

Are There Differences Between the Secretion Characteristics of NGF and BDNF? Implications for the Modulatory Role of Neurotrophins in Activity-Dependent Neuronal Plasticity

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ABSTRACT In previous experiments the activity-dependent secretion of nerve growth factor (NGF) from native hippocampal slices and from NGF-cDNA transfected hippocampal neurons showed unusual characteristics [Blöchl and Thoenen (1995) *Eur J Neurosci* 7:1220–1228; (1996) *Mol Cell Neurosci* 7:173–190]. In both hippocampal slices and cultured hippocampal neurons the activity-dependent secretion proved to be independent of extracellular calcium, but dependent on the release of calcium from intracellular stores. Under different experimental conditions, Goodman et al. [(1996) *Mol Cell Neurosci* 7:222–238] reported that the high potassium-mediated secretion of brain-derived neurotrophic factor (BDNF) from hippocampal cultures was dependent on extracellular calcium. Mowla et al. [(1997) *Proc 27th Annu Meet Soc Neurosci New Orleans* 875.10] reported on even further-reaching differences between NGF and BDNF secretion, namely, that in hippocampal neurons and in pituitary cell lines NGF was secreted exclusively according to the constitutive pathway, whereas BDNF was exclusively sorted according to the activity-dependent regulated pathway. In view of the crucial importance of such potential differences between the processing, sorting, and secretory mechanisms of different neurotrophins for their modulatory roles in activity-dependent neuronal plasticity, a thorough analysis under comparable experimental conditions was mandatory. We demonstrate that in native hippocampal slices and adenoviral-transduced hippocampal neurons there are no differences between NGF and BDNF with respect to the subcellular distribution and mechanism of secretion; that the activity-dependent secretion of both NGF and BDNF is dependent on intact intracellular calcium stores; and that the differences between our own observations and those of Goodman et al. (*ibid.*) regarding the dependence on extracellular calcium do not reflect differences between NGF and BDNF sorting and secretion, but reflect the differing experimental conditions used. *Microsc. Res. Tech.* 45:262–275, 1999. © 1999 Wiley-Liss, Inc.

INTRODUCTION

The modulatory role of neurotrophins in activity-dependent neuronal plasticity represents a new facet in the ever-growing spectrum of biological actions of this gene family (see Bonhoeffer, 1996; Schuman, 1997; Thoenen, 1995). The physiological importance of the modulatory role of neurotrophins is evident from the observation that the development of the activity-dependent formation of ocular dominance in the visual cortex of several species can be disrupted not only by the local administration of neurotrophins (see Bonhoeffer, 1996; Cellerino and Maffei, 1996), but also—and most importantly—by blocking agents, i.e., anti-neurotrophin antibodies (Berardi et al., 1994) and Trk receptor bodies (IgG fusion proteins in which the variable domains of IgGs are replaced by the extracellular domains of different Trk receptors; see Cabelli et al., 1997; Figurov et al., 1996).

However, the relatively complex system of the visual cortex (see Bonhoeffer, 1996; Katz and Schatz, 1996) is not suitable for a detailed analysis of the underlying cellular and molecular mechanisms. Therefore, comple-

mentary analyses have been performed in appropriate *in vitro* systems, including synaptosomes (Knipper et al., 1994a,b; Sala et al., 1998), primary neuronal cultures (Lessmann et al., 1994; Lessman and Heumann, 1998; Levine et al., 1995; Suen et al., 1997), co-cultures of *Xenopus* spinal neurons and myocytes (Lohof et al., 1993; Wang and Poo, 1997; Wang et al., 1998), and acute and chronic slices of different brain regions (Akaneya et al., 1997; Figurov et al., 1996; Kang and Schuman, 1995; Kang et al., 1997; McAllister et al., 1995, 1996). The administration of exogenous neurotrophins modulates synaptic transmission by both presynaptic and postsynaptic mechanisms. Presynaptically, neurotrophins enhance the activity-dependent secretion of conventional neurotransmitters (Gottschalk

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et al., 1998; Lessmann et al., 1994; Lessmann and Heumann, 1998; Li et al., 1998; Lohof et al., 1993). Postsynaptically, they enhance transmission through N-Methyl-D-aspartic acid (NMDA) receptors (Levine et al., 1995, 1998), promoting the phosphorylation of the NMDA receptor subunit-1 (Suen et al., 1997). Conversely, brain-derived neurotrophic factor (BDNF) attenuates the transmission via GABA_A receptors (Tanaka et al., 1997). It is important to emphasize that these pre- and postsynaptic effects are not ubiquitous, but depend on the expression of the corresponding Trk receptors that are preferentially assigned to the individual neurotrophins: TrkA to nerve growth factor (NGF), TrkB to BDNF and NT-4/5, and TrkC to NT-3 (see Barbacid, 1994; Bothwell, 1995; Lewin and Barde, 1996). Moreover, at the CA1-Schaffer collateral synapses of hippocampal slices—a well-analyzed *in vitro* system—BDNF has been shown to be important in the formation of long-term potentiation (LTP) (Korte et al., 1995; Patterson et al., 1996), a paradigm for learning and memory (see Bliss and Collingridge, 1993; Collingridge and Bliss, 1995). LTP was drastically reduced in hippocampal slices of BDNF knockout mice. Interestingly, homo- and heterozygous mice showed the same impairment (Korte et al., 1995; Patterson et al., 1996), indicating that a critical quantity of BDNF has to be available in this system for LTP formation. Long-lasting (3–6 hours) LTP seems to be even more dependent on BDNF, since it is totally abolished in BDNF knockout mice (Korte et al., 1996b). Since LTP can be reconstituted either by adenoviral-mediated overexpression of BDNF in the CA1 region (Korte et al., 1996a) or by bath application of BDNF (Patterson et al., 1996), the reduced BDNF availability is responsible for these effects rather than subtle cumulative developmental changes in the knockout animals. The importance of BDNF for the formation of LTP is further supported by the observation that treatment of hippocampal slices with TrkB receptor bodies inhibited the generation of theta-burst stimulation induced LTP (Kang et al., 1997). The natural onset of LTP expression during development coincides with the rapid increase of BDNF levels in the hippocampus (Figurov et al., 1996; Gottschalk et al., 1998). Before this developmental stage, exogenous administration of BDNF permitted the induction of LTP (Figurov et al., 1996). All these experiments demonstrate that BDNF availability is crucial for the formation of this specific kind of neuronal plasticity.

In order to integrate the different effects of neurotrophins into a physiological context, it is essential to identify the mechanisms that determine the availability of neurotrophins under physiological conditions, i.e., the regulation of synthesis and secretion of neurotrophins. It is now well established that the regulation of synthesis of neurotrophins, particularly that of BDNF and NGF, occurs in a very rapid manner and is mediated by the interplay of excitatory and inhibitory neurotransmitters (see Lindholm et al., 1994; Thoenen, 1995). The positive regulation is mediated by the influx of calcium, which then—via activation of calmodulin—results in CREB phosphorylation (Tao et al., 1998), regulating the synthesis of BDNF via specific promoter sites (Shieh et al., 1998). However, in addition to the site, extent, and rate of the regulation of synthesis, the

mechanism(s) and site(s) of secretion are of primary importance. It has been demonstrated that NGF is secreted from both hippocampal slices and NGF-transfected hippocampal cultures along the constitutive and activity-dependent pathway (Blöchl and Thoenen, 1995, 1996). The activity-dependent NGF secretion showed unconventional characteristics in that it is independent of extracellular calcium but depends on intact intracellular calcium stores (Blöchl and Thoenen 1995, 1996). Similar characteristics have also been demonstrated for neurotrophin-mediated neurotrophin secretion occurring as a consequence of Trk receptor activation (Canossa et al., 1997). However, discordant results have been reported for high potassium-mediated secretion of BDNF from virus-transduced primary cultures of hippocampal neurons and AtT-20 and PC12 cells (Goodman et al., 1996). These authors observed that the high potassium-mediated secretion of BDNF was dependent on extracellular calcium under their (“static”) experimental conditions. Based on these observations, they suggested that one possible explanation for this discrepancy could be differences between the secretory mechanisms of NGF and BDNF. Even more striking differences were reported in an abstract by Mowla et al. (1997), who claimed that the secretion of NGF occurs in different cellular systems exclusively according to the constitutive pathway, whereas BDNF is secreted exclusively according to the regulated pathway. Such potential differences between the mechanisms of sorting and secretion of different neurotrophins are of paramount importance for the function of these neurotrophins in modulating activity-dependent neuronal plasticity and will, therefore, be addressed in this article. We demonstrate that the reported differences between the secretion characteristics of BDNF and NGF do not reflect differences between the subcellular distribution and mechanism of secretion of the two neurotrophins, but reflect the different experimental conditions (“static” vs. “perfusion” system) under which these results were obtained.

MATERIALS AND METHODS

Acute Hippocampal Slices

Slices (350 μ m) were prepared from the hippocampi of adult Wistar rats (either sex) in cold, oxygenated modified Hanks buffer (Blöchl and Thoenen, 1995) using a MacIlwain tissue chopper. Slices obtained from one hippocampus were placed in a perfusion chamber and constantly perfused with Hanks buffer equilibrated with 95% O₂ and 5% CO₂. After an equilibration period of 30 minutes at a flow rate of 0.1 or 0.5 ml/min, release experiments were started.

Primary Cultures of Hippocampal Neurons

Hippocampal neurons were prepared from E17–18 Wistar rats according to the procedure of Zafra et al. (1990). Briefly, neurons were plated at a density of 300,000 per well (48-well dish) on glass coverslips coated with poly-DL-ornithine (0.5 mg/ml) (Brewer and Cotman, 1989). After 7–8 days in culture, neurons were transduced with approximately 10⁷–10⁸ pfu of AdCMV-NGF (Ad = adenovirus; CMV = cytomegalovirus promoter) or AdCMV-BDNF_{myc} in a reduced volume of 300 μ l complete medium (Griesbeck et al., 1997). Neu-

rons were used for the release experiments on the following day.

Adenoviral Vectors

Viral vectors were constructed by homologous recombination in 293 cells (Graham and Prevec, 1991; McGrory et al., 1988). AdCMV-NGF contained the sequence of mouse preproNGF and AdCMV-BDNFmyc the sequence of mouse preproBDNF. In order to add the myc epitope of 10 amino acids, the three C-terminal amino acids of the BDNF coding region were removed (Canossa et al., 1997; Heymach and Shooter, 1995).

Characteristics of the Perfusion System

Acute slices and hippocampal neurons cultured on glass coverslips were placed in a perfusion chamber (Minucell and Minutissue, Bad Abbach, Germany) and constantly perfused with Hanks buffer at 37.8°C. Fractions of 0.5 ml volume were collected for 1 minute at a flow rate of 0.5 ml/min or 5 minutes at a flow rate of 0.1 ml/min and the samples analyzed by ELISA. A pulse of 1 or 5 minutes of bovine serum albumin (BSA) or the vital dye Cochenille Red (0.1%, Sigma, Deisenhofen, Germany) was collected as a defined peak, indicating that the liquid stream is laminar in these chambers (data not shown). The dead volume of the perfusion system was lower than 0.5 ml, corresponding to a perfusion time of less than 1 minute at a flow rate of 0.5 ml/min and between 4 and 5 minutes at a flow rate of 0.1 ml/min.

Release Experiments and Statistical Analysis

Cultured hippocampal neurons or hippocampal slices were perfused with modified Hanks buffer in a perfusion system as described by Canossa et al. (1997). Briefly, cells were placed in perfusion chambers and equilibrated with calcium-containing modified Hanks buffer at a perfusion rate of 0.1 ml/min or 0.5 ml/min for 20 to 30 minutes before samples were collected. After two basal values of 1 or 5 minutes, stimulations were initiated for 1 or 5 minutes and three more fractions were collected after stimulation. After 30 minutes of recovery, samples were again collected and a second stimulation was initiated. Stimulations were performed either by replacing 50 mM NaCl with 50 mM KCl in the perfusion medium or by using glutamate at a concentration of 50 μ M. In order to distinguish between the roles played by extracellular and intracellular calcium, either the high affinity calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), its membrane-permeable ester 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid pentaacetoxymethyl ester (BAPTA/AM) (Tsien, 1980, 1981) or thapsigargin and caffeine were added at a concentration of 10 μ M. After a first stimulation in calcium-containing perfusion buffer serving as a control, calcium was omitted from the perfusion buffer and BAPTA or BAPTA/AM was added (thapsigargin and caffeine were added to the perfusion buffer in the presence of calcium). After a further equilibration period of 30 minutes, a second stimulation was initiated.

BDNF and NGF were quantified by ELISA and expressed in pg/ml as shown for representative examples in Figures 1, 2, 3, 5, and 6A. These experiments include examples of differing perfusion rates. For statis-

tical analysis, in order to be able to compare BDNF secretion characteristics obtained in slices and transduced cultures, the data are presented as a percentage of control of at least four independent experiments in which the quantity of BDNF release by the second stimulus was expressed as a percentage of the first reference stimulus (Fig. 4).

BDNF secretion was also quantified under "static" conditions. In these experiments, hippocampal neurons infected with AdCMV-BDNFmyc were equilibrated with calcium-containing modified Hanks buffer for 30 minutes before samples were collected. After two basal collection values of 10 minutes, the cultures were stimulated for 10 minutes replacing 50 mM NaCl with 50 mM KCl in the medium. In order to distinguish between the roles played by extracellular and intracellular calcium originating from the calcium stores, the high-affinity calcium chelator BAPTA was added to the calcium-free medium, or the cultures were treated with thapsigargin (10 μ M) and caffeine (3 mM) in calcium-containing medium during the equilibration time before samples were collected. The data presented are the mean \pm SEM of at least four independent experiments (Fig. 6B).

Release of ³H-Glutamate From Hippocampal Neurons

To study glutamate release, hippocampal neurons (300,000 on a glass coverslip) were loaded for 1 hour with 1 μ Ci ³H-glutamate in a 48-well dish at 37°C; cells were placed in a perfusion chamber equilibrated with calcium-containing modified Hanks buffer. Cells were then perfused for 10 minutes before samples were collected. After two basal values of 5 minutes, the neurons were depolarized with 50 mM KCl for 5 minutes and four additional fractions of 5 minutes each were collected (Fig. 3C).

Enzyme Immunoassays (ELISAs)

Two different enzyme immunoassays for BDNF were used. Before anti-BDNF monoclonal antibodies became available, preliminary experiments were performed using an enzyme immunoassay according to Nawa et al. (1995). However, the data presented in this article are based on the assay described by Canossa et al. (1997). Briefly, two monoclonal antibodies (#1 and #9) were affinity-purified. The #1 antibody was used as a first antibody; #9 was conjugated with peroxidase (Boehringer Mannheim, Germany) and used as a second antibody. ELISA plates were coated overnight at 4°C with the first antibody in 50 mM sodium carbonate buffer (pH 9.7), blocked overnight with ELISA buffer consisting of Hanks buffer, 0.1% Triton X-100, and 2% BSA. Samples and standards of recombinant BDNF (0.5–1,000 pg/ml) were transferred to the plates together with the second antibody and incubated overnight at 48°C. After washing, the quantity of bound peroxidase-coupled second antibody was determined by incubation with TMB-peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) for 30 minutes. After stopping the reaction with 1 M H₂SO₄, the absorptions were determined. The ELISAs showed a sensitivity of 0.5 pg/ml of BDNF. NGF was quantified according to the method of Korsch-

ing and Thoenen (1987), as modified by Spranger et al. (1990).

Calcium Imaging

We measured free intracellular calcium in both "static" and "perfusion" conditions. Hippocampal neurons were loaded for 40 minutes with 2 μ M Fura2-AM, rinsed, and incubated in fresh culture medium for 10 minutes before the measurements. The cells were kept in modified Hanks buffer during analysis. Cells were visualized with a Zeiss Fluor 40 \times 1.30 oil objective by using an inverted microscope (Axiovert 100, Zeiss). Fluorescence was determined at the excitation wavelengths of 340/380 nm, monitoring the emission at 510 nm with an intensified charge coupled device camera (Hamamatsu), and images were processed with Argus 50/CA software. Images were taken at a sampling rate of 0.75/sec; eight frames were averaged for each image.

Immunohistochemistry and Confocal Microscopy

For intracellular detection of NGF or BDNFmyc, hippocampal neurons were fixed for 20 minutes with 4% paraformaldehyde in PBS. Cells were permeabilized using 0.2% Triton X-100. Background fluorescence was quenched by 0.1 M glycine for 20 minutes. Unspecific binding sites were blocked by incubating the neurons for 1 hour with 20% normal goat serum (NGS). All antibodies were diluted in 1% NGS in PBS. The first antibody was added overnight at 4°C; all other steps were performed at room temperature. For the detection of BDNFmyc, a 9E10 hybridoma supernatant (dilution 1:10) recognizing the 10 amino acid myc epitope was used. For NGF, a rabbit anti-NGF antiserum (Calbiochem-Novabiochem, San Diego, CA, USA) was used at a dilution of 1:1,000.

The secondary antibody was biotinylated anti-mouse IgG (1:2,000; Jackson ImmunoResearch, West Grove, PA, USA), in combination with anti-rabbit lissamine-rhodamine antibody (1:150; Dianova, Hamburg, Germany). Biotin was visualized by incubation with streptavidin-conjugated FITC (1:500; Dianova) for 30 minutes. After extensive washing, coverslips were mounted and analyzed by confocal microscopy.

RESULTS

Comparison Between the Mechanisms of Secretion of NGF and BDNF in Transduced Hippocampal Neurons and Native Hippocampal Slices

The characteristics of the 50 mM potassium-induced BDNF and NGF secretion from native hippocampal slices are very similar (Fig. 1A,B), although a comparison of absolute values is not possible, since the basal levels of NGF secretion are much lower than those of BDNF, reflecting the substantially lower levels of NGF mRNA and protein in the rat hippocampus compared to those of BDNF (Hofer et al., 1990; Korsching et al., 1985; Nawa et al., 1995). Similar results were obtained by stimulation of hippocampal slices with 50 μ M glutamate (Canossa et al., 1997). Thus, virtually identical features are shown by the high potassium- and glutamate-mediated NGF and BDNF secretion from native hippocampal slices.

In dissociated primary cultures of hippocampal neurons, a 50 mM potassium-mediated release of BDNF could occasionally be observed, but the levels were always at the detection limit and therefore unreliable (data not shown). Since the levels of NGF secretion in native hippocampal neurons were in any case below the detection limit, further analyses in dissociated hippocampal cultures were performed after transduction with adenoviral vectors carrying the cDNAs of BDNF or NGF. This procedure also permitted expression of NGF and BDNF in comparable quantities.

Adenovirus-Mediated Gene Transfer Increases Levels of Basal and Regulated Secretion of NGF and BDNF

Basal levels of NGF and BDNF secretion were similar and reliably detectable after infection with appropriate titers of corresponding adenoviral constructs for about 16 hours (Fig. 1C,D). Both BDNF and NGF concentrations in the perfusate increased 3–5-fold by depolarization with 50 mM potassium as compared to basal values (Fig. 1C,D). The time course of NGF and BDNF secretion was very similar to that of endogenous NGF from native hippocampal slices (Fig. 1A,B). Thus, the basic characteristics of secretion are not changed by adenovirus-mediated overexpression of NGF and BDNF compared to those of nontransduced hippocampal slices.

In order to compare the results obtained for NGF and BDNF secretion from hippocampal slices and adenoviral-transduced hippocampal cultures resulting in highly differing absolute quantities of neurotrophins in the perfusion media, the evolving results were also presented as a percentage of control as the mean \pm SEM of at least four independent experiments (presented in Fig. 4).

Characteristics of BDNF Secretion From Hippocampal Neurons Induced by High Potassium at Different Perfusion Rates

In order to obtain a higher temporal resolution of release, we analyzed the neurotrophin secretion at a higher flow rate of 0.5 ml/min. The time course of BDNF secretion from hippocampal slices (Fig. 2A) and BDNF-transduced primary cultures (Fig. 2B,C) demonstrated that BDNF secretion occurs immediately after depolarization (the time lag corresponds to the dead volume of less than 1 minute; see Materials and Methods). Depolarization-initiated intracellular calcium increase showed a time course similar to that obtained for BDNF secretion (Fig. 2B), as demonstrated by calcium imaging with Fura-2. Although prolonged (5 minutes) depolarization caused a sustained calcium increase throughout the stimulation period (Fig. 2C), the time course of BDNF secretion remained essentially the same as that following high potassium stimulation for 1 minute only.

If the high potassium-mediated secretion was initiated at shorter time intervals than 30 minutes, the quantity of BDNF secreted by subsequent stimulations gradually decreased, possibly resulting from a gradual depletion of the available stores (Fig. 3A). In contrast, after the normal recovery period of 30 minutes between two subsequent stimulations, comparable quantities of secreted BDNF were obtained (Fig. 3B).

We further compared the characteristics of activity-dependent neurotrophin secretion with those of conven-

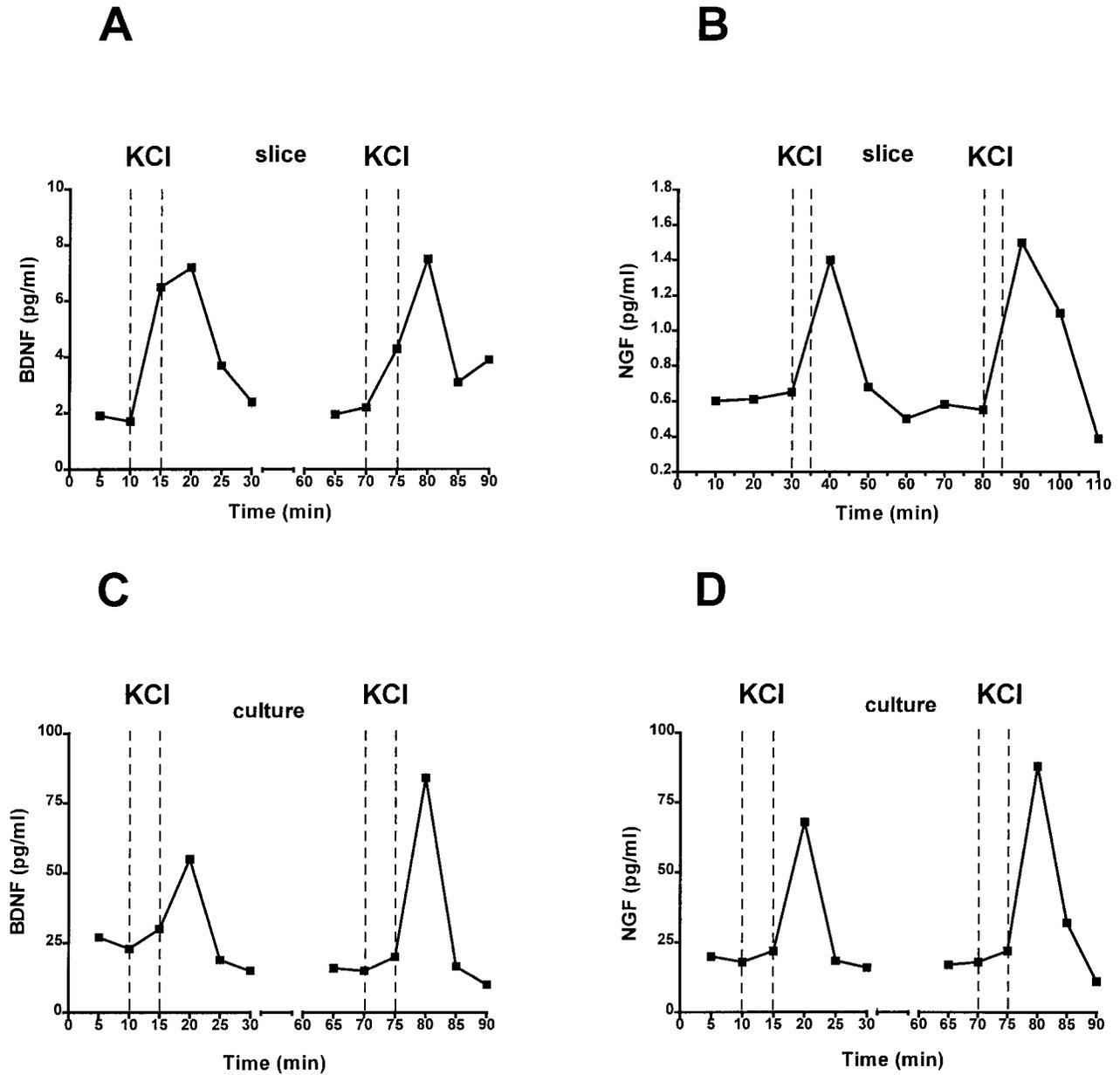


Fig. 1. Representative examples of depolarization-mediated NGF and BDNF secretion: comparison between native hippocampal slices and AdCMV-BDNFmyc or AdCMV-NGF infected cultures of hippocampal neurons. **A:** Time course of BDNF secretion induced by two consecutive stimulations with 50 mM KCl in native hippocampal slices perfused at a flow rate of 0.1 ml/min. Fractions were collected over periods of 5 minutes and BDNF concentrations of each fraction were quantified. **B:** Time course of NGF secretion induced by two consecutive stimulations with 50 mM KCl in native hippocampal slices perfused at a flow rate of 0.1 ml/min. Samples were collected over periods of 10 minutes and their NGF concentrations were determined. Data adapted from Blöchl and Thoenen (1995). **C:** Time course of BDNF secretion induced by two consecutive depolarizations with 50 mM KCl in cultured hippocampal neurons infected with

AdCMV-BDNFmyc and perfused at a flow rate of 0.1 ml/min. Fractions were collected over periods of 5 minutes and BDNF concentrations were quantified. **D:** Time course of NGF secretion induced by two consecutive depolarizations with 50 mM KCl in cultured hippocampal neurons infected with AdCMV-NGF and perfused at a flow rate of 0.1 ml/min. Fractions were collected over periods of 5 minutes and the NGF concentrations were quantified. The data show representative examples of BDNF and NGF secretion quantified by ELISA and expressed in pg/ml. The statistical analysis of BDNF secretion induced by high potassium and glutamate is shown in Figure 4 and expressed as the mean \pm SEM of at least four independent experiments. For details of slice preparations, culture and perfusion procedures, and ELISAs, see Materials and Methods.

tional neurotransmitters. After loading of BDNF-transduced hippocampal neurons with ^3H -glutamate, the time courses of both ^3H -glutamate and BDNF secretion were determined (Fig. 3C). The greater propor-

tion of ^3H -glutamate released was already present in the fraction collected during the time of stimulation, whereas BDNF secretion peaked immediately after depolarization. Similar results were obtained in native

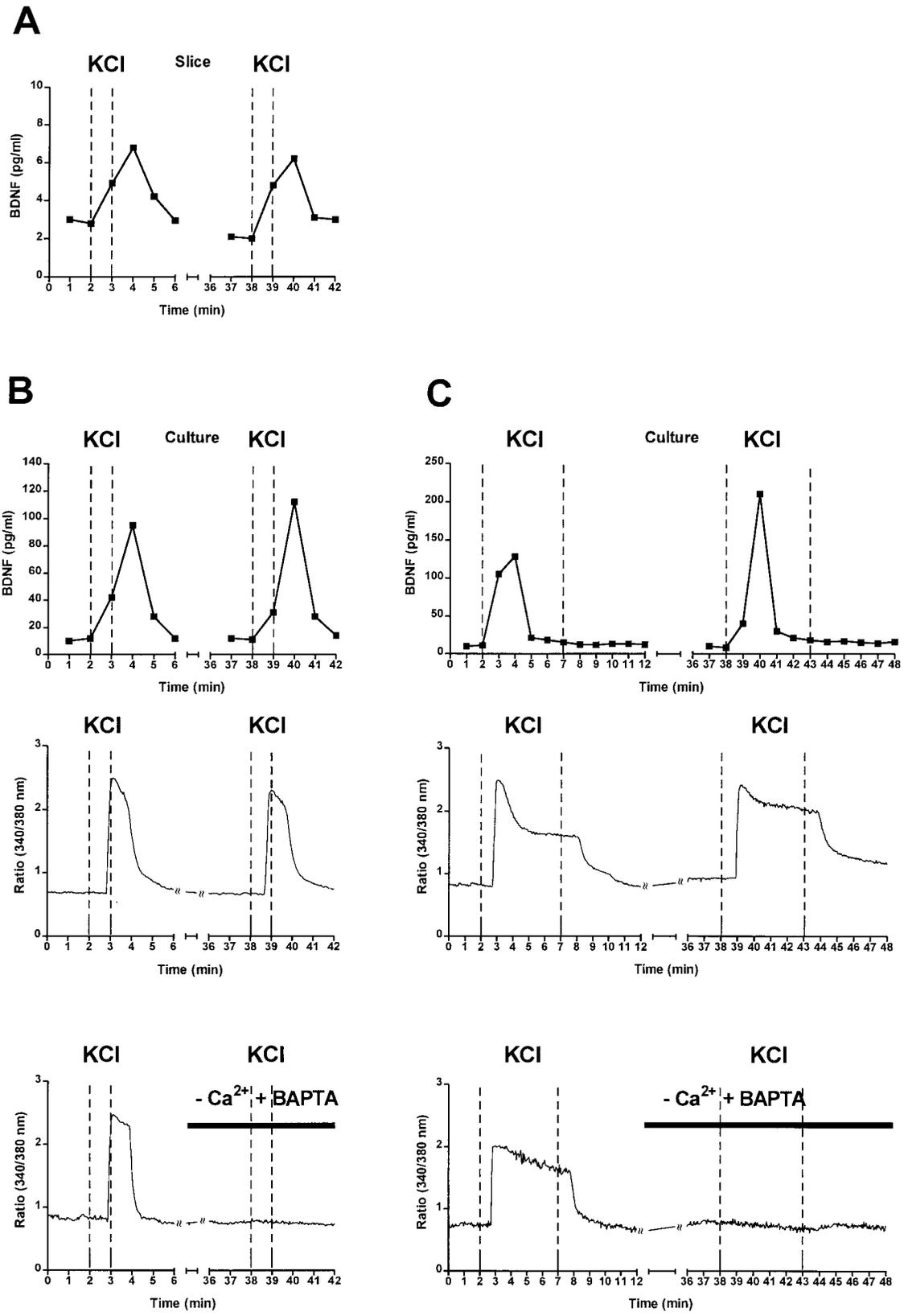


Fig. 2.

hippocampal slices loaded with ^3H -glutamate (data not shown). Thus, although BDNF secretion rapidly follows high potassium depolarization, it is nevertheless delayed compared to depolarization-mediated ^3H -glutamate release.

Role Played by Extracellular Calcium in the Mechanism of Activity-Dependent Secretion of Neurotrophins: Comparison Between "Perfusion" and "Static" Conditions

In previous experiments, Blöchl and Thoenen (1995) demonstrated that the activity-dependent secretion of NGF is independent of extracellular calcium, but depends on intact intracellular calcium stores. In contrast, Goodman et al. (1996) reported that BDNF secretion depends on extracellular calcium and suggested that one possible explanation for this discrepancy could be different mechanisms of NGF and BDNF secretion. We now demonstrate that the discordant results reflect differing experimental conditions rather than differences between the mechanisms of NGF and BDNF secretion. We first compared the role played by extracellular calcium in high potassium-initiated BDNF secretion in acute hippocampal slices and transduced primary cultures of hippocampal neurons in our perfusion system. After a first depolarizing stimulus in calcium-containing medium, neurons were perfused for 30 minutes with calcium-free buffer containing 10 μM BAPTA; this was followed by initiation of the second stimulus. In both native hippocampal slices and BDNF-transduced hippocampal cultures, the KCl- and glutamate-mediated secretion of BDNF was unaffected by the absence of extracellular calcium (Figs. 4A,B, 5A,B). As a control for the presence of glutamate in the perfusion system, glutamate was quantified in each of the collected fractions (Fig. 5D). Similar to the effect of high potassium on intracellular calcium levels (Fig.

2B,C), glutamate increased intracellular calcium in hippocampal neurons in calcium-containing medium. However, in contrast to high potassium-mediated depolarization there was a clearly detectable residual calcium signal, also in the absence of extracellular calcium, most likely reflecting mobilization of calcium from intracellular stores (Fig. 5C).

Having demonstrated that NGF and BDNF showed essentially the same characteristics of secretion under our own experimental conditions, we next compared the results evolving from these conditions with those of Goodman et al. (1996). The differences were as follows: 1) Goodman et al. (1996) performed their experiments under "static" conditions, while we used a perfusion system (see Discussion for reasons for using a perfusion system); 2) high potassium stimulation for 30 minutes, compared to 1 or 5 minutes in our experiments; 3) calcium concentrations 5 mM, compared to 1 mM in our experiments; and 4) use of the irreversible esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) in order to prevent proteolytic degradation of BDNF during the long stimulation period. The experimental conditions chosen by Goodman et al. (1996) were dictated by the necessity to obtain sufficiently high concentrations of secreted BDNF in order to permit its identification by Western blot analysis. Although we used gentler experimental conditions (omitting PMSF and 1 mM calcium, duration of stimulation 5 or 10 minutes), we nevertheless confirmed that under static conditions the high potassium-mediated secretion was dependent on extracellular calcium (Fig. 6B), in contrast to perfusion conditions (Fig. 4).

Calcium Mobilization From Intracellular Stores Is Necessary for Depolarization-Mediated Neurotrophin Secretion Under All Experimental Conditions

In view of the discordant results concerning the role of extracellular calcium under static as compared to perfusion conditions, we evaluated the importance of calcium mobilization from intracellular stores for activity-dependent neurotrophin secretion in the presence of extracellular calcium. Figure 6A demonstrates that the addition of thapsigargin and caffeine, which themselves initiate BDNF release (resulting from calcium mobilization), abolishes the subsequent BDNF release by high potassium in the presence of extracellular calcium. Similar results were obtained by stimulation with 50 μM glutamate (data not shown). Interestingly, the depletion of calcium stores by thapsigargin and caffeine also abolished high potassium-mediated BDNF secretion under static conditions in the presence of extracellular calcium (Fig. 6B), demonstrating that intact intracellular calcium stores are in any case of primary importance.

Localization of BDNF and NGF in Hippocampal Neurons Infected With AdCMV-BDNFmyc or AdCMV-NGF

Since under all experimental conditions no differences between NGF and BDNF secretion characteristics could be detected, it was interesting to compare the localization of NGF and BDNF in individual hippocampal neurons co-infected with adenoviral vectors coding for BDNFmyc and NGF. As demonstrated in Figure 7, no differences between the intracellular localizations of

Fig. 2. Comparison of different durations of high potassium depolarization on the time course of BDNF secretion and calcium transients. **A:** Representative example of the effect of two consecutive 50 mM KCl depolarizations on BDNF secretion from hippocampal slices. The duration of the depolarizing stimulus was 1 minute; a flow rate of 0.5 ml/min was chosen in order to obtain a higher resolution. Fractions were collected over periods of 1 minute and BDNF concentrations were quantified by ELISA. **B:** Representative example of the time course of BDNF secretion from cultures of hippocampal neurons infected with AdCMV-BDNFmyc. The perfusion rate was 0.5 ml/min and fractions were collected over periods of 1 minute. BDNF concentrations were quantified by ELISA (upper panel). Effect of 50 mM KCl depolarization for 1 minute on calcium transients in hippocampal neurons. Neurons were loaded with fura-2/AM and intracellular calcium concentrations were calculated from the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm, respectively (middle and lower panels). In the middle panel, the calcium transients were determined in the presence of calcium in the perfusion fluid; in the lower panel they were determined in the absence of calcium and presence of BAPTA. **C:** Representative example of the time course of BDNF secretion from AdCMV-BDNFmyc infected hippocampal neurons induced by 5 minutes depolarization with 50 mM KCl. The cultures were perfused at a flow rate of 0.5 ml/min and fractions were collected over periods of 1 minute each. BDNF concentrations were quantified by ELISA. In contrast to **A** and **B**, the duration of the KCl depolarization was 5 minutes. The perfusates were collected in fractions of 1 minute each and the BDNF concentrations were determined by ELISA (upper panel). In parallel experiments, the effects of 5 minutes 50 mM KCl depolarization on calcium transients were determined in the presence (middle panel) and absence (lower panel) of extracellular calcium, as in **B**. The data show representative examples of BDNF secretion obtained from at least four independent experiments. For experimental details, see Material and Methods.

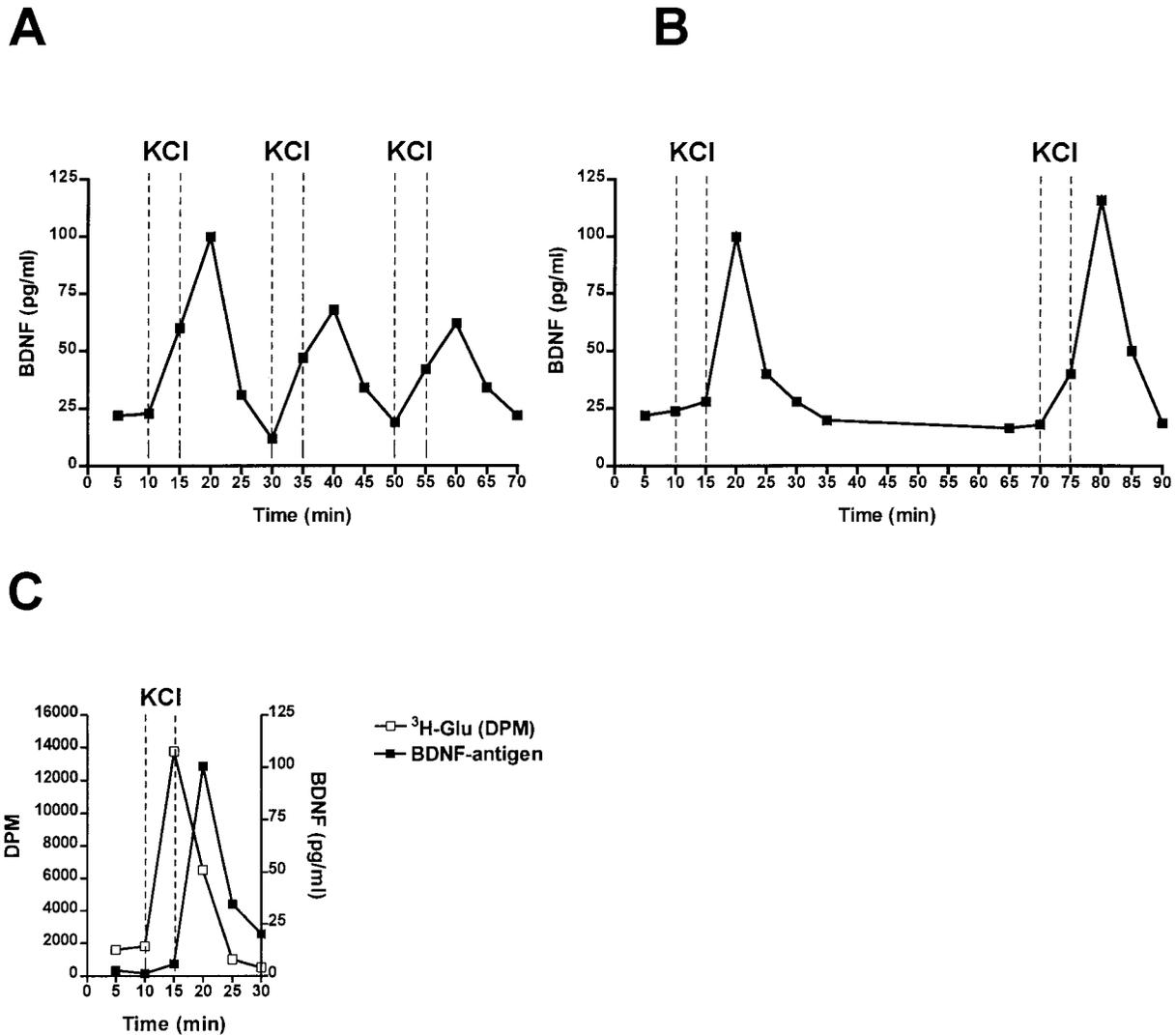


Fig. 3. Significance of different time intervals between consecutive 50 mM KCl depolarizations for the extent of BDNF secretion. Comparison between the time course of 50 mM KCl-mediated ³H-glutamate and BDNF secretion. **A:** Primary cultures of hippocampal neurons infected with AdCMV-BDNFmyc, perfused at a flow rate of 0.1 ml/min and fractions collected over periods of 5 minutes. In contrast to the usual time interval of 30 to 40 minutes between two consecutive 50 mM KCl stimulations, the cultures were stimulated at intervals of 15 minutes for 5 minutes by 50 mM KCl. **B:** In contrast to **A**, the time interval between the first and the second stimulation was extended to

60 minutes. Periods of 50 mM KCl depolarizations, collection procedures, and determinations of BDNF concentrations as in **A**. **C:** Comparison between the time courses of high potassium depolarization-mediated ³H-glutamate and BDNF secretion. Hippocampal neurons infected with AdCMV-BDNFmyc were loaded with ³H-glutamate and perfused at a flow rate of 0.1 ml/min. Depolarization with 50 mM KCl was initiated for 5 minutes and samples were assayed for BDNF and ³H-glutamate concentrations. The data show representative examples of BDNF secretion obtained from four independent experiments. For experimental details, see Material and Methods.

the two neurotrophins were detectable. Both NGF and BDNF were localized in relatively high concentrations in the perikaryon and in a punctuated dispersed pattern in all neuronal processes with virtually identical localization, as demonstrated by the overlay of two confocal scanning pictures at the same level representing BDNF and NGF immunoreactivity (Fig. 7).

DISCUSSION

Against our expectations, the initial analysis of activity-dependent secretion of NGF from native hippocampal slices and NGF-transfected hippocampal neurons showed unusual characteristics, indicating that the activity-dependent secretion of NGF (Blöchl and

Thoenen, 1995, 1996) is different from that of conventional neurotransmitters (Hanson et al., 1997; Neher, 1998; Sudhof, 1995). The secretion of NGF elicited by 50 mM potassium or 50 μM glutamate was independent of extracellular calcium but depended on the release of calcium from intact intracellular calcium stores. In a different set of experiments, Goodman et al. (1996) demonstrated that the high potassium-mediated release of BDNF from hippocampal neurons transduced with a BDNF-expressing herpes simplex virus depended on extracellular calcium. This led those authors to conclude that one possible explanation for this discrepancy could be differences between the mechanisms of NGF and BDNF secretion. Even more funda-

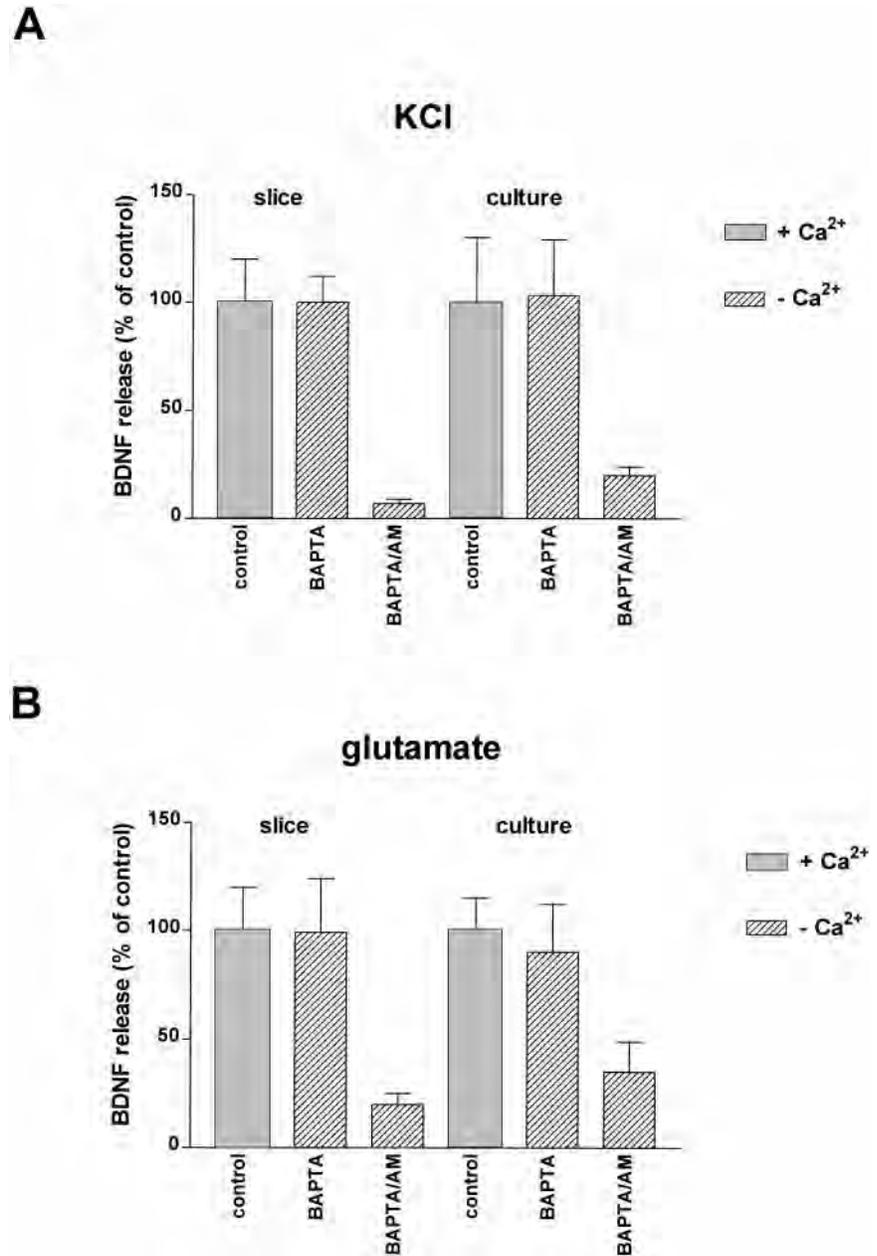


Fig. 4. Influence of extracellular and intracellular calcium on depolarization-mediated BDNF release from native hippocampal slices and AdCMV-BDNFmyc-infected hippocampal neurons. **A:** The effect of a first depolarization (50 mM KCl) on hippocampal slices or cultured hippocampal neurons infected with AdCMV-BDNFmyc is compared with that of a second depolarization in the absence of extracellular calcium and the addition of the extracellular calcium chelator BAPTA (10 μ M) or the intracellular chelator BAPTA/AM (10 μ M). **B:** Same experimental procedure as in **A** but, in place of 50 mM KCl, stimulations were performed by 50 μ M glutamate. The data show the mean \pm SEM of at least four independent experiments. In order to compare BDNF secretion characteristics obtained in slices and cultures more conveniently, the second stimulus was expressed as a percentage of the first reference stimulus. For experimental details, see Materials and Methods.

mental differences were reported by Mowla et al. (1997), suggesting that NGF and BDNF were sorted and secreted in a distinctly different manner in hippocampal neurons and in pituitary AtT-20 and GH3 cell lines. If, indeed, such differences exist with respect to the processing, sorting, and mechanism of secretion of different members of the neurotrophin family, this would have important implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. It would add an additional facet of complexity to the differing sites and extent of the expression of the different neurotrophins and their corresponding signal-transducing Trk receptors.

In view of these far-reaching consequences, a comparative analysis of the secretion of neurotrophins under comparable experimental conditions seemed mandatory. Here, we demonstrate that under the same

experimental conditions BDNF and NGF show the same subcellular distribution and the same characteristics of secretion initiated by high potassium and glutamate.

There Are No Differences Between the Mechanisms of Secretion of NGF and BDNF in Native Hippocampal Slices and Transduced Hippocampal Neurons

The analysis of the mechanism of activity-dependent secretion of neurotrophins is hampered by their low expression in vivo and in cultured neurons in vitro. Moreover, neuroendocrine cell lines, which are preferentially used for the analysis of sorting and secretion, do not express neurotrophins at all. Therefore, for many

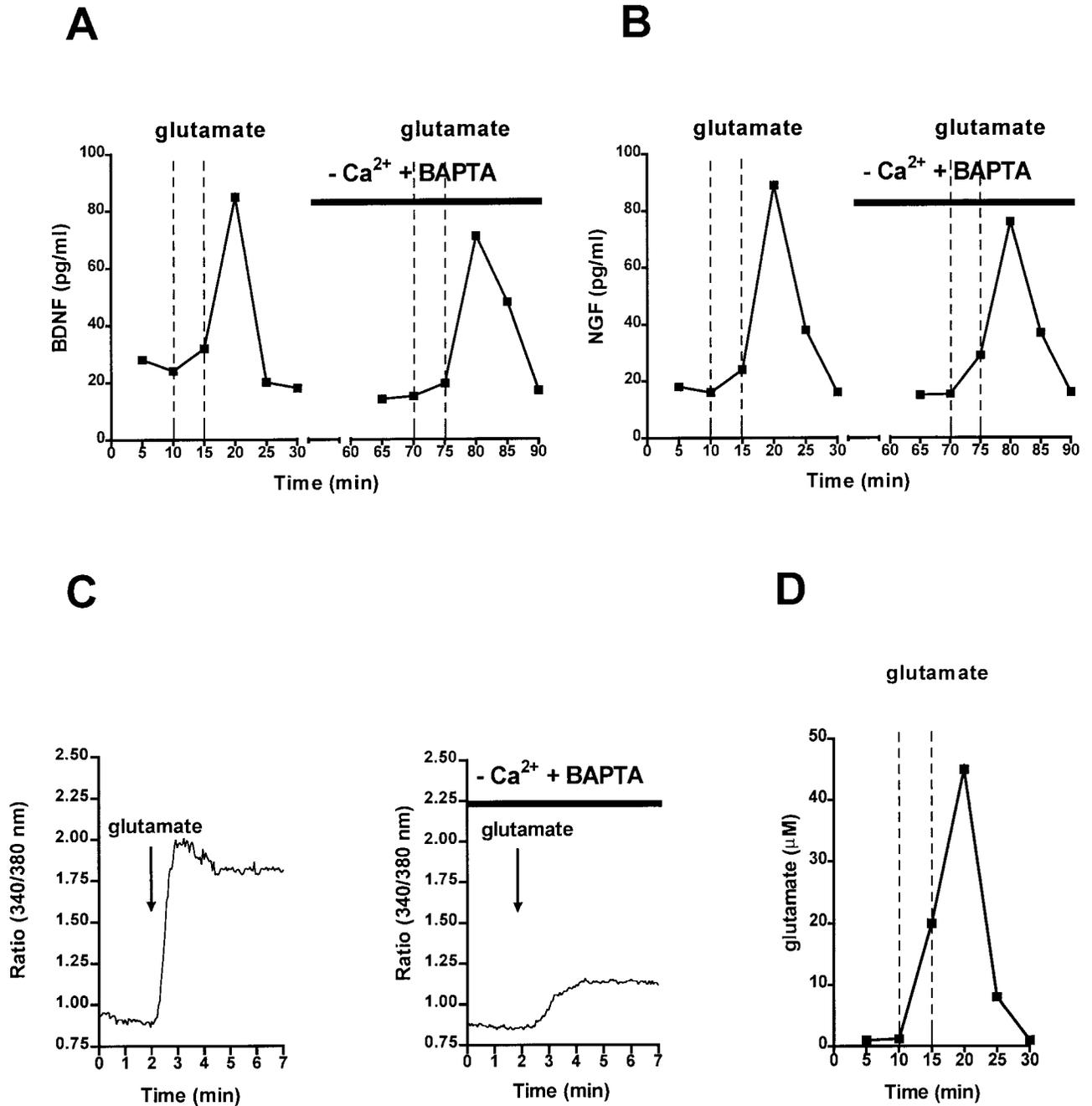
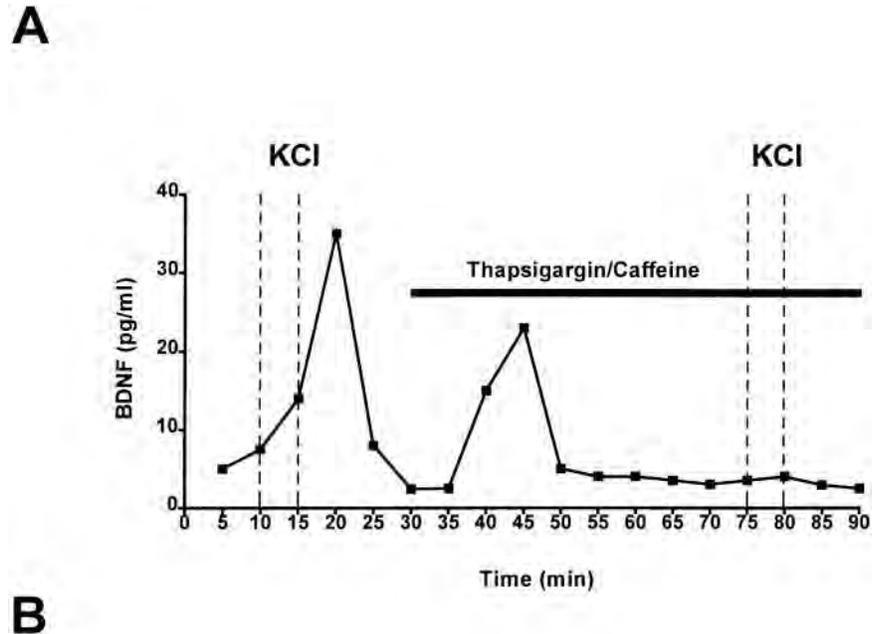


Fig. 5. Comparison between the presence and absence of extracellular calcium on glutamate-mediated BDNF and NGF secretion and calcium transients. **A:** Cultured hippocampal neurons were infected with AdCMV-BDNFmyc and perfused at a flow rate of 0.1 ml/min. After a first 5-minute stimulation with 50 µM glutamate, a second stimulation was initiated after a 30-minute perfusion in the absence of extracellular calcium and the addition of BAPTA (10 µM). Fractions were collected over 5-minute periods and BDNF concentrations were determined. The data show a representative example of BDNF secretion quantified by ELISA and expressed in pg/ml. The statistical analysis relative to glutamate-induced BDNF secretion is shown in Figure 4 and expressed as the mean ± SEM of at least four independent experiments. **B:** The same experimental procedures as in **A** but,

in place of AdCMV-BDNFmyc, AdCMV-NGF was used for infecting hippocampal neurons. **C:** Hippocampal neurons were loaded with fura-2/AM and the calcium transients were determined in the presence (left panel) or absence (right panel) of extracellular calcium. The calcium-free perfusion medium was supplied with the extracellular calcium chelator BAPTA. $[Ca^{2+}]_i$ was determined from the ratio of the fluorescence at excitation wavelengths of 340 and 380 nm, respectively. For experimental details, see Materials and Methods. **D:** Time course of glutamate concentration in the perfusate of hippocampal cultures, perfused at a flow rate of 0.1 ml/min. The perfusate was supplied by 50 µM glutamate for 5 minutes and the glutamate content was determined in 5-minute fractions. For experimental details, see Materials and Methods.

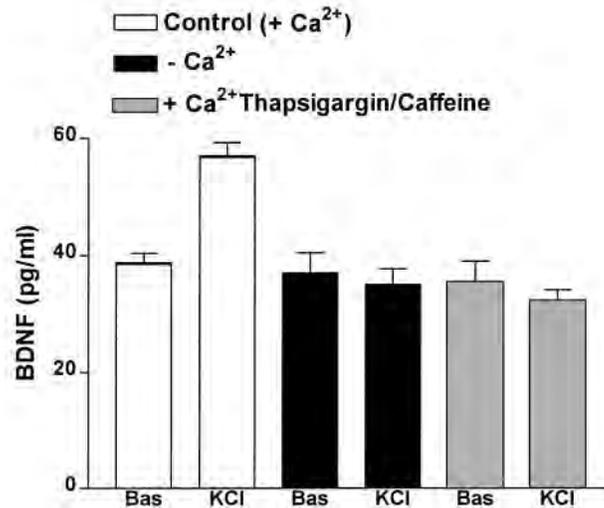
experimental approaches (Blöchl and Thoenen, 1996; Canossa et al., 1997; Goodman et al., 1996; Heymach and Shooter, 1995; Heymach et al., 1996) an overexpression of the neurotrophins to be investigated was un-

avoidable, bearing in mind the danger that the results obtained did not reflect the physiological situation. In the present experiments, aimed at the comparison of the mechanisms of secretion of NGF and BDNF, it was



B

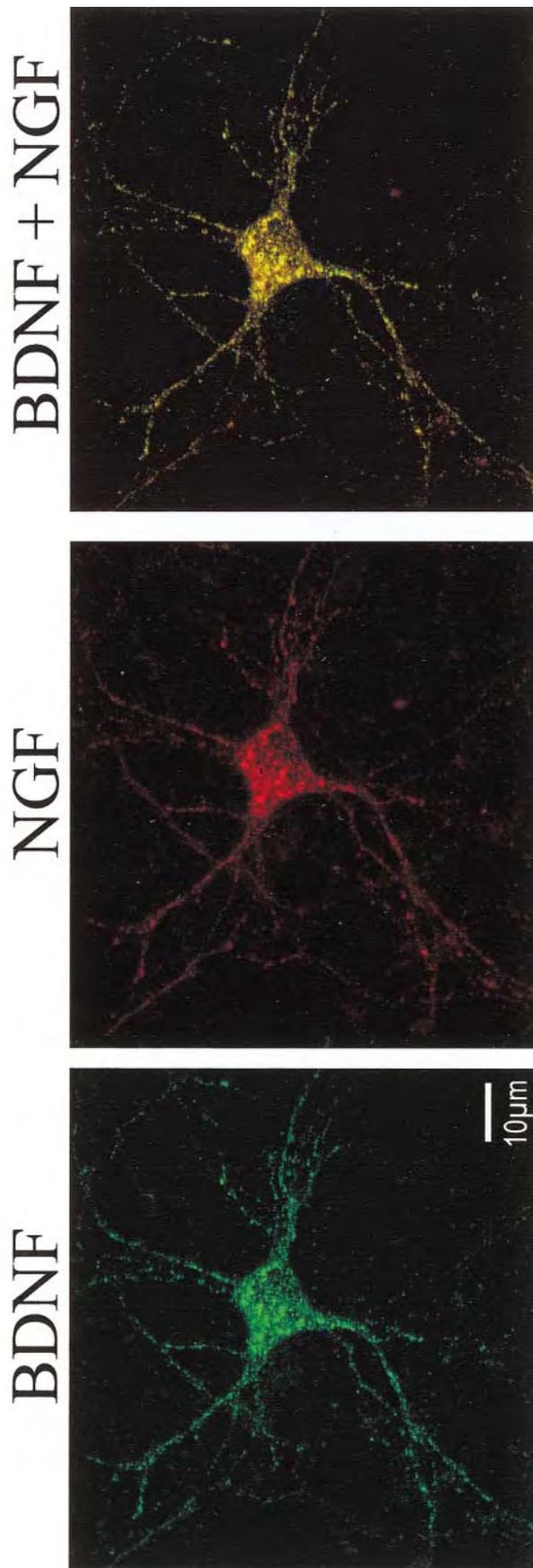
Fig. 6. Influence of the depletion of calcium stores on high potassium-mediated BDNF secretion in the presence of extracellular calcium under perfusion and static conditions. **A:** AdCMV-BDNFmyc-infected hippocampal cultures were perfused at a flow rate of 0.1 ml/min. A first 5-minute stimulation with 50 mM KCl was followed by the addition of thapsigargin (10 μ M) and caffeine (3 M each) to the perfusion fluid, with the initiation of a second 5-minute KCl stimulation after 35 minutes of thapsigargin/caffeine perfusion. Perfusion fluid was collected over periods of 5 minutes and BDNF concentrations were determined by ELISA. The data show a representative example of BDNF secretion obtained from at least four independent experiments. **B:** In contrast to **A**, the effect of thapsigargin/caffeine-mediated depletion of calcium stores was performed under "static" conditions. Hippocampal neurons, infected with AdCMV-BDNFmyc, were depolarized for 10 minutes with 50 mM KCl. The effects of KCl depolarizations on the release of BDNF are compared with respect to the absence of extracellular calcium and the presence of the extracellular calcium after depletion of intracellular stores by thapsigargin/caffeine. The data presented are the mean \pm SEM of at least four independent experiments.



comforting to see that the transduction procedures used did not change the characteristics of secretion compared to those of native hippocampal slices. This was especially true for the time course of secretion initiated by high potassium and glutamate, the independence of extracellular calcium, and the dependence on intact intracellular calcium stores. Moreover, using the same transduction procedure, BDNF and NGF showed a virtually identical intracellular localization in differentiated hippocampal neurons as demonstrated by confocal fluorescence microscopy. All these observations are in agreement with the notion that neurotrophins show a high degree of amino acid conservation in domains that are essential for processing and appropriate folding (Heymach et al., 1996).

Basis for the Controversial Results Reported by Goodman and Co-Workers in Comparison With Our Results

In order to obtain a reproducible time resolution of neurotrophin release, we developed a perfusion system that permitted the comparison of the release from native hippocampal slices and—since the levels of NGF were too low—that from NGF-transduced hippocampal cultures (Blöchl and Thoenen, 1995, 1996; Canossa et al., 1997). The use of a perfusion system was also recommended by the virtually exclusive use of perfusion systems for the quantitative analysis of neurotransmitter release from neurons and brain slices, which are particularly sensitive to suboptimal metabolic condi-



tions (Malcangio et al., 1997). The prerequisite for the analysis of NGF and BDNF secretion in a perfusion system was the availability of highly sensitive specific ELISAs for the two neurotrophins.

The BDNF release experiments by Goodman et al. (1996) were based on the identification of BDNF secretion by Western blot analysis and, accordingly, required prolonged and intense stimulation procedures that opened up the possibility of neuronal damage. This explanation for the discordant results proved to be wrong since we confirmed that, under static conditions, high potassium-mediated BDNF secretion is dependent on extracellular calcium even with stimulation times of only 5 minutes using 1 mM calcium and omitting PMSF (Fig. 6). Thus, the reported differences between the secretion characteristics of BDNF and NGF do not reflect differences between the subcellular distribution and mechanism of secretion of the two neurotrophins, but are dependent on the different experimental conditions under which these results were obtained, namely "static" versus "perfusion" conditions.

A further analysis of the mechanism of neurotrophin secretion, conducted under "perfusion" conditions, revealed that calcium release from intracellular stores is required even in the presence of extracellular calcium. After depletion of intracellular stores by pretreatment with caffeine and thapsigargin, the KCl-mediated secretion was also blocked in the presence of extracellular calcium (Fig. 6A). Similar results were obtained when the secretion was stimulated with glutamate (data not shown). Particularly interesting was the observation that intact calcium stores are also required for neurotrophin secretion under "static" conditions in the presence of extracellular calcium (Fig. 6B).

In view of the importance of intact calcium stores for neurotrophin secretion, we further analyzed the calcium transients resulting from glutamate and high potassium stimulation, both in the presence and absence of extracellular calcium. In confirmation of previous experiments (Canossa et al., 1997), glutamate (50 M) produced a marked increase in the calcium transients in hippocampal neurons, which were reduced but not abolished by the omission of extracellular calcium and addition of 10 M BAPTA (Fig. 5C). In contrast, calcium transients resulting from 50 mM potassium depolarization were completely abolished by the absence of extracellular calcium under both perfusion (Fig. 2B,C) and static (data not shown) conditions. Nevertheless, the high potassium-mediated neurotrophin secretion seems to depend on calcium release from intracellular stores, since it can be blocked by BAPTA/AM (Fig. 4A) and by the depletion of intracellular calcium stores by caffeine and thapsigargin (Fig. 6). Most likely the calcium imaging procedure used—despite its high sensitivity (but limited spatial resolution)—does not permit the detection of calcium at

Fig. 7. Comparison between the intracellular distribution of BDNF and NGF in hippocampal neurons using confocal microscopy. Hippocampal neurons kept in culture for 9 days were co-infected with AdCMV-BDNFmyc and AdCMV-NGF and simultaneously immunostained with an antibody (9E10) recognizing the myc epitope and antibodies against NGF as described in Materials and Methods. The upper left panel (in green) depicts FITC fluorescence of BDNFmyc immunoreactivity; the upper right panel (in red) depicts the NGF immunoreactivity (Lissamine Rhodamine fluorescence). The lower panel shows the overlay of the NGF and BDNFmyc at the same confocal level. For experimental details, see Material and Methods.

specific strategic sites necessary to initiate the regulated secretion of neurotrophins.

Since at the light microscopic level the storage compartments for neurotrophins in all neuronal processes correspond to the endoplasmic reticulum (Blöchl and Thoenen, 1996; Gärtner et al., 1998), it seems conceivable that the calcium and neurotrophin stores are closely associated or even identical.

Although the voltage-gated calcium influx is well established and generally accepted as the crucial triggering mechanism for the fusion of synaptic vesicles with the plasma membrane (Berridge, 1998; Neher, 1998; Sudhof, 1995), the role of calcium released from intracellular stores in the regulation of exocytosis is receiving increasing attention. In gonadotrophs of the pituitary, exocytosis is triggered by calcium from internal stores (Tse et al., 1993, 1997). It is thought that calcium is released locally from intracellular stores in close association with exocytotic sites. Moreover, in melanotrophs (Lee, 1996), bag cell neurons of *Aplysia* (Jonas et al., 1997), and pancreatic acinar cells (Kasai and Augustine, 1990), calcium release from intracellular stores seems to be responsible for exocytosis. In view of all these observations, a precise ultrastructural localization of storage sites in neurons at different stages of differentiation is mandatory. In PC12 cells transduced with BDNF_{myc}, immunoreactivity has been localized in large dense core vesicles (Möller et al., 1997). This is of particular interest, given that neurotrophin secretion from PC12 cells also occurs in the absence of extracellular calcium (Canossa et al., 1997), suggesting an additional storage compartment other than the large dense core vesicles. In hippocampal neurons, the storage compartment from which neurotrophins are secreted must be different, since there are virtually no large dense core vesicles detectable. However, in axon terminals of dorsal root ganglion neurons projecting to the spinal cord, endogenous BDNF has been localized in large dense core vesicles (Michael et al., 1997). Therefore, it could well be that activity-dependent secretion of the BDNF moiety localized in the large dense core vesicles of sensory neurons and in large dense core vesicles of PC12 cells depends on extracellular calcium.

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REFERENCES

- Akaneya Y, Tsumoto T, Kinoshita S, Hatanaka H. 1997. Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. *J Neurosci* 17:6707–6716.
- Barbacid M. 1994. The Trk family of neurotrophin receptors. *J Neurobiol* 25:1386–1403.
- Berardi N, Cellerino A, Domenici L, Fagiolini M, Pizzorusso T, Cattaneo A, Maffei L. 1994. Monoclonal antibodies to nerve growth factor affect the postnatal development of the visual system. *Proc Natl Acad Sci USA* 91:684–688.
- Berridge MJ. 1998. Neuronal calcium signaling. *Neuron* 21:13–26.
- Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31–39.
- Blöchl A, Thoenen H. 1995. Characterization of nerve growth factor (NGF) release from hippocampal neurons: evidence for a constitutive and an unconventional sodium-dependent regulated pathway. *Eur J Neurosci* 7:1220–1228.
- Blöchl A, Thoenen H. 1996. Localization of cellular storage compartments and sites of constitutive and activity-dependent release of nerve growth factor (NGF) in primary cultures of hippocampal neurons. *Mol Cell Neurosci* 7:173–190.
- Bonhoeffer T. 1996. Neurotrophins and activity-dependent development. *Curr Opin Neurobiol* 6:119–126.
- Bothwell M. 1995. Functional interactions of neurotrophins and neurotrophin receptors. *Annu Rev Neurosci* 18:18223–18253.
- Brewer GJ, Cotman CW. 1989. Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. *Brain Res* 494:65–74.
- Cabelli RJ, Shelton DL, Segal RA, Shatz CJ. 1997. Blockade of endogenous ligands of trkB inhibits formation of ocular dominance columns. *Neuron* 19:63–76.
- Canossa M, Griesbeck O, Berninger B, Campana G, Kolbeck R, Thoenen H. 1997. Neurotrophin release by neurotrophins: implications for activity-dependent neuronal plasticity. *Proc Natl Acad Sci USA* 94:13279–13286.
- Cellerino A, Maffei L. 1996. The action of neurotrophins in the development and plasticity of the visual cortex. *Prog Neurobiol* 49:53–71.
- Collingridge GL, Bliss TV. 1995. Memories of NMDA receptors and LTP. *Trends Neurosci* 18:54–56.
- Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B. 1996. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. *Nature* 381:706–709.
- Gärtner A, Shostak Y, Thoenen H. 1998. Ultrastructural analysis of activity-dependent neurotrophin release in hippocampal primary cultures transduced by adenovirus. In: *Proceedings of 28th Annual Meeting of the Society for Neuroscience*, Los Angeles, 875.10.
- Goodman LJ, Valverde J, Lim F, Geschwind M, D, Federoff HJ, Geller AI, Hefti F. 1996. Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. *Mol Cell Neurosci* 7:222–238.
- Gottschalk W, Pozzomiller LD, Figurov A, Lu B. 1998. Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. *J Neurosci* 18:6830–6839.
- Graham FL, Prevec L. 1991. Manipulation of adenoviral vectors. *Methods Mol Biol* 7:109–128.
- Griesbeck O, Korte M, Gravel C, Bonhoeffer T, Thoenen H. 1997. Rapid gene transfer into cultured hippocampal neurons and acute hippocampal slices using adenoviral vectors. *Mol Brain Res* 44:171–177.
- Hanson PI, Heuser JE, Jahn R. 1997. Neurotransmitter release—four years of SNARE complexes. *Curr Opin Neurobiol* 7:310–315.
- Heymach JV Jr, Shooter EM. 1995. The biosynthesis of neurotrophin heterodimers by transfected mammalian cells. *J Biol Chem* 270:12297–12304.
- Heymach JV Jr, Krüttgen A, Suter U, Shooter EM. 1996. The regulated secretion and vectorial targeting of neurotrophins in neuroendocrine and epithelial cells. *J Biol Chem* 271:25430–25437.
- Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde Y-A. 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J* 9:2459–2464.
- Jonas EA, Knox RJ, Smith TC, Wayne NL, Connor JA, Kaczmarek LK. 1997. Regulation by insulin of a unique neuronal Ca²⁺ pool and of neuropeptide secretion. *Nature* 385:343–346.
- Kang H, Schuman EM. 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* 267:1658–1662.
- Kang H, Welcher AA, Shelton D, Schuman EM. 1997. Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. *Neuron* 19:653–664.
- Kasai H, Augustine GJ. 1990. Cytosolic Ca²⁺ gradients triggering unidirectional fluid secretion from exocrine pancreas. *Nature* 348:735–738.
- Katz LC, Shatz CJ. 1996. Synaptic activity and the construction of cortical circuits. *Science* 274:1133–1138.
- Knipper M, da Penha Berzaghi M, Blöchl A, Breer H, Thoenen H, Lindholm D. 1994a. Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived neurotrophic factor in the rat hippocampus. *Eur J Neurosci* 6:668–671.
- Knipper M, Leung LS, Zhao D, Rylett RJ. 1994b. Short-term modulation of glutamatergic synapses in adult rat hippocampus by NGF. *NeuroReport* 5:2433–2436.
- Korsching S, Thoenen H. 1987. Two-site enzyme immunoassay for nerve growth factor. In: Barnes D, editor. *Methods in enzymology: peptide growth factors*. Part B. Orlando, FL: Academic Press. p 167–185.

- Korsching S, Auburger G, Heumann R, Scott J, Thoenen H. 1985. Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J* 4:1389-1393.
- Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T. 1995. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc Natl Acad Sci USA* 92:8856-8860.
- Korte M, Griesbeck O, Gravel C, Carroll P, Staiger V, Thoenen H, Bonhoeffer T. 1996a. Virus-mediated gene transfer into CA1 region restores LTP in BDNF mutant mice. *Proc Natl Acad Sci USA* 93:12547-12552.
- Korte M, Staiger V, Griesbeck O, Thoenen H, Bonhoeffer T. 1996b. The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. *J Physiol (Paris)* 90:157-164.
- Lee AK. 1996. Dopamine (D2) receptor regulation of intracellular calcium and membrane capacitance changes in rat melanotrophs. *J Physiol* 495:627-640.
- Lessmann V, Heumann R. 1998. Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures—presynaptic enhancement depends on pre-established paired-pulse facilitation. *Neuroscience* 86:399-413.
- Lessmann V, Gottmann K, Heumann R. 1994. BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultures of hippocampal neurons. *NeuroReport* 6:21-25.
- Levine ES, Dreyfus CF, Black IB, Plummer MR. 1995. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. *Proc Natl Acad Sci USA* 92:8074-8077.
- Levine ES, Black IB, Plummer MR. 1998. Neurotrophin modulation of hippocampal synaptic transmission. *Adv Pharmacol* 42:921-924.
- Lewin GR, Barde YA. 1996. Physiology of the neurotrophins. *Annu Rev Neurosci* 19:289-317.
- Li YX, Xu YF, Ju DS, Lester HA, Davidson N, Schuman EM. 1998. Expression of a dominant negative TrkB receptor T1, reveals a requirement for presynaptic signaling in BDNF-induced synaptic potentiation in hippocampal neurons. *Proc Natl Acad Sci USA* 95:10884-10889.
- Lindholm D, Castrèn E, Berzaghi M, Blöchl A, Thoenen H. 1994. Activity-dependent and hormonal regulation of neurotrophin mRNA levels in the brain—implications on neuronal plasticity. *J Neurobiol* 25:1362-1373.
- Lohof AM, Ip NY, Poo M-M. 1993. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature* 363:350-353.
- Malcangio M, Garrett NE, Cruwys S, Tomlinson DR. 1997. Nerve growth factor- and neurotrophin-3-induced changes in nociceptive threshold and the release of substance P from the rat isolated spinal cord. *J Neurosci* 17:8459-8467.
- McAllister AK, Lo DC, Katz LC. 1995. Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15:791-803.
- McAllister AK, Katz LC, Lo DC. 1996. Neurotrophin regulation of cortical dendritic growth requires activity. *Neuron* 17:1057-1064.
- McGrory WJ, Bautista DS, Graham FL. 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 163:614-617.
- Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DL, Yan Q, Priestley JV. 1997. Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *J Neurosci* 17:8476-8490.
- Möller JC, Kruttgen A, Heymach JV Jr, Ghori N, Shooter EM. 1998. Subcellular localization of epitope-tagged neurotrophins in neuroendocrine cells. *J Neurosci Res* 51:463-472.
- Mowla SJ, Fawcett JP, Pareek S, Seidah NG, Sossin WS, Murphy RA. 1997. Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. *Proceedings of 27th Annual Meeting of the Society for Neuroscience, New Orleans, 875.10.*
- Nawa H, Carnahan J, Gall C. 1995. BDNF protein measured with a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels. *Eur J Neurosci* 7:1527-1535.
- Neher E. 1998. Vesicle pools and Ca²⁺ microdomains: new tools for understanding their roles in neurotransmitter release. *Neuron* 20:389-399.
- Patterson SL, Abel T, Deuel TAS, Martin KC, Rose JC, Kandel ER. 1996. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 16:1137-1145.
- Sala R, Viegi A, Rossi FM, Pizzorusso T, Bonanno G, Raiteri M, Maffei L. 1998. Nerve growth factor and brain-derived neurotrophic factor increase neurotransmitter release in the rat visual cortex. *Eur J Neurosci* 10:2185-2191.
- Schuman EM. 1997. Synapse specificity and long-term information storage. *Neuron* 18:339-342.
- Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A. 1998. Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron* 20:727-740.
- Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Näher-Noé M, Thoenen H. 1990. Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system: comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. *Eur J Neurosci* 2:69-76.
- Sudhof TC. 1995. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* 375:645-653.
- Suen PC, Wu K, Levine ES, Mount HT, Xu JL, Lin SY, Black IB. 1997. Brain-derived neurotrophic factor rapidly enhances phosphorylation of the postsynaptic N-methyl-D-aspartate receptor subunit 1. *Proc Natl Acad Sci USA* 94:8191-8195.
- Tanaka T, Saito H, Matsuki N. 1997. Inhibition of GABA_A synaptic responses by brain-derived neurotrophic factor (BDNF) in rat hippocampus. *J Neurosci* 17:2959-2966.
- Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. 1998. Ca²⁺ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* 20:709-726.
- Thoenen H. 1995. Neurotrophins and neuronal plasticity. *Science* 270:593-598.
- Tse A, Tse FW, Almers W, Hille B. 1993. Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotrophs. *Science* 260:82-84.
- Tse FW, Tse A, Hille B, Horstmann H, Almers W. 1997. Local Ca²⁺ release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron* 18:121-132.
- Tsien RY. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19:2396-2404.
- Tsien RY. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* 290:527-528.
- Wang XH, Poo MM. 1997. Potentiation of developing synapses by postsynaptic release of neurotrophin-4. *Neuron* 19:825-835.
- Wang XH, Berninger B, Poo MM. 1998. Localized synaptic actions of neurotrophin-4. *J Neurosci* 18:4985-4992.
- Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D. 1990. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *EMBO J* 9:3545-3550.