soma and dendrites (5). Application of exogenous neurotrophins in synaptosomal preparations or cell cultures of the hippocampus induces a rapid increase in the second messengers cAMP, inositol 1,4,5-trisphosphate, and Ca^{2+} (6, 7) as well as the immediate release of acetylcholine and glutamate (8, 9). Electrophysiological recordings revealed that neuronal activity of motor neurons (10) and glutamatergic transmission in hippocampal neurons (11) are stimulated by BDNF[1] and that intrahippocampal injections of BDNF in freely moving rats rapidly induce high frequency spiking (12). In hippocampal slices an increase of synaptic transmission at the Schaffer colateral-CA1 synapses could be seen after applications of BDNF and NT-3 (13). Korte and collaborators (14) observed that in hippocampal slices of the BDNF knockout mouse long term potentiation was drastically reduced; this effect was visible even in heterozygous mutant mice. Taken together, these data suggest a role of neurotrophins as specific retrograde messengers modulating synaptic transmission and their involvement in neuronal plasticity (for review see Ref. 15).

Two types of receptors might be involved in neurotrophin-mediated neurotransmission, the Trk receptor family and p75^{Lntr}. The tyrosine kinase-coupled receptors TrkA, TrkB, and TrkC are specifically recognized by NGF, BDNF, and neurotrophin-3 (NT-3), respectively, and belong to the tyrosine kinase receptor family (16–20; for review see Refs. 21, 22). They show high affinity properties with a slow off-rate (for review see Ref. 22). p75^{Lntr} is a low affinity receptor binding all three neurotrophins (with a high off-rate; $K_D = 2 \times 10^{-9}$ to 4×10^{-9}, *i.e.* about 20 to 50 ng/ml; see Refs. 18, 23). The biological activity of neurotrophins can be attributed to an induction of one of the Trk receptors, whereas p75^{Lntr} is thought to cooperate with TrkA receptors to form a high affinity receptor for NGF (24). p75^{Lntr} is not necessary for NGF-mediated signaling and is discussed to form heterodimers with any other member of the Trk family (for review see Ref. 25). Up to now, p75^{Lntr} could not be related to a biological activity independent of Trk receptors, but it has been shown that p75^{Lntr} coexpressed with Trk receptors increases the sensitivity to neurotrophins (26, 27) and leads to clearer discrimination between the neurotrophins (28). Although p75^{Lntr} activation induces the sphingomyelin pathway in cell lines expressing solely p75^{Lntr} (29, 30), no biological effect has yet been attributed to this activation.

In this context, we were interested whether the modulation of synaptic transmission is restricted to cholinergic and glutamatergic systems, or if it is a general effect of neurotrophins, and how p75^{Lntr} might be involved in this modulation. Since the dopaminergic neurons of the substantia nigra of the rat express BDNF as well as its specific receptor TrkB (31) but not NGF and TrkA receptors (for review see Ref. 22), and since the survival and differentiation of mesencephalic dopaminergic neurons in culture is improved by BDNF and NT-3 (32–34), these neurons represent a good system to study the effects of

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[1] The abbreviations used are: BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NGF, nerve growth factor.

neurotrophins on dopamine release. Neuronal cell cultures develop networks with spontaneous depolarization and intact intracellular signal transduction in contrast to other *in vitro* preparations. Cell cultures are less complex than slices used for electrophysiological analysis, but they allow the biochemical examination of the signal transduction in specific populations of neurons.

Because of the trophic function of neurotrophins, particularly during development, the dominant view has been that neurotrophins act over longer periods. Several authors analyzed the effect of chronic neurotrophin application (up to 2 weeks) on dopaminergic neurons *in vivo* (34, 35) and *in vitro* (36–38). They observed an increased dopamine uptake (34) and elevated levels of dopamine and its metabolites (36–38), indicating an increased dopamine turnover. But prolonged treatment with neurotrophins also increased the sprouting of dopaminergic neurons (34, 35) and the density of varicosities and of binding sites of dopamine uptake (35). Also a desensitization of TrkB receptors has been observed after only a 24-h treatment with BDNF (39). Apparently long term treatment with neurotrophin concentrations usually used alters the equilibrium of neurons. In the last years also short term effects of neurotrophins have been investigated (for review see Refs. 2, 15). After the demonstration of regulated, depolarization-induced neurotrophin release (3, 4), fast local variation of neurotrophin concentrations seems possible. Therefore, we were interested particularly in the short term or immediate effects that neurotrophins might have on the release of dopamine and on receptors mediating these effects.

We observed a stimulation of dopamine release induced by both BDNF and NGF, but the signal transduction induced by the two neurotrophins was different. While BDNF stimulated the release of dopamine exclusively via the TrkB receptor, NGF activated the p75^{Lntr} receptor and thereafter stimulated the sphingomyelin pathway.

EXPERIMENTAL PROCEDURES

Serum-free medium (Neurobasal) was purchased from Life Technologies, Inc. (Eggenstein, Germany). All other reagents were obtained from Sigma (Munich, Germany) if not otherwise indicated.

Cell Culture and Dopamine Detection—Dopaminergic neurons of the mesencephalon (predominantly the substantia nigra) from embryonic Wistar rats (E14) were cultured according to Voorn *et al.* (40), with the following modifications. After dissociation, cells were preplated on uncoated tissue culture dishes (Falcon; Becton Dickinson, Heidelberg, Germany) for 2 h. Then the neurons, which do not attach to the culture dish, were removed and seeded on coverslips precoated with poly-L-ornithine (0.5 mg/ml) in defined, serum-free medium at a density of about 300,000 cells per 10-mm (diameter) coverslip. The cells were used 14 days after dissection in a perfusion system (modified perfusion chambers of Minucell and Minutissue, Bad Abbach, Germany) as described elsewhere (3). The amount of dopamine released into the perfusion buffer (modified Hank's buffer: 125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 μM $ZnCl_2$, 10 mM glucose, 0.25% bovine serum albumin, 1 nM tyrosine, 25 mM HEPES, pH 7.4) was measured by using high pressure liquid chromatography and electrochemical detection (Waters, Eschborn, Germany) as described by Eriksson and Persson (41). Dihydrobenzylamine was added as an internal standard to calculate the amount of dopamine in each sample. A first depolarization (D1), which served as a reference, was induced by substitution of 30 mM NaCl with KCl (depolarization buffer). The conditions for D1 were constant in all experiments. A second stimulation (D2) was induced 60 min later, either by depolarization buffer (control) or by addition of neurotrophins (BDNF, NGF, or NT-3) to the depolarization buffer, or by addition of ceramide, sphingomyelinase, or neurotrophins to the perfusion buffer (without KCl; see also Fig. 1). The tyrosine kinase inhibitors K252a or K252b (Calbiochem-Novabiochem, Bad Soden, Germany) or the supernatant of hybridoma cells, producing the monoclonal antibody against p75^{Lntr} MC192, were added 30 min prior to D2 to the perfusion buffer. The proportion D2/D1 was used to calculate the effect of a stimulation of the cultures by neurotrophins or the effect of a treatment with K252a/b or MC192. The results are expressed as percentage of the controls, which received two depolarizations with 30 mM KCl (for details see Ref. 3).

Dopamine uptake was measured in 12-day-old cultures. The neurons were stimulated with neurotrophins for 15 min at 37 °C. The uptake was analyzed as described by Beck *et al.* (34).

Autophosphorylation of TrkB—14-day-old cultures (about 10^7 cells) were incubated for 5 min with BDNF (100 ng/ml) at 37 °C. The cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 250 μM *p*-nitrophenol phosphate, 1 mM ATP, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM aprotinin, and 21 μM leupeptin. From 1 mg of protein of the lysed cells TrkB or TrkA receptors were immunoprecipitated either with rabbit TrkB-antiserum or rabbit TrkA-antiserum (both purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with a pan Trk antiserum (a generous gift of D. Kaplan) and analyzed for autophosphorylation in a Western blot, using a mouse monoclonal anti-phosphotyrosine-antibody (UBI, Hamburg, Germany), a peroxidase-coupled anti-mouse IgG-antibody (Boehringer Mannheim, Germany), and the BM chemiluminescence system (Boehringer Mannheim, Germany) for detection.

Ceramide Production—Neuronal mesencephalic cultures (about 3×10^6 cells) were used at day 14 in culture. Prior to the measurement of ceramide, cells were washed three times. 3 h after the change of the medium, the cultures (except the controls) were stimulated with neurotrophins (100 ng/ml). Pretreatment with K252a or the supernatant of hybridoma cells, producing the monoclonal anti-p75 antibody MC192, was started 30 min prior to stimulation. 15 min after stimulation, cells were fixed in 1 ml of methanol, and lipids were extracted, and the amount of ceramide was quantified as described by Van Veldhoven *et al.* (42).

Immunohistochemistry—Immunohistochemical analysis was performed in 14-day-old cultures of the substantia nigra.

Colocalization of p75^{Lntr} and Tyrosine Hydroxylase—The cells were first stained against p75^{Lntr} with the monoclonal antibody MC192 (1:10 dilution of conditioned medium from hybridoma cells), using a biotinylated second antibody (dilution of 1:1000; Dianova, Hamburg, Germany) and a streptavidin-Cy3 conjugate (dilution 1:2500; Dianova, Hamburg, Germany) for the detection of the antigen. Subsequently the cells were fixed and permeabilized with 5% acetic acid in 95% ethanol and stained for tyrosine hydroxylase using a mouse monoclonal anti-tyrosine hydroxylase antibody at a dilution of 1:10 followed by fluorescein-coupled, affinity purified anti-mouse antiserum at a dilution of 1:50 (both antibodies from Boehringer Mannheim, Germany) to visualize the antigen.

Colocalization of TrkB or TrkA and Tyrosine Hydroxylase—Cells were fixed and permeabilized with 100% methanol. For TrkB immunolabeling, a serum raised against the extracellular domain of TrkB (43); for TrkA a rabbit anti-TrkA serum (purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used, and for the tyrosine hydroxylase labeling the monoclonal antibody described above was used. The staining was detected by using affinity purified, cy3-coupled antibodies from a goat anti-rabbit antiserum (Dianova, Hamburg, Germany) at a dilution of 1:2500 or fluorescein-coupled anti-mouse antibodies described above. For a control, the culture medium of hybridoma cells was used with respect to the mouse monoclonal antibodies and a normal rabbit serum with respect to the rabbit antisera. Since dopaminergic mesencephalic neurons do not express TrkA receptors, staining the primary cultures against TrkA gave an additional negative control.

RESULTS

Neurotrophins Stimulate the Release of Dopamine—Dopaminergic neurons of the mesencephalon continuously released small amounts of dopamine into the perfusion buffer. This basal release is probably due to spontaneous depolarization within the neuronal network (suppressed by applications of tetrodotoxin, data not shown). To stimulate the dopamine release we used 30 mM KCl, which induced an approximate half-maximal response, to allow for possible amplification of the release of dopamine by additional pharmacological interventions. 30 mM KCl stimulated the basal release of dopamine to about 5–6-fold within the depolarization period (*i.e.* 5 min; Fig. 1*A*). Since the dead volume of the perfusion system was 200 μl (corresponding to 3 min of perfusion time), the release occurred within 2 min. A second depolarization after 1 h of

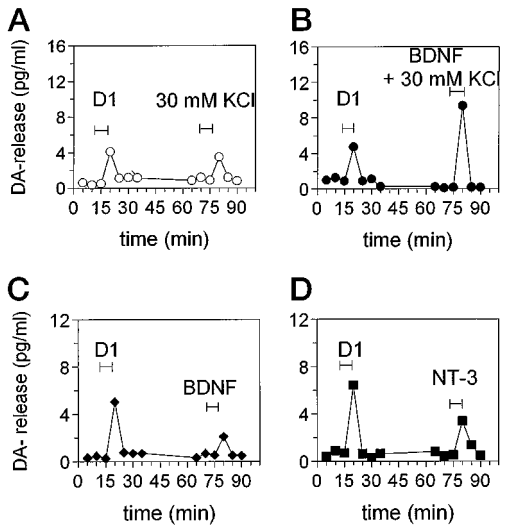

FIG. 1. **Time course of four single perfusion experiments.** Neuronal cultures of rat mesencephalon (E14) were perfused after 14 days in culture, and the amount of released dopamine in each sample was analyzed as described under "Experimental Procedures." The figure shows a first depolarization with 30 mM KCl (5 min, D1, duration as indicated by the *bar*). 60 min later, the release of dopamine was induced either by 30 mM KCl (*A*), 30 mM KCl + 20 ng/ml BDNF (*B*), 100 ng/ml BDNF (*C*), or 100 ng/ml NT-3 (*D*).

recovery resulted in a somewhat lower release of dopamine (about 65% of the first depolarization). BDNF (2, 20, and 100 ng/ml), given simultaneously with a depolarization induced by 30 mM KCl, stimulated the release of dopamine in cultured mesencephalic neurons in a dose-dependent manner (Figs. 1*B* and 2*A*). Heat-inactivated BDNF (100 ng/ml, 1 h at 100 °C) did not induce a significant effect (Fig. 2*C*). We observed the dopamine releasing effect of BDNF during depolarization within the time resolution of the perfusion system (*i.e.* 2 min, Fig. 1*B*). BDNF also stimulated the release of dopamine without additional depolarization by KCl (Table I, Fig. 1*C*, and Fig. 3).

NT-3 (100 and 20 ng/ml) increased the basal release of dopamine comparable with BDNF (Fig. 1*D*, and Table I).

NGF (both, recombinant and purified 2.5 S NGF from mouse salivary gland) increased KCl-induced (Fig. 2*A*) and basal (Table I, Fig. 3) release of dopamine only at high doses (100 ng/ml); lower doses (20 ng/ml) reduced the release slightly (Fig. 2*A*). We used for our experiments recombinant NGF (Boehringer Mannheim) or purified NGF (2.5 S) from the mouse salivary gland. Both substances gave essentially the same results (Table I). This excludes the possibility that a contamination of the purified NGF could have caused the observed effects.

To ensure that the neurotrophin-induced increase of dopamine in the collected samples is due to an increased release and not to a diminished reuptake, the effect of neurotrophins on the uptake of [^{3}H]dopamine was analyzed. BDNF (100 ng/ml) increased the uptake of [^{3}H]dopamine into mesencephalic neurons significantly (Table II). Preincubation of the neurons with K252a (100 nM) inhibited this effect. NGF did not influence the [^{3}H]dopamine uptake.

The Dopamine Releasing Effect Follows Different Pathways for NGF and BDNF—Since mesencephalic dopaminergic neurons express TrkB and TrkC receptors, which directly activate the tyrosine kinase, it should be expected that the same signal transduction is involved in the release of dopamine induced by BDNF and NT-3, respectively. Therefore, we performed all experiments, analyzing tyrosine kinase activation, only with BDNF. To investigate an involvement of Trk receptors in the observed effects, we inhibited the tyrosine kinase with K252a or K252b. Pretreatment of the cultures with K252a (100 nM, Table I, Fig. 3) or K252b (1.5 μM, Fig. 2*B*), given 30 min before stimulation, partially reduced the dopamine releasing effect of 100 ng/ml BDNF (to about 40% of the BDNF induced release); the effect of 20 ng/ml BDNF was completely blocked by K252b (Fig. 2*B*). K252a or K252b did not influence the dopamine releasing effect of 100 ng/ml NGF (Figs. 3 and 2*B*).

To study whether signaling is triggered by NGF via p75^{Lntr}, we pretreated the cells with supernatant (in a 1:10 dilution) of cultured hybridoma cells producing the monoclonal anti-p75 antibody MC192. The antibody inhibited the effect of NGF on dopamine release (Fig. 2*C*). To exclude any unspecific effects of the supernatant on the dopamine release, an equally pretreated control group received two depolarizations with 30 mM KCl. The KCl-induced dopamine release was not substantially changed compared with a control group receiving two depolarizations but no pretreatment with the supernatant (Fig. 2*C*). Compared with untreated neurons, the BDNF-induced release of dopamine was not impaired by MC192 treatment of the neurons (Fig. 2*C*).

To reveal a potential activation of the sphingomyelin pathway, which is induced by p75 receptor stimulation (29, 30), we analyzed the effect of sphingomyelinase and C_2-ceramide, an analogue of the second messenger ceramide of this pathway, on the release of dopamine. Both sphingomyelinase (0.1 unit/ml) and ceramide (1 μM) induced a release of dopamine similar to 30 mM KCl (Table III).

The production of ceramide was elevated in cultures treated 15 min with NGF (100 ng/ml) compared with untreated cultures. Pretreatment of the cultures with MC192 completely blocked the effect of NGF (Fig. 4). BDNF (100 ng/ml) failed to induce ceramide production. Blocking the tyrosine kinase activation and thereby the TrkB-mediated signal transduction increased the basal ceramide production in BDNF-stimulated neurons slightly (Fig. 4; about 45% increase compared with untreated, BDNF-stimulated neurons).

Effect of Simultaneous Application of BDNF and NGF—NGF and BDNF were not additive with regard to the release of dopamine (Fig. 3). BDNF, given simultaneously with NGF, even reduced the release to 65% compared with stimulation by NGF alone. After pretreatment of the cultures with K252a and subsequent stimulation with both neurotrophins together, the release of dopamine reached again levels comparable with that produced by NGF alone (Fig. 3).

Autophosphorylation of Trk Receptors by Treatment with BDNF or NGF—To establish the involvement of Trk receptors, we analyzed the tyrosine kinase-mediated autophosphorylation of these receptors during treatment of the cell cultures with BDNF (20 ng/ml; 100 ng/ml) or NGF (100 ng/ml). As demonstrated in Fig. 5, *A* and *B*, both doses of BDNF phosphorylated the receptor (within 5 min) but NGF did not cross-react with the TrkB receptor. Immunoprecipitation with pan-Trk antibodies (which recognize all Trk receptors) indicated also that only BDNF treatment resulted in an autophosphorylation of Trk receptors (Fig. 5*C*).

In contrast to PC12 cells, which express TrkA, we could not observe phosphorylated TrkA receptors by treatment of the neuronal mesencephalic culture with NGF (Fig. 5*D*, a TrkA-antibody was used for immunoprecipitation).

Presence of p75^{Lntr} and TrkB Receptors in the Mesencephalic Neuronal Culture—Immunohistochemical staining revealed that both p75^{Lntr} (Fig. 6, *G* and *H*) and TrkB receptors (Fig. 6, *E* and *F*) were located on dopaminergic neurons that expressed tyrosine hydroxylase, the key enzyme of dopamine synthesis. In a negative control, instead of MC192 the culture medium of hybridoma cells was used (Fig. 6, *I* and *J*). TrkA receptors could not be found with immunohistochemical analysis (Fig. 6, *C* and

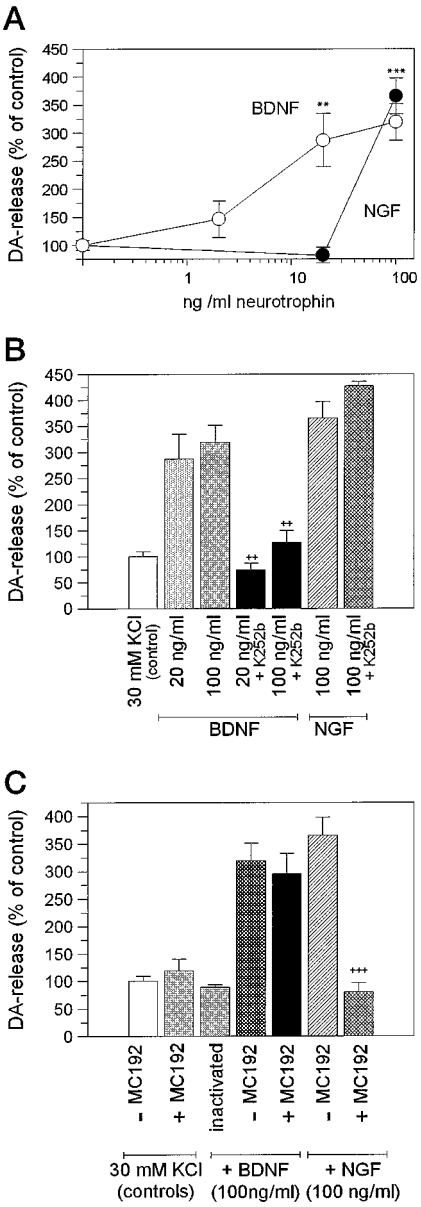

FIG. 2. **Effect of BDNF and NGF on the release of dopamine from neuronal cultures of rat mesencephalon.** Neuronal cultures of rat mesencephalon (E14) were perfused after 14 days in culture, and the amount of released dopamine was analyzed as described under "Experimental Procedures." For an internal control in each experiment a first depolarization was induced by 30 mM KCl added for 5 min to the perfusion buffer. 60 min later, a second depolarization was induced by 30 mM KCl and simultaneously by different doses of NGF or BDNF as indicated. The data are presented as ratio of the second depolarization to the first depolarization in percentage of the controls, which received two depolarizations with 30 mM KCl, as mean ± S.E. *A*, dose-dependent stimulation of the release of dopamine by BDNF (*open circles*) or NGF (*solid circles*) added to the depolarization buffer (*i.e.* with 30 mM KCl). Data represent mean ± S.E.M. ** = $p < 0.01$, *** = $p < 0.001$, neurotrophins *versus* controls (number of experiments varies between 5 and 7); calculated according to Bonferroni (64). *B*, involvement of tyrosine kinase activation in neurotrophin-induced dopamine release. The tyrosine kinase inhibitor *K252b* (1.5 μM) was added 30 min prior to the second depolarization completed with BDNF (20 ng/ml or 100 ng/ml) or NGF (100 ng/ml). ++ = $p < 0.01$, K252b/BDNF$_{(20\ ng/ml)}$ *versus* BDNF$_{(20\ ng/ml)}$, and K252b/BDNF$_{(100\ ng/ml)}$ *versus* BDNF$_{(100\ ng/ml)}$; calculated according to Bonferroni (64) (n = 5–7). *C*, involvement of p75^{Lntr} in neurotrophin-induced dopamine release. The monoclonal antibody MC192 (supernatant of hybridoma cells in a 1:10 dilution) was added 30 min prior to the second depolarization completed with BDNF (100 ng/ml) or NGF (20 ng/ml or 100 ng/ml). As additional controls, cultures pretreated with supernatant of hybridoma cells were also depolarized twice with 30 mM KCl to exclude possible effects of the supernatant on the dopamine release, and the effect of inactivated BDNF (100 ng/ml, incubated 60 min at 100 °C) added to the depolarization buffer (*i.e.* with 30 mM KCl) was analyzed. +++ = $p < 0.001$, MC192/NGF *versus* NGF, 100 ng/ml; calculated according to Bonferroni (n = 4–6).

TABLE I

Effect of neurotrophins on the basal dopamine release

Neuronal cultures of rat mesencephalon (E14) were perfused after 14 days in culture (see "Experimental Procedures"). Depolarization was induced by 30 mM KCl added for 5 min to the perfusion buffer (modified Hank's buffer). After 60 min recovery BDNF, NT3, or NGF (rec = recombinant, 2.5 S = purified NGF from the mouse salivary gland) at the indicated dose were added to the perfusion buffer for 5 min. The results are expressed as percentage (mean ± S.E.M.) of the control (two depolarizations with 30 mM KCl), the number n of independent experiments in different cell preparations is given in parentheses.

	Dopamine release
	% of control
30 mM KCl (control)	100 ± 0.30 (n = 6)
NT-3, 20 ng/ml	71 ± 23.0 (n = 4)
NT-3, 100 ng/ml	167 ± 13.4 (n = 4)[a]
BDNF, 20 ng/ml	85 ± 11.9 (n = 6)
BDNF, 100 ng/ml	150 ± 13.4 (n = 6)[b]
NGF$_{rec}$, 100 ng/ml	175 ± 18.7 (n = 4)[a]
NGF$_{2.5\ S}$, 100 ng/ml	185 ± 17.1 (n = 4)[a]

[a] $p < 0.01$, neurotrophins *versus* control.
[b] $p < 0.05$, BDNF *versus* control.

D), although in PC12 cells the receptor could be detected by the antiserum we used (Fig. 6, *A* and *B*). Fig. 6*D* also shows that no unspecific staining occurs.

DISCUSSION

In the present analysis, we show that the neurotrophins BDNF, NT-3, and NGF can induce a release of dopamine in neuronal cultures of the mesencephalon within minutes after addition. The neurotrophins modulate the release triggered by 30 mM KCl as well as the basal release of dopamine. The main difference between the effects of BDNF or NT-3 and NGF is the dosage needed to induce a release of dopamine. While BDNF is effective at doses as low as 2 ng/ml, we observed an effect of NGF only at high doses (100 ng/ml).

The elevated dopamine concentration in the perfusion buffer could arise from increased dopamine release and/or reduced dopamine inactivation. We therefore analyzed the effect of neurotrophins on the uptake of dopamine, which is the main inactivation pathway of the neurotransmitter. It is known that long-term treatment (over 5 days) with BDNF increases the uptake of [^{3}H]dopamine (34). In our experiments the uptake of [^{3}H]dopamine is already elevated 15 min after treatment of the culture with BDNF. This effect was mediated by TrkB, since it could be blocked by K252a and was not seen during stimulation by NGF (Table II). Therefore, the increase of dopamine in the perfusion buffer after stimulation with neurotrophins should be due to an elevated release and not to a reduced inactivation of dopamine.

One aim of this study was to reveal a possible involvement of the p75^{Lntr} receptor in neurotrophin-mediated dopamine release. Dopaminergic neurons of the substantia nigra express TrkB and TrkC receptors but not the TrkA receptor (31, 33, for review see Ref. 22), and these neurons do not depend on NGF for survival in culture (32, 44, 45). With immunohistochemical staining we observed a colocalization of TrkB or p75^{Lntr} receptors with the tyrosine hydroxylase, the key enzyme of dopamine synthesis. Therefore, BDNF should act via stimulation of TrkB receptors, while in the absence of TrkA NGF should induce a biological action either via cross-reaction with TrkB or TrkC or through activation of p75^{Lntr}.

In our system, BDNF (and NT-3) increased the release of dopamine in a dose-dependent manner effectively at doses as

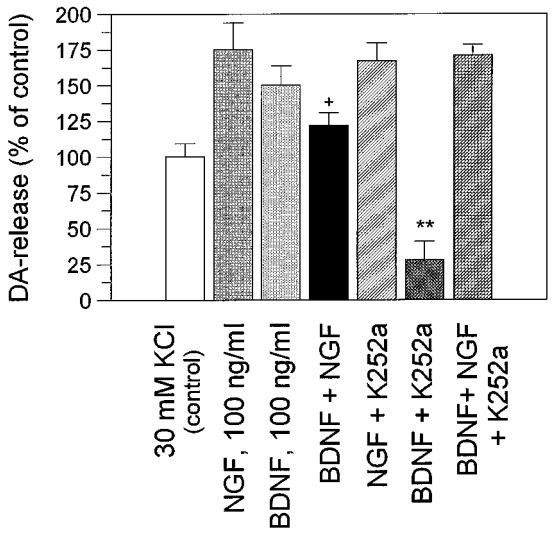

FIG. 3. **Effect of simultaneous application of BDNF and NGF on the basal dopamine release.** Same experimental procedure as in Fig. 2. Instead of a second depolarization the neurotrophins BDNF or NGF (100 ng/ml each) or both neurotrophins were added to the perfusion buffer for 5 min (without additional KCl). Pretreatment of cultures with K252a (100 nM) was started 30 min prior to the application of neurotrophins. The results are expressed as percentage of the control (two depolarizations with 30 mM KCl) as mean ± S.E.M.; numbers of independent experiments in different cell preparations were between 5 and 6. + = $p < 0.05$, BDNF + NGF *versus* NGF; ** = $p < 0.01$, BDNF + K252a *versus* BDNF; calculated according to Bonferroni.

TABLE II
Effect of neurotrophins on the [^{3}H]dopamine uptake

14-day-old cultures were preincubated for 15 min with BDNF (100 ng/ml). K252a (100 nM) was given 20 min prior to the BDNF (100 ng/ml) incubation. The uptake of [^{3}H]dopamine was analyzed as described by Beck *et al.* (34). The data represent mean ± S.E.M., *n* represents the number of independent experiments in different cell preparations.

	[^{3}H]Dopamine uptake pmol/h
Control (untreated)	2.565 ± 0.11 (n= 6)
BDNF	6.856 ± 0.44 (n=3)[a]
K252a/BDNF	2.670 ± 0.03 (n= 5)
NGF	2.590 ± 0.05 (n= 3)

[a] $p < 0.001$. BDNF *versus* control; calculated according to Bonferroni (64).

low as 2 ng/ml (Fig. 2*A*), which suggests that high affinity receptors might be activated. Activation of a member of the Trk receptor family involves the activation of tyrosine kinase. This enzyme can be blocked by K252a or K252b (46). Since K252a is known to be partially agonistic we also used K252b, which does not show agonistic effects (47). We did not observe any increase of the basal dopamine release during the treatment with K252a or K252b, and we therefore assume that at the doses we used, hypothetical agonistic effects are negligible. Both drugs, given 30 min prior to the depolarization buffer completed with 100 ng/ml BDNF, diminished the effect of BDNF, and the effect of 20 ng/ml was completely blocked (Fig. 2*B*). Activation of tyrosine kinase leads to an autophosphorylation of Trk receptors. We found phosphorylated TrkB receptors after stimulation of the neuronal culture for 5 min with BDNF (Fig. 5). These results justify the assumption that BDNF elevates the release of dopamine mainly by stimulation of the specific TrkB receptor.

With high NGF doses, we think that no Trk receptors are involved but that the effect of NGF is mainly due to an activation of p75^{Lntr} receptors. The following arguments support this hypothesis. 1) No TrkA receptors are expressed in dopaminergic neurons, and the neurons do not depend on NGF (32, 44, 45). Any effect seen with NGF must therefore be due to an activation of other receptors, and, apart from TrkB, we could demonstrate the presence of p75^{Lntr} in dopaminergic neurons

TABLE III
Effect of sphingomyelinase and ceramide on the release of dopamine without additional depolarization by KCl

Depolarization was induced by 30 mM KCl added for 5 min to the perfusion buffer (modified Hank's buffer, for details see "Experimental Procedures"). After 60 min recovery 30 mM KCl (control), or sphingomyelinase, or ceramide was added to the perfusion buffer for 5 min. The results are expressed as percentage (mean ± S.E.M.) of the control, the number *n* of independent experiments in different cell preparations is given in parentheses.

	Dopamine release
	% of control
30 mM KCl (control)	100 ± 9.30 (n = 6)
Sphingomyelinase, 0.1 unit/ml	100 ± 34.0 (n = 4)
Ceramide, 1 μM	146 ± 26.1 (n = 4)

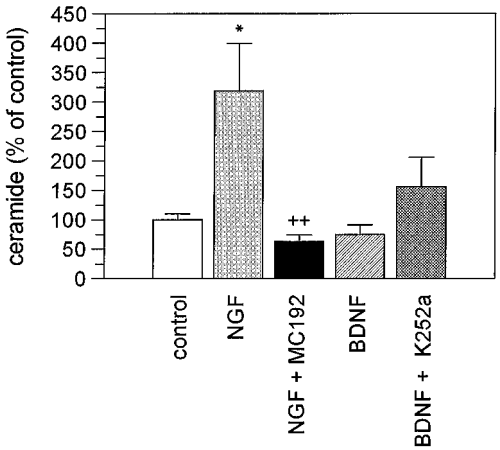

FIG. 4. **Ceramide production in mesencephalic neurons of the rat (E14).** Neuronal cultures were stimulated for 15 min with NGF (100 ng/ml) or BDNF (100 ng/ml). Pretreatment with MC192 or K252a (100 nM) was started 30 min prior the stimulation. Stimulation was stopped with 1 ml of methanol, and ceramide was analyzed from extracted lipids using a standard curve with C_2-ceramide. The data are presented as mean ± S.E. given in percentage of the control, which received no treatment. The number of experiments was between 4 and 6. * = $p < 0.05$, NGF *versus* control; + = $p < 0.05$, MC192/NGF *versus* NGF; calculated according to Bonferroni.

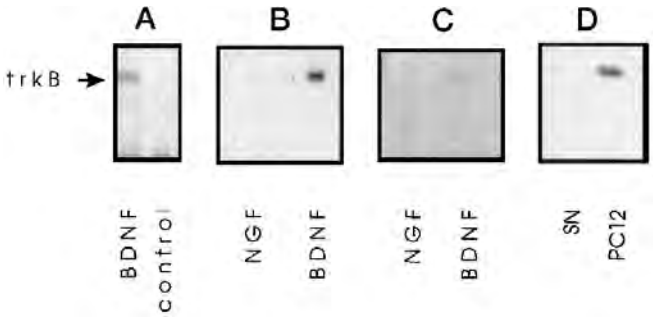

FIG. 5. **Phosphorylation of Trk receptors by treatment with BDNF and NGF.** Cultured mesencephalic neurons of embryonal Wistar rats (E14) were incubated at day 14 with BDNF (20 ng/ml, *A* and *C*; or 100 ng/ml, *B*) or NGF (100 ng/ml) for 5 min. In *A* and *B*, TrkB receptors were immunoprecipitated using anti-TrkB antibodies. In *C*, neurons were stimulated with NGF or BDNF, but for immunoprecipitation a pan-Trk antiserum, recognizing all neurotrophins, was used, and in *D* PC12 cells or mesencephalic neurons of the substantia nigra (*SN*) were stimulated with NGF, and the samples were immunoprecipitated with TrkA antibodies. The samples were analyzed in a Western blot, using anti-phosphotyrosine antibodies and the BM chemiluminescence system for detection.

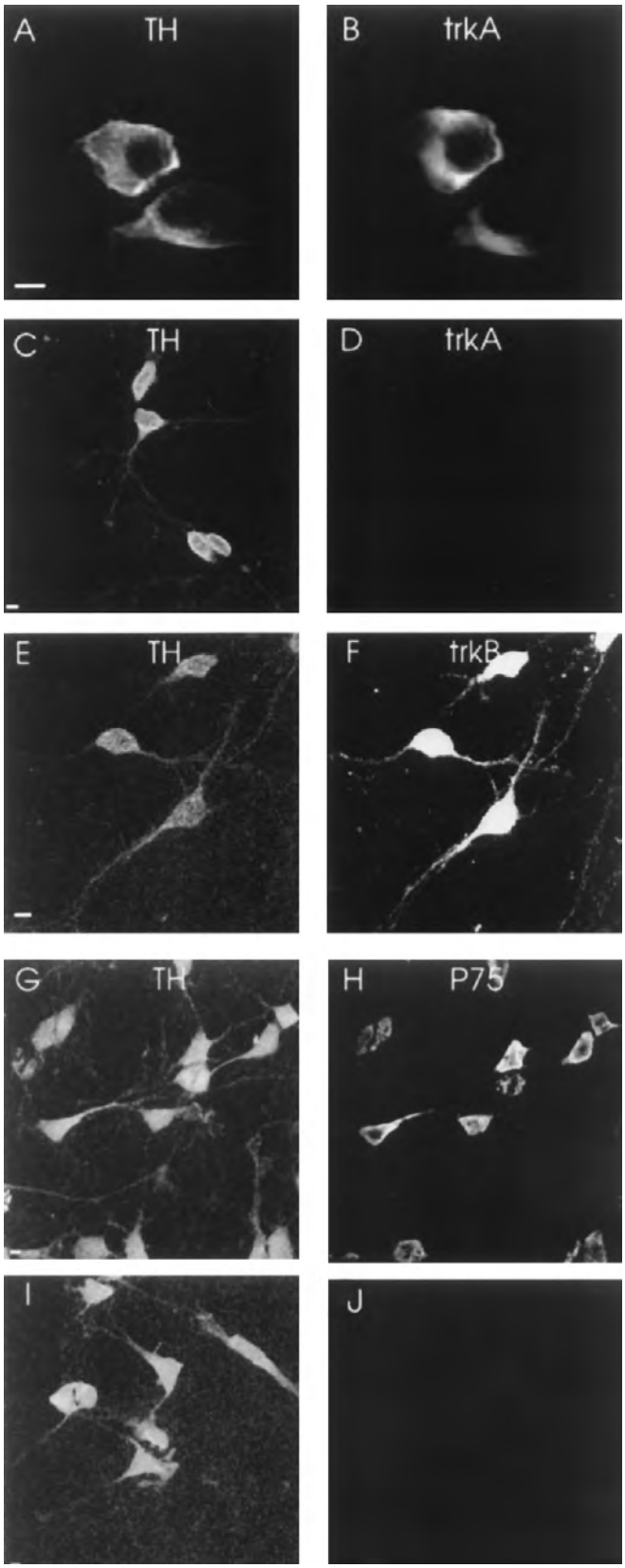

FIG. 6. **Colocalization of neurotrophin receptors and tyrosine hydroxylase.** Immunohistochemical analysis was performed in 14-day-old cultures of the substantia nigra. Colocalization of TrkA (*B*, *D*) or TrkB (*F*) and tyrosine hydroxylase (*TH*) (*A*, *C*, *E*). Cells were fixed and permeabilized with 100% methanol. The primary antibodies (antiserum raised against the extracellular domain of TrkB and a monoclonal anti-tyrosine hydroxylase antibody) were detected by using anti-rabbit IgG coupled to Cy3 (*B*, *D*, *F*) or anti-mouse IgG coupled to fluorescein (*A*, *C*, *E*). Colocalization of p75^{Lntr} (*H*) and tyrosine hydroxylase (*G*). The cells were first stained for p75^{Lntr} (supernatant of hybridoma cell line MC192) using a biotinylated second antibody and a streptavidin-Cy3 conjugate (*H*, *J*). Then the cells were fixed and permeabilized with 5% acetic acid in 95% ethanol and stained for tyrosine hydroxylase using fluorescein-coupled second antibodies to visualize the antigen (*G*, *I*). For a control, instead of the supernatant of the hybridoma cells, producing MC192, the culture medium for hybridoma cells was used (*I*, *J*). *Bar* = 5 μm.

in our neuronal culture. 2) p75^{Lntr} has been suggested to interact with each member of the Trk receptor family (48). Therefore, it could be possible to observe a tyrosine phosphorylation by NGF binding first to p75^{Lntr} and subsequently interacting with TrkB or TrkC, in the absence of TrkA receptors. However, we did not observe phosphorylation of TrkB or any other Trk receptor (immunoprecipitation with pan Trk and TrkA antibodies) after NGF stimulation of the cultures, which indicates that tyrosine kinase had not been activated (Fig. 5). 3) The tyrosine kinase inhibitor K252a or K252b did not diminish the effect of 100 ng/ml NGF on the release of dopamine, but the effect could be blocked by pretreatment of the culture with MC192 (Fig. 2). This antibody reduces the effect of NGF in PC12 cells (49) and in neurons (50). MC192 does not block the binding of NGF to the receptor, but it interrupts the interaction between TrkA and p75 (51). It is not clear whether this effect is due to a possible loss of heterodimerization between TrkA and p75^{Lntr} or to a probable inhibition of the signal transduction of p75^{Lntr}. Other studies showed that MC192 reduces NGF-mediated induction of c-*fos* mRNA (52) and of immediate early genes (51). In these studies the results were interpreted as a disruption of the interaction between p75^{Lntr} and TrkA. To our knowledge no data are available concerning the effect of MC192 in cells that express p75^{Lntr} and not TrkA. In our system, MC192 blocked the production of ceramide, which is considered to be a second messenger of p75^{Lntr} (29, 30). This supports the idea that p75^{Lntr}- activated signal transduction may be inhibited by the monoclonal antibody MC192.

Recently, p75^{Lntr} has been suggested to induce the sphingomyelin pathway (29, 30). In this pathway the enzyme sphingomyelinase hydrolyzes sphingomyelin to ceramide and phosphocholine (for review see Ref. 53). Ceramide is a second messenger involved in various pathways (for review see Refs. 54, 55). It stimulates protein phosphatases and kinases (56–58). Ceramide can be phosphorylated by a calcium-dependent protein kinase located in synaptic vesicles (59) to ceramide 1-phosphate, and a ceramide-1-phosphate phosphatase has been described by Boudker and Futerman (60). Therefore Chao (55) discussed the possibility that ceramide phosphorylation is involved in neurotransmitter release, for example via phosphorylation of synaptic vesicle proteins like synapsin I. Knipper *et al*. (9) showed in synaptosomes of rat hippocampus that NGF and BDNF induced a phosphorylation of synapsin I.

We found an elevated level of ceramide production after NGF treatment, whereas MC192 blocked this NGF-induced effect completely (Fig. 4). Conversely, in our experiments C_2-ceramide and sphingomyelinase increased the release of dopamine similar to 30 mM KCl (Table III). Therefore, we conclude that the effect of NGF is probably linked to a stimulation of the sphingomyelin pathway by interaction of NGF with the p75^{Lntr} receptor.

We did not observe an additive effect of BDNF and NGF but instead a reduction compared with NGF alone (Fig. 3). After pretreatment of the cultures with K252a, simultaneous application of BDNF and NGF increased the release of dopamine comparable with the effect of NGF alone, but still we did not observe an additive effect. Since in K252a-pretreated cultures even high doses of BDNF induced only a small release of

dopamine, the missing additive effect is not due to a competition of BDNF and NGF at the p75^{Lntr} receptor. In addition, we did not observe induction of the sphingomyelin pathway by high doses of BDNF and only a borderline increase (Fig. 4) in cultures pretreated with K252a to block the signal transduction of TrkB. Surprisingly, dopamine release after simultaneous application of BDNF and NGF was lower than after stimulation with NGF alone; stimulation of the TrkB receptor seems to inhibit partially the p75^{Lntr}-induced dopamine release (Fig. 3). The monoclonal antibody against p75^{Lntr}, MC192, also did not impair BDNF-stimulated dopamine release (Fig. 2*C*). Therefore, in neuronal mesencephalic cultures, BDNF does not seem to be capable of activating the p75^{Lntr} receptors as observed for NGF. Dobrowsky *et al.* (30) analyzed the induction of the sphingomyelin pathway by neurotrophins in NIH 3T3 cells, which express solely p75^{Lntr}. The authors observed only marginal differences between BDNF and NGF in stimulating the sphingomyelin hydrolysis at the dose of 100 ng/ml, but in PC12 cells, which express p75^{Lntr} and TrkA receptors, NGF did not activate the sphingomyelin pathway. After pretreatment of the PC12 cells with K252a, they again observed an increase in sphingomyelin hydrolysis by NGF stimulation, which was still reduced compared with stimulation by NT-3 alone. Although it is known that p75^{Lntr} recognizes different neurotrophins as similar, but not identical molecules (61), these results of Dobrowsky and coauthors (30) demonstrate that in principle all neurotrophins are capable of inducing p75^{Lntr}-activated signal transduction with similar kinetics. It is not clear why BDNF had only a small effect on the dopamine release in K252a-pretreated neuronal substantia nigra cultures, but it is possible that the ratio of TrkB to p75 receptors might influence the signal transduction either by binding site competition or by direct interference.

The observed stimulation of dopamine release by BDNF should have physiological significance, since BDNF is present in the substantia nigra, the neurons express the TrkB receptors specific for BDNF, and concentrations within the range of the binding capacity of the TrkB receptors (17) induce dopamine release. The unexpected dopamine release induced by NGF via p75^{Lntr} activation is more difficult to judge with respect to physiological significance. Until recently (29, 30) p75^{Lntr} was only considered to modulate the activity of Trk receptors, especially TrkA; Dobrowsky *et al.* (30) demonstrated that neurotrophins (BDNF and NT-3 even in lower concentrations than NGF) can trigger signaling via the p75^{Lntr} receptor, activating the sphingomyelin pathway. Our data show that this signaling can stimulate neurotransmitter release in dopaminergic neurons. Although the amount of NGF detected in the substantia nigra is quite low (between 50 and 200 pg/g tissue) (62), nothing can be said about the local (extracellular) concentration which could not be determined yet with the techniques available. There are also dopaminergic projections of the substantia nigra, *e.g.* into the striatum where NGF is present in moderate concentrations (up to 800 pg/g tissue; 62). Here, NGF could indeed modulate the dopamine release from substantia nigra projections. In any case, our study demonstrates the real possibility that neurotrophins may influence neurotransmitter release via p75^{Lntr}, which could be of interest in the context of neuronal plasticity.

Okazawa *et al.* (63) describes in *in vivo* experiments an up-regulation of the expression of BDNF in the dopaminergic system by levodopa. We observed the release of dopamine by BDNF in neuronal cultures of the substantia nigra. It is therefore possible that in the nigrostriatum there is a regulation mechanism similar to that in the hippocampus. In this region, the synthesis of BDNF is regulated by glutamatergic and GABAnergic inputs, and BDNF increases the release of glutamate (Ref. 9, for review see Ref. 2). The present data show that modulation of neurotransmission by neurotrophins is not restricted to the hippocampus but may also occur in other brain areas.

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