The Journal of Neuroscience, August 1993, 13(8): 3394–3405

Cultured Hippocampal Neurons Show Responses to BDNF, NT-3, and NT-4, but Not NGF

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To investigate the possibility of neurotrophins acting directly on hippocampal neurons, we first examined expression of the *trk* receptors in sections of adult rat brain and in cultures of embryonic rat hippocampus, and then investigated general and specific responses of cultured hippocampal neurons to each of the neurotrophins. *In situ* hybridization studies indicated high levels of expression of *trk*B and *trk*C but not *trk*A in pyramidal cells, dentate granule neurons, and scattered interneurons. Cultures of embryonic day 18 (E18) hippocampal neurons were also found by Northern analysis to express *trk*B and *trk*C but not *trk*A, indicating potential responsiveness to brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4, but not NGF. Phosphorylation experiments indeed showed that BDNF, NT-3, and NT-4 produced rapid tyrosine phosphorylation of Trk proteins, as detected by immunoprecipitation using a pan-Trk-specific antibody, whereas NGF produced no detectable tyrosine phosphorylation in hippocampal cultures. Similarly, all of the neurotrophins, except NGF, induced expression of c-*fos* mRNA and c-*fos* protein in these cultures. c-*Fos* protein induction was detectable in approximately 40–50% of the cells. While we observed no major effect of any of the neurotrophins upon the survival of E18 hippocampal neurons, BDNF, NT-3, and NT-4, but not NGF, produced marked increases in the number of neurons expressing detectable levels of either calbindin or AChE. NT-3 produced the greatest increase in the number of calbindin-positive neurons, whereas BDNF and NT-4 produced the greater increase in the number of AChE-positive neurons. Our results suggest that several of the neurotrophins have important effects in the differentiation and maintenance of function of subpopulations of hippocampal neurons.

[Key words: NGF, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4, hippocampus, Trk receptors, neurotrophins, calbindin]

Tissue culture has been widely used to define the responses and requirements of developing nerve cells for factors that influence their survival, differentiation, and phenotypic expression. Such studies have been most successful with neurons of the PNS—sensory, sympathetic, and parasympathetic—where cells are present in discrete ganglia and where methods to purify neurons from non-neuronal cells have been well established (Davies, 1989). The use of cultured dorsal root ganglion neurons, for example, has been instrumental in the identification of NGF and brain-derived neurotrophic factor (BDNF), and was essential as a bioassay for their subsequent purification (Barde et al., 1982; Levi-Montalcini, 1987; Leibrock et al., 1989). *In vitro* studies with CNS neurons have generally been more problematic than those with peripheral neurons, especially when trying to define the effects of potential neurotrophic factors on specific populations of CNS neurons. For the most part, CNS neurons can be obtained only as heterogeneous cultures of neurons and non-neuronal cells, where identification of specific neuronal populations requires immunocytochemical or histochemical markers. The relatively large size and ease of isolation of the hippocampal formation have made it one of the most studied CNS regions *in vitro*. Elegant studies by Banker and Cowan (1977, 1979) established excellent baseline conditions for maintaining embryonic hippocampal neurons at low density *in vitro*. Subsequent studies have focused mainly on the morphological and electrophysiological aspects of developing hippocampal neurons, and characterization of their neurochemical phenotype (Bartlett and Banker, 1984a,b). More recently, hippocampal cultures have been used by several groups to explore the neurotoxicity of β-amyloid and excitatory amino acids such as glutamate, and, more importantly, to search for neuroprotective agents (Mattson, 1989; Mattson and Kater, 1989; Yankner et al., 1990a,b; Mattson and Rychlik, 1990; Skaper et al., 1991; Cheng and Mattson, 1992).

The early finding that the survival and differentiation of cultured hippocampal neurons were greatly enhanced in the presence of a soluble factor from cocultured astrocytes raised the possibility that glial cells might provide trophic support for developing hippocampal neurons (Banker, 1980). In support of this, it has recently been shown that cultures of hippocampal astrocytes express transcripts for several well-characterized neurotrophic factors including ciliary neurotrophic factor (CNTF), NGF, and neurotrophin-3 (NT-3; Rudge et al., 1992). To date, however, there have been very few studies defining effects of well-characterized neurotrophic factors on hippocampal neurons; fibroblast growth factor, γ-interferon, and CNTF have thus far been the only purified trophic factors reported to have effects on the survival or differentiation of these neurons (Walicke et al., 1986; Barish et al., 1991; Ip et al., 1991).

The recent molecular cloning of BDNF (Leibrock et al., 1989) uncovered the close homology of this protein to NGF and led to the discovery of an even larger family of NGF-related neurotrophic factors, termed the neurotrophins. In addition to NGF

Received Oct. 7, 1992; revised Dec. 21, 1992; accepted Feb. 10, 1993.

We thank Dr. L. S. Schleifer and other colleagues at Regeneron for their enthusiastic support. We particularly thank Paul Wright and Li Pan for their expert assistance with the *in situ* hybridization studies. We also thank Dr. Ralph Alderson for providing mouse NGF, Dr. James Miller (Amgen Inc., Thousand Oaks, CA) for recombinant BDNF and NT-3, and Dr. James Fandl for recombinant NT-4.

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and BDNF, this family now includes NT-3 (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990a; Rosenthal et al., 1990); and a factor alternatively referred to as NT-4 or NT-5 (from hereon referred to as NT-4; Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992). There is now convincing evidence that each of these neurotrophins may have specific actions on distinct subpopulations of developing and/or mature sensory neurons (Maisonpierre et al., 1990a; Hallböök et al., 1991; DiStefano et al., 1992; Lindsay, 1993; Ruit et al., 1992; Hory-Lee et al., 1993). In the CNS, individual neurotrophins exhibit distinct patterns of expression, but interestingly, the highest levels of expression of transcripts for NGF, BDNF, and NT-3 are all in the hippocampus (Ernfors et al., 1990; Maisonpierre et al., 1990b; Phillips et al., 1990). The finding of high levels of NGF within the hippocampus strongly supports the role of this neurotrophin as a target-derived neurotrophic factor for innervating cholinergic neurons of the basal forebrain. This role of NGF has long been predicted from both *in vitro* and *in vivo* studies (Snider and Johnson, 1989). The finding of high levels of BDNF within the hippocampus would similarly support recent findings that this neurotrophin may also be a target-derived neurotrophic factor for septohippocampal cholinergic neurons (Alderson et al., 1990; Knüsel et al., 1991; Morse et al., in press). Recent retrograde transport studies with radiolabeled neurotrophins and neurotrophin receptor binding studies strongly suggest that other hippocampal afferents are likely to be responsive to BDNF and NT-3 (Wiegand et al., 1991; DiStefano et al., 1992; Altar et al., 1993). In addition to roles as target-derived factors for neurons projecting to the hippocampus, the above-mentioned studies also predict that BDNF and NT-3 are likely to have local actions within the hippocampus.

The recent identification of the *trk* gene family of tyrosine kinase receptors as neurotrophin receptors allows for a better understanding of how the neurotrophins act on responsive cells (Klein et al., 1989; Martin-Zanca et al., 1989; Lamballe et al., 1991; Middlemas et al., 1991). Studies that have explored the specificity with which the neurotrophins interact with their receptors reveal that NGF is the ligand specific for the TrkA receptor, BDNF and NT-4 are the preferred ligands for the TrkB receptor, while NT-3 is the preferred ligand for the TrkC receptor, although NT-3 can also activate TrkB, albeit less efficiently (Cordon-Cardo et al., 1991; Glass et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991, 1992; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1992, 1993). Although it has been reported that both TrkB and TrkC are present at high levels in the hippocampus of the mouse (Klein et al., 1989, 1990; Lamballe et al., 1991), similar studies have not been performed in the rat.

In this study, we have examined the expression of TrkA, TrkB, and TrkC in the rat hippocampus, and have characterized the effects of neurotrophins on cultured hippocampal neurons. A number of approaches were used, including induction of Trk phosphorylation and c-*fos* by the neurotrophins, to obtain initially a broad view of the specificity of the neurotrophins, followed by a more detailed characterization of responsive neuronal populations within the cultures. We conclude from our results that under the experimental paradigms used, neurotrophins did not appear to promote survival of embryonic day 18 (E18) hippocampal neurons, but rather acted to regulate the function of specific subpopulations, especially the expression of certain phenotypic markers. The responsiveness of cultured hippocampal neurons to the neurotrophins BDNF, NT-3, and NT-4, but not to NGF, appears to correlate with the expression and activation of specific Trk receptors in these cultures.

Materials and Methods

Hippocampal cell cultures. Hippocampal cultures from embryonic day 18 rats were prepared as previously described (Ip et al., 1991), with modifications. Briefly, dissociated hippocampal neurons were plated onto polyornithine-laminin (10 μg/ml)–coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After 4 hr of culture, the medium was changed to DMEM plus 1 mg/ml BSA, 1 mM pyruvate, and N2 media supplement (Bottenstein and Sato, 1979). Under such culture conditions, we have established that the percentage of astroglial cells present in the cultures following 8 d in culture is approximately 3%. In cultures maintained for more than 2 d, medium was changed every 3 d, as was growth factor when present.

RNA analysis of embryonic hippocampal cultures. RNA was prepared from cultured hippocampal neurons following various treatments with neurotrophins, by guanidinium thiocyanate extraction as previously described (Chomczynski and Sacchi, 1987). RNA analysis, performed as previously described (Maisonpierre et al., 1990b), utilized randomly labeled DNA fragments derived from either kinase-encoding domains of *trk*A, *trk*B, and *trk*C (Valenzuela et al., in press), c-*fos* [1 kilobase (kb) Pst I fragment], or calbindin [780 base pair (bp), prepared by polymerase chain reaction from published sequence using rat brain cDNA (Nordquist et al., 1988)].

In situ *hybridization.* DNA fragments (1.5 kb, 0.3 kb, and 0.8 kb for *trk*A, *trk*B, and *trk*C, respectively) encoding the kinase domain region were subcloned into Bluescript (KS$^+$) for generating RNA probes. Radiolabeled antisense- or sense-strand probes were transcribed off of linearized plasmids using a transcription kit from Promega. Coronal sections (10 μm thick) from adult rat brain were thawed and mounted onto polylysine-coated slides, and *in situ* hybridization was performed as described previously (Friedman et al., 1992). In all cases, sense control probes resulted in no specific hybridizing signals.

Tyrosine phosphorylation assays. Immunoprecipitations, immunoblotting, and tyrosine phosphorylation assays were performed as previously described (Nye et al., 1992). Briefly, cells were starved for 60 min in defined medium prior to a 5 min treatment with various neurotrophins (50 ng/ml) and subsequent lysis in RIPA buffer. The lysates were immunoprecipitated with purified pan-Trk-specific polyclonal antisera RG22 (derived in rabbits by immunizing with a peptide derived from the C-terminal portion of TrkA that is highly conserved in all the known Trks) in combination with an anti-rabbit secondary antibody conjugated to agarose (Sigma Chemical).

Immunohistochemical staining. Immunohistochemical staining for calbindin, GABA, or neuron-specific enolase (NSE) was performed as previously described (Ip et al., 1991). Similarly, histochemical staining for acetylcholinesterase was carried out as described previously (Ip et al., 1990). For c-*fos* staining, cells were incubated with normal goat serum with 1% bovine serum albumin/0.4% Triton, prior to incubation with primary antibody against c-*fos* (1:2000; Oncogene Science, Long Island, NY) for 2 d. Cells were then incubated with biotinylated secondary antibody (1:1500), and c-*fos*–immunoreactive cells were visualized using the Vectastain ABC kit with nickel(II) sulfate intensification. Omission of primary antibodies did not result in any detectable signals. ELISA assay for neurofilament protein was performed as previously described (Ip et al., 1991).

GABA uptake and glutamate uptake. Assays for high-affinity GABA or glutamate uptake were performed as previously described (Ip et al., 1991).

Neurotrophins. NGF was mouse NGF purified from adult male mouse salivary gland (provided by Dr. Ralph Alderson, Regeneron Pharmaceuticals). BDNF and NT-3 were recombinant human BDNF and NT-3, produced and generously provided by Dr. James Miller (Amgen, Inc.; DiStefano et al., 1992), whereas NT-4 was recombinant human NT-4 (Ip et al., 1993).

Results

Expression of mRNA for Trk receptors in adult rat hippocampus and embryonic rat hippocampal cultures

Expression of transcripts for each of the Trk receptors (TrkA, TrkB, and TrkC) was examined in coronal sections of adult rat

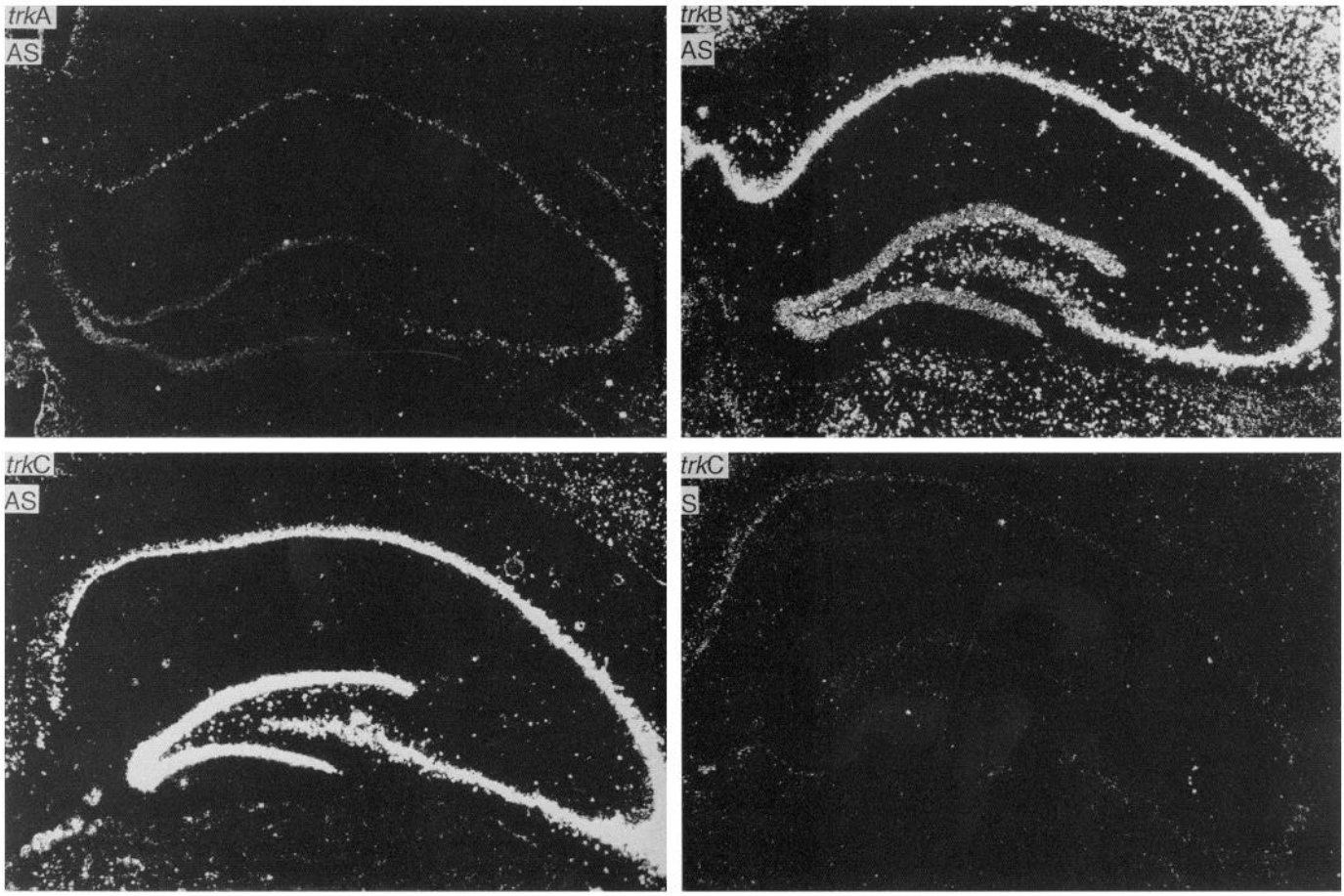

Figure 1. *In situ* hybridization of *trk*A, -B, and -C in adult rat hippocampus. Coronal sections from adult rat brain were hybridized to antisense (*AS*) probes encoding kinase domains of TrkA, TrkB, and TrkC. Sense (*S*) control probes on adjacent sections showed no signal. Dark-field photomicrographs (6.4×) of representative sections are depicted.

brain using *in situ* hybridization. In agreement with previous reports using adult mouse brain sections, abundant message for both *trk*B and *trk*C, but not *trk*A, was observed in the pyramidal cell layers, as well as in the dentate gyrus (Fig. 1). Prominent signals for *trk*B and *trk*C were also detected in scattered interneurons. The hybridization signal for *trk*C was particularly predominant in the granule cells of the dentate gyrus. High-power views of adjacent sections (not shown) indicated that the majority of the hippocampal neurons expressed *trk*B as well as *trk*C, suggesting that these two Trk receptors may be coexpressed by a substantial proportion of the hippocampal neurons.

We next examined expression of *trk* receptors in embryonic rat (E18) hippocampal cultures by Northern analysis, using cDNA probes to the kinase domains of TrkA, TrkB, and TrkC. Similar to the *in situ* hybridization results observed in adult hippocampus, there was significant message for *trk*B and *trk*C, but not *trk*A, in cultured hippocampal neurons (Fig. 2*A*). Expression profiles of the *trk*s in both embryonic and adult hippocampal neurons would suggest that these neurons have the capacity to respond to BDNF, NT-3, and NT-4, but not NGF, throughout much of their lifetime.

Induction of Trk phosphorylation by BDNF, NT-3, and NT-4, but not NGF, in hippocampal cultures

Given that we utilized the probes to the kinase domains of the Trk receptors in the above Northern analysis, which yielded the appropriate size expected for full-length transcripts (Valenzuela et al., in press), the *trk* receptor mRNAs found in cultured hippocampal neurons should reflect expression of functional receptors for BDNF, NT-3, and NT-4. To verify this, each of the neurotrophins was examined for the ability to induce tyrosine phosphorylation of each of the Trk receptors. A 5 min treatment of hippocampal cultures with either BDNF, NT-3, or NT-4 resulted in each case with rapid Trk phosphorylation, as detected by immunoprecipitation using a pan-Trk-specific antibody (Fig. 2*B*). NGF treatment was without effect, consistent with the lack of *trk*A expression in these cultures.

*Induction of c-*fos *expression by specific neurotrophins in hippocampal cultures*

Growth factor activation of receptor-mediated signal transduction pathways universally results in the rapid and transient activation of a class of genes referred to as immediate-early response genes (Lau and Nathans, 1985), which include the proto-oncogene c-*fos* (Morgan and Curran, 1989; Sheng and Greenberg, 1990). Activation of c-*fos* has often served as a useful marker of a functional interaction between a given ligand and its cognate receptor (Sheng and Greenberg, 1990). Thus, the ability of the neurotrophins to induce c-*fos* expression in hippocampal cultures was examined. As would be predicted from the Trk expression and phosphorylation studies, NGF did not induce c-*fos* mRNA, while the other three neurotrophins, BDNF, NT-3, and NT-4, were all able to induce c-*fos* mRNA expression

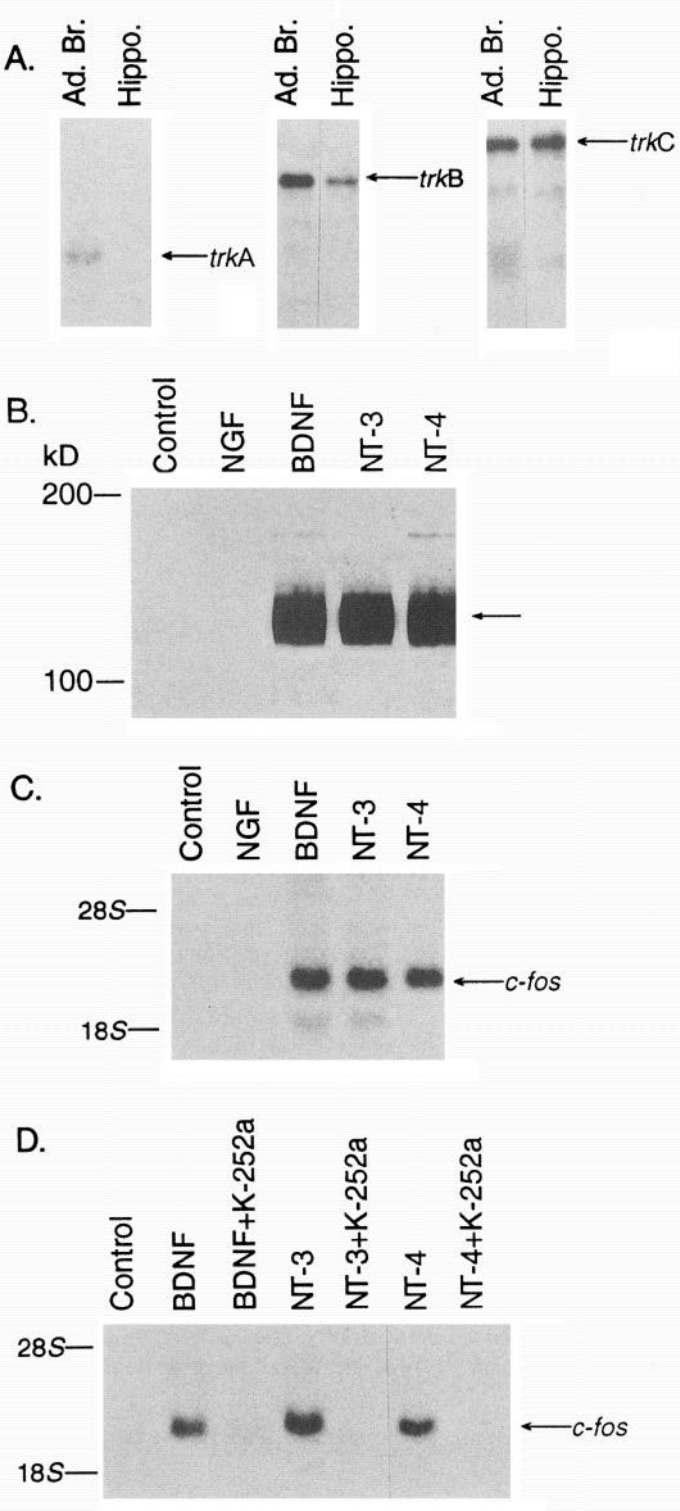

Figure 2. Expression of Trk receptors mRNA, induction of tyrosine phosphorylation of Trk receptors, and c-*fos* mRNA induction by the neurotrophins in cultures derived from E18 hippocampus and maintained in culture for 2 d. *A,* RNA blot analysis of mRNA levels for *trk*A, *trk*B, and *trk*C in hippocampal cultures from E18 rat. The transcript size for *trk*B and *trk*C was estimated to be 9 kb and 14 kb, respectively. *B,* Trk phosphorylation induced by 5 min of treatment with 50 ng/ml of BDNF, NT-3, and NT-4. Molecular weight standards are indicated on the *left,* while *arrow on the right* denotes phosphorylated Trk species. *C,* Hippocampal cultures were treated for 1 hr with 50 ng/ml of various neurotrophins prior to examination of c-*fos* mRNA levels. Migration of ribosomal bands is indicated on the *left. D,* Hippocampal cultures were pretreated with (+) or without (−) K-252a for 30 min prior to the addition of 50 ng/ml of BDNF, NT-3, or NT-4 for a 1 hr incubation period. c-*fos* mRNA levels were examined.

(Fig. 2*C*). K-252a, a compound that has recently been shown to be a specific and potent inhibitor of the *trk* family of receptor protein kinases (Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992), completely blocked the increases in c-*fos* induced by BDNF, NT-3, and NT-4 (Fig. 2*D*).

The K-252a data, together with the appropriate correlation between the expression of a particular Trk and responsiveness to its cognate ligands, provide evidence that the induction of c-*fos* in hippocampal neurons is mediated by Trk receptors. Thus, responses to BDNF and NT-4 were presumably mediated by TrkB receptors, whereas those to NT-3 were presumably mediated primarily by TrkC receptors; although NT-3 has been shown to activate TrkB under certain conditions, this only occurs at concentrations of NT-3 well above those used here (Ip et al., 1993). For most of the following experiments, BDNF was used to examine TrkB responses while NT-3 was used to explore TrkC-mediated processes, although in some cases experiments were also performed with NT-4 to verify that it elicited effects similar to those of BDNF.

Immunohistochemical localization of c-fos *responses defines overlapping subpopulations of responding cells*

A time course study of induction of c-*fos* mRNA by BDNF or NT-3 revealed that the increase apparently peaked at about 1 hr after the addition of the factors (Fig. 3). The increase in c-*fos* mRNA was followed by an increase in c-*fos* protein as detected immunocytochemically (Fig. 4). c-*fos* protein levels peaked at about 2 hr following addition of BDNF, and persisted for up to at least 6 hr. Similar results were observed with NT-3 (not shown). Based on observed differences in the size and morphology of the responsive cells (indicated by large and small arrows in Fig. 4, 1h), it is probable that heterogeneous cell types in the hippocampus have the ability to respond to the neurotrophins. Furthermore, quantitation of the percentage of responsive cells revealed that approximately 40–50% of the hippocampal cells in culture were found to respond to either BDNF or NT-3 by c-*fos* induction. Similar results were obtained with NT-4, as would be expected if NT-4 utilized the same receptors as BDNF (not shown). Most, if not all, of the cells showing a c-*fos* response were assessed to be neurons, given that at least 95% of the cells in our cultures were determined to be neurons by comparison of the number of cells that stained with the neuronal marker NSE and glial marker glial fibrillary acidic protein (data not shown).

To examine whether the cells responsive to either BDNF or NT-3 represent overlapping cell populations, additivity experiments were performed. The total number of cells that showed a c-*fos* response in the presence of both BDNF and NT-3, or both NT-4 and NT-3, was essentially the same as with individual factor alone (Fig. 5). These results indicate that the majority of cells that are responsive to BDNF or NT-4 (and thus express TrkB) overlap with those that respond to NT-3 (and thus express TrkC). The coexpression of TrkB and TrkC had also been suggested by the *in situ* analysis described above.

Neurotrophins do not support survival of E18 hippocampal neurons

There was no obvious effect of any of the neurotrophins on the number of neurons surviving in the E18 hippocampal cultures

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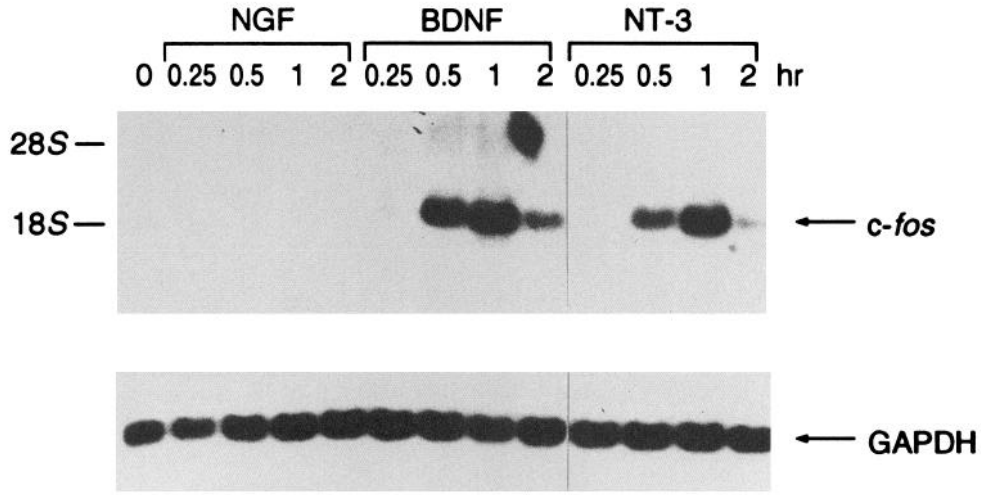

Figure 3. Time course of induction of c-*fos* mRNA and protein by the neurotrophins in cultures derived from E18 hippocampus and maintained in culture for 2 d. Time course of increase in c-*fos* mRNA levels induced by NGF, BDNF, and NT-3. E18 hippocampal cultures were treated for 0.25–2 hr with the indicated neurotrophins prior to isolation of RNA and Northern analysis using a c-*fos*–specific probe. Equal loading of total cellular RNAs is indicated by the relatively constant levels of GAPDH.

after 8 d, as determined by immunocytochemical staining with antibodies to NSE (Table 1). Whereas it is possible that the neurotrophins may act as survival factors for a minor cell population too small to be reflected in total cell counts, it seems likely that these factors affect the majority of responding cells (as defined by phosphorylation and c-*fos* responses) in ways that are distinct from sustaining their survival. For example, we have noted that both BDNF and NT-3 (but not NGF) produced significant increases in the total neurofilament content of the E18 hippocampal cell population (Table 1).

Effects of the neurotrophins on AChE- and calbindin-containing neurons

To begin to define possible subpopulations of hippocampal neurons that were among the c-*fos*–responsive cells, we used a variety of biochemical and immunocytochemical markers to assess responses to each of the neurotrophins. Using the assays indicated in Table 1, none of the neurotrophins examined appeared to have any survival effect on two of the major neuronal populations within the hippocampus, GABAergic and glutamatergic neurons. Neither the high-affinity uptake of GABA and glutamate nor the number of GABA-immunopositive neurons was changed in the presence of the neurotrophins NGF, BDNF, or NT-3. As discussed above, it is still possible that BDNF and NT-3 may have other effects on these cells not detected by these assays.

The neurotrophins, however, were found to effect the expression of the phenotypic markers of two minor neuronal populations present in hippocampal culture, AChE- and calbindin-containing cells (Fig. 6). We found that BDNF produced a fivefold increase in the number of AChE-positive cells, while NT-3 produced a smaller (twofold) effect (Fig. 6*A*). As expected from the receptor specificities that BDNF and NT-4 share, NT-4 produced similar effects as BDNF (Fig. 6*A*).

NT-3, BDNF, and NT-4 also affected the expression of the phenotypic marker in the other minor hippocampal cell population examined, calbindin-containing neurons. Double labeling with both c-*fos* and calbindin have indicated that calbindin-immunopositive cells were among the neurons that showed a c-*fos* response to the neurotrophins (data not shown). NT-3 was observed to produce a substantial increase in the number of cells containing detectable calbindin levels. BDNF, but not NGF, was also found to increase the number of calbindin-containing neurons, albeit with effects smaller than that of NT-3 (Fig. 6*B*). Once again, NT-4 was observed to have effects similar to those of BDNF (Fig. 6*B*).

Difference in the time course and magnitude of calbindin induction by NT-3 and BDNF

As mentioned above, a large increase in the number of calbindin-positive neurons was observed in hippocampal cultures (isolated from either E16 or E18) treated with NT-3 (Fig. 7*A,B*). In addition to increasing the number of neurons expressing detectable levels of calbindin, NT-3 treatment produced more intense calbindin immunoreactivity within individual cells, indicative perhaps of an induction of calbindin protein. The in-

Table 1. E18 hippocampal cultures maintained in the presence or absence of NGF, BDNF, or NT-3 for 8 d

	Immunocytochemistry			Neurotransmitter uptake	
	NSE (staining) (cells/well)	NF (ELISA) (OD_{490})	GABA (staining) (cells/well)	GABA uptake (cpm/10 min/well)	Glutamate uptake (cpm/5 min/well)
Control	9837 ± 932	0.351 ± 0.028	712 ± 102	11,635 ± 940	36,411 ± 1930
NGF	9107 ± 561	0.360 ± 0.022	729 ± 89	8,684 ± 1063	35,308 ± 993
BDNF	9224 ± 132	0.616 ± 0.057	613 ± 53	11,386 ± 911	40,577 ± 198
NT-3	9604 ± 773	0.611 ± 0.083	675 ± 43	11,136 ± 749	32,562 ± 3246

At the end of the culture period, the cells were either fixed and stained for NSE or GABA, or assayed for neurofilament (NF) high-affinity neuron-specific GABA or glutamate uptake as described in Materials and Methods. In all cases, data represent the mean ± SEM of replicate cultures, $n = 5$.

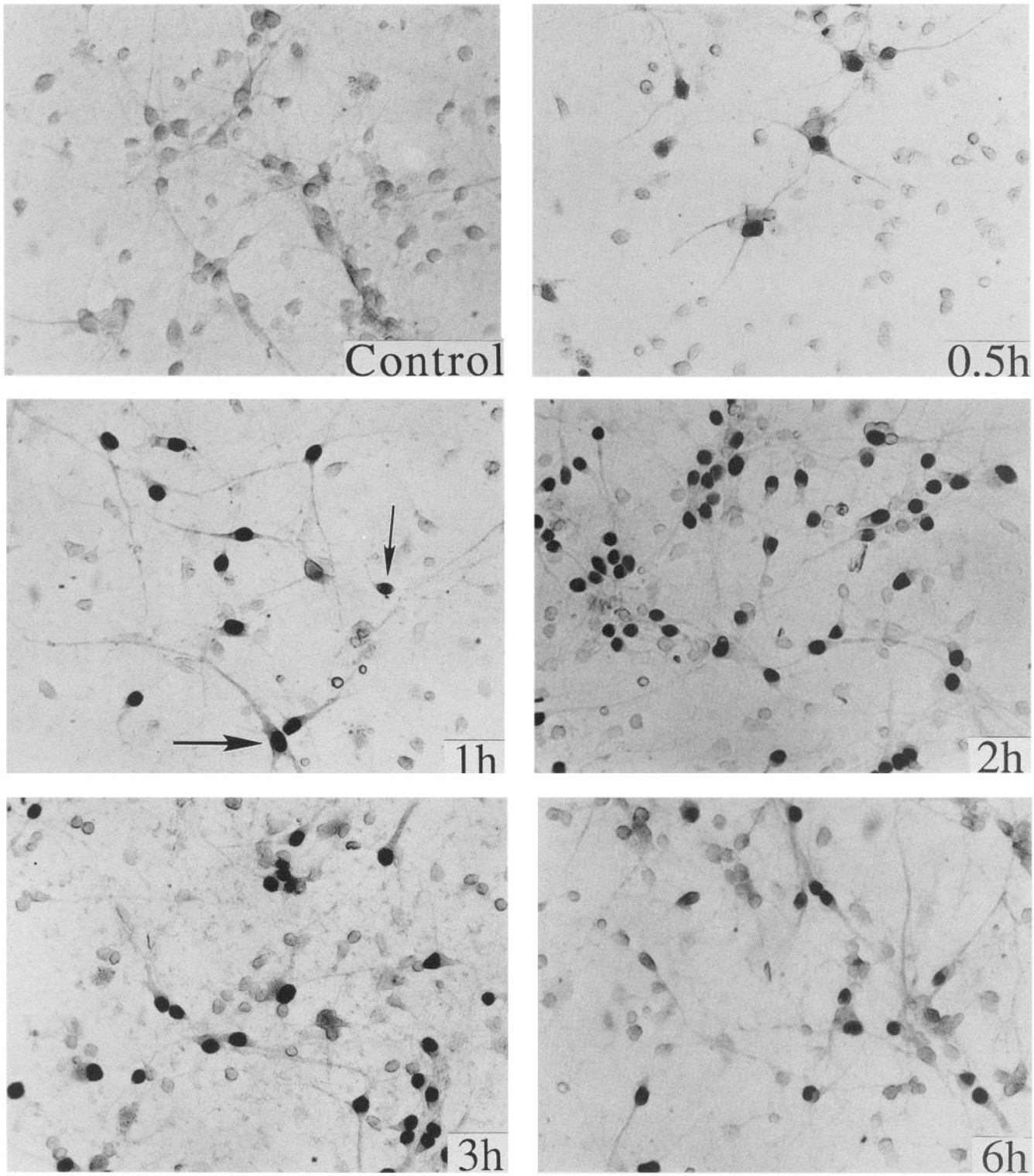

Figure 4. Time course of change in c-*fos* protein induced by BDNF. Hippocampal cultures were treated with BDNF (50 ng/ml) for 0.5–6 hr, and subsequently stained with a c-*fos*–specific antibody. The *large and small arrows* indicate that the induction of c-*fos* occurs in large and small neurons.

crease in the number of calbindin-positive cells was preceded by an increase in the level of calbindin mRNA in cultures treated with NT-3 (Fig. 7*C*).

At all time points examined, including our standard 8 d assay, NT-3 produced the largest increase in calbindin induction. From a time course study, it was also evident that NT-3 produced the earliest induction of calbindin, as shown by the large increase in the number of detectable calbindin-positive cells after only 3 d of exposure to NT-3 (Fig. 8*A*). Although the effect of BDNF was much smaller than NT-3 at all times up to 8 d, the effects of BDNF and NT-3 were very similar after a total of 10 d in culture.

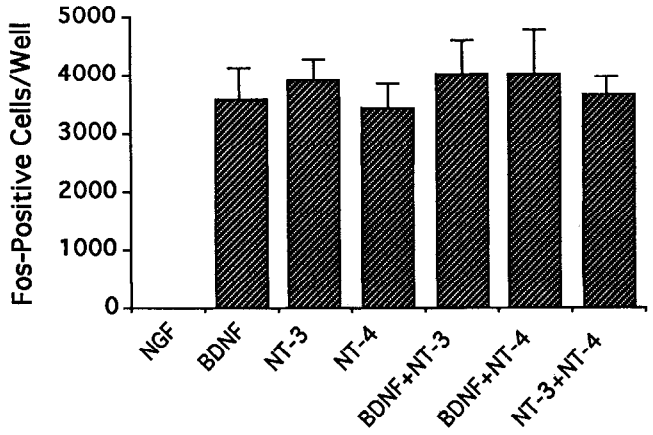

Figure 5. Overlap of the effects of BDNF, NT-3, and NT-4 in c-*fos* induction. Hippocampal cultures derived from E18 hippocampus were cultured for 2 d and were then treated for 2 hr with 50 ng/ml of either BDNF, NT-3, or NT-4 alone, or different combinations of two factors. The total number of c-*fos*–positive cells were counted. Data are the mean ± SEM of replicate cultures, $n = 5$.

Dose–response studies performed in hippocampal cultures treated with various neurotrophins for 3 d revealed that a relatively low concentration of NT-3 (EC_{50} of 1 ng/ml) was able to produce a striking increase of 20-fold in the number of detectable calbindin-immunopositive cells (Fig. 8*B*). On the other hand, BDNF had a much smaller effect of only about twofold. This dramatic difference between BDNF and NT-3 was observed at all concentrations of the neurotrophins examined. Once again, as expected from the similar receptor specificity of BDNF and NT-4, NT-4 produced similar effects as BDNF at all concentrations tested (not shown).

To distinguish between the possibilities of a survival-promoting effect and a phenotypic effect of NT-3 on the calbindin marker, addition of NT-3 to cultures was delayed for 4 d. Delaying the addition of NT-3 to E18 cultures did not appear to affect the subsequent increase in the number of detectable calbindin-positive cells following NT-3 treatment for 3 d (Fig. 9). Thus, the effect of NT-3 on calbindin-immunopositive cells in E18 hippocampal cultures did not appear to represent an increase in neuronal survival, but rather phenotypic induction of calbindin.

Discussion

There has recently been a wave of circumstantial evidence implicating major roles for several of the neurotrophins in the development and maintenance of the hippocampal formation and its afferents. Abundant levels of NGF, BDNF, and NT-3 mRNA have been detected in the developing and adult hippocampus and localized to subpopulations of pyramidal and dentate granule neurons (Ernfors et al., 1990; Hofer et al., 1990; Maisonpierre et al., 1990b; Phillips et al., 1990). High levels of NGF within the hippocampus are very consistent with the role of this neurotrophin as a target-derived neurotrophic factor for cholinergic neurons of the septohippocampal pathway (Snider and Johnson, 1989). The absence of TrkA expression within the hippocampus and the absence of retrograde transport of radiolabeled NGF from the hippocampus, other than by cholinergic neurons (DiStefano et al., 1992), suggest that the single role of NGF within the hippocampus may be that of a target-derived factor for cholinergic neurons of the basal forebrain. Whereas

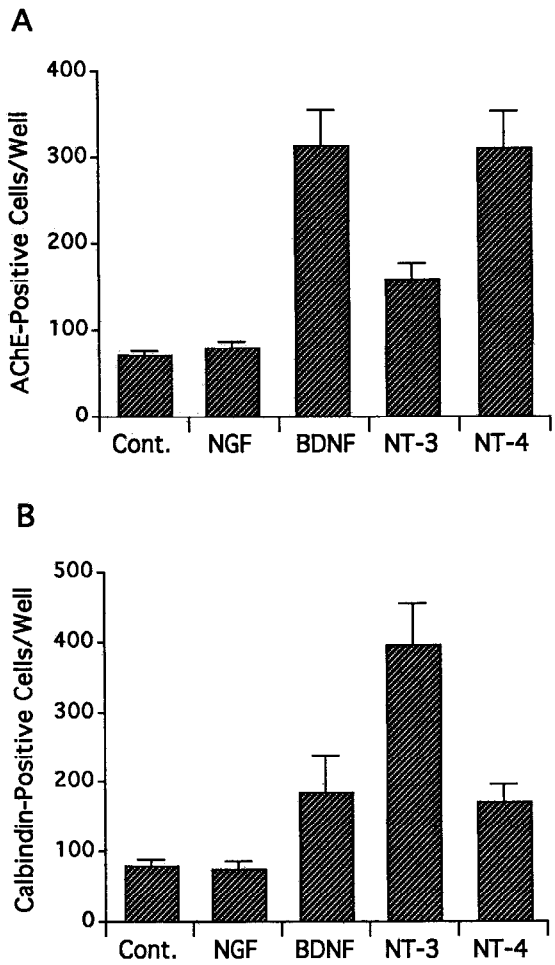

Figure 6. Effects of neurotrophins on AChE- or calbindin-positive cells in E18 hippocampal cultures maintained *in vitro* for 8 d. Hippocampal cultures were incubated in the presence of 50 ng/ml of each of the neurotrophins (NGF, BDNF, NT-3, and NT-4) for 8 d. Medium was changed every 3 d, and neurotrophins were re-added when appropriate. The total number of AChE-positive cells (*A*) or calbindin-immunopositive cells (*B*) was counted. Data are the mean ± SEM of replicate cultures, $n = 5$.

in vitro and *in vivo* studies indicate that BDNF in the hippocampus may play a role similar to that of NGF, there is growing evidence that BDNF (and NT-3) is likely to affect a much broader spectrum of responsive neurons than NGF. Thus, the abundant expression of TrkB and TrkC receptors within the mouse hippocampus (Klein et al., 1989, 1990; Lamballe et al., 1991), the recent demonstration of distant and local transport of radiolabeled neurotrophins when injected into the hippocampus (DiStefano et al., 1992), and the detection of high-affinity neurotrophin binding sites in hippocampal sections (Altar et al., 1993) all suggest a role for the neurotrophins not only as target-derived neurotrophic factors for hippocampal afferents, but also as locally acting, possibly autocrine or paracrine, factors for hippocampal neurons.

Our study has demonstrated functional responses to several of the neurotrophins in primary CNS neurons that appear to correlate with the expression and activation of specific Trk receptors in these cultures. We first used molecular approaches to establish the presence and functional capabilities of neurotrophin receptors in these cultures. *In situ* and Northern analysis indicated the presence of transcripts for *trk*B and *trk*C, but not

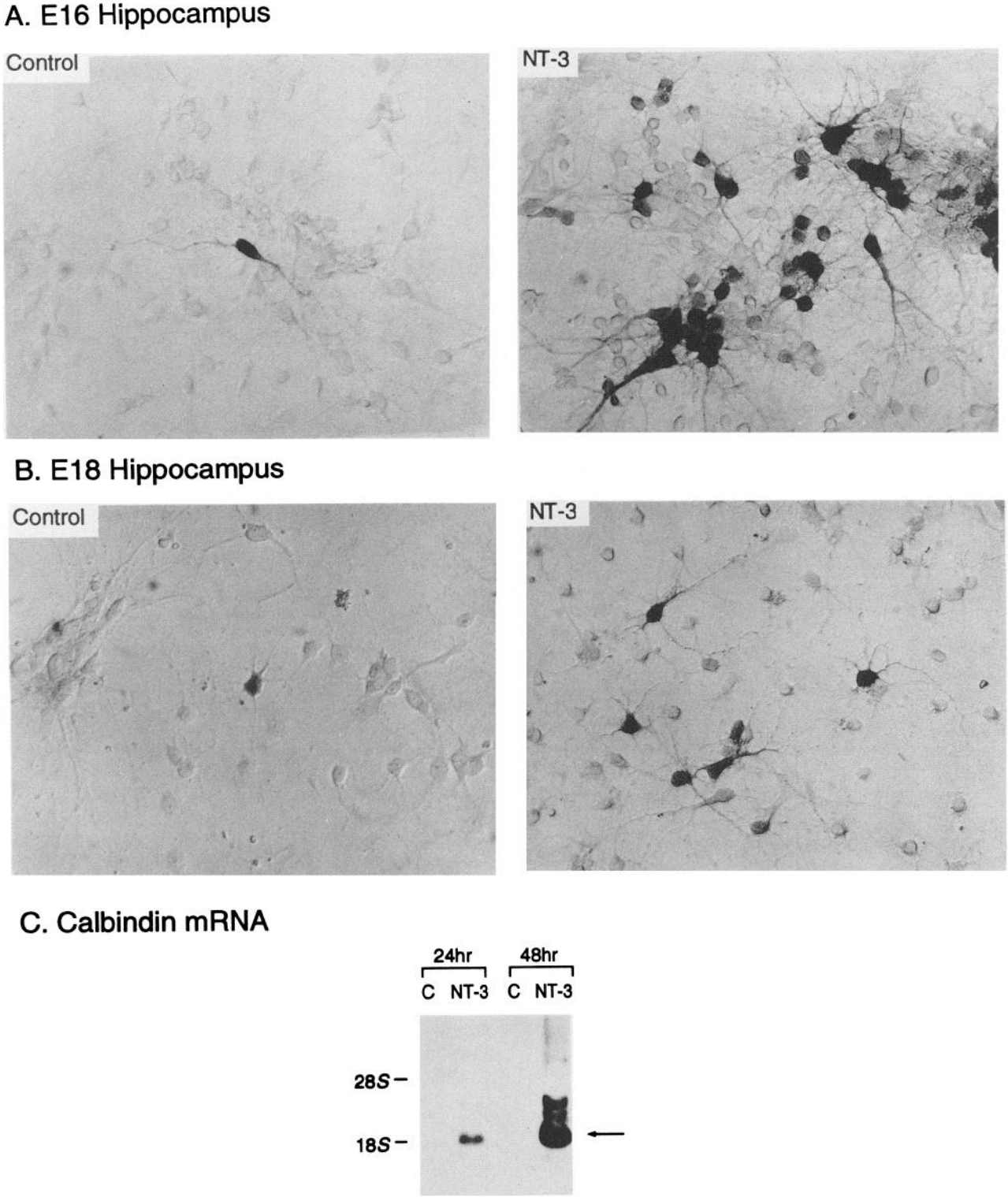

Figure 7. Immunocytochemical detection of calbindin-positive neurons and Northern analysis of calbindin mRNA in hippocampal cultures treated with NT-3. *A* and *B* depict neurons stained for calbindin in control cultures or in NT-3–treated cultures derived from E16 or E18 embryos and cultured for 8 d. *C,* Induction of calbindin mRNA in NT-3–treated cultures. E18 hippocampal cultures were treated for 24 or 48 hr with 50 ng/ml NT-3, and calbindin mRNA expression was determined by Northern analysis as described in Materials and Methods.

*trk*A, mRNA in E18 hippocampal cultures as well as in sections of adult rat hippocampus. Confirming this expression pattern, functional responses (tyrosine phosphorylation of the appropriate Trk receptors, as well as subsequent c-*fos* induction) of cultured neurons were observed with BDNF, NT-3, and NT-4, but not NGF. This approach was then followed by more specific experiments to begin to establish specific phenotypic effects of each of the neurotrophins. In contrast to a number of

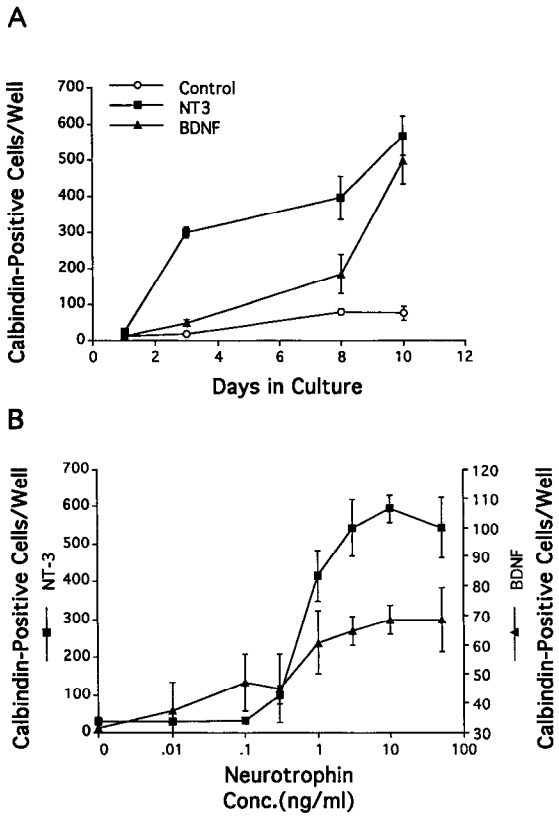

Figure 8. Time course and dose dependency of the effects of BDNF and NT-3 on calbindin-positive cells in E18 hippocampal cultures. *A*, Time course of the increase in calbindin-positive cells in hippocampal cultures treated with BDNF or NT-3. Hippocampal neurons isolated from E18 hippocampus were treated with 50 ng/ml of BDNF or NT-3 for 1–10 d, prior to determination of the total number of calbindin-immunopositive cells. Medium and growth factors were replenished every 3 d. *B*, Dose dependency of the effects of BDNF and NT-3. Hippocampal cultures were treated with various concentrations of BDNF or NT-3 for 3 d, and the total number of calbindin-positive cells was determined. Data for *A* and *B* are the mean ± SEM of replicate cultures, $n = 5$.

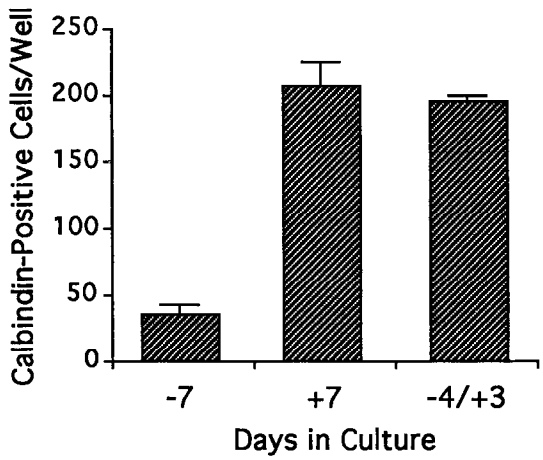

Figure 9. Effect of delaying the addition of NT-3 on the NT-3–induced increase in number of calbindin-positive cells in E18 hippocampal cultures. Hippocampal cultures were cultured for 7 d in the absence (*−7*) or presence (*+7*) of NT-3. To some cultures, addition of NT-3 was delayed for 4 d, prior to subsequent incubation with NT-3 for 3 d (*−4/+3*). The number of calbindin-positive cells was counted at the end of the culture period. Data are the mean ± SEM of replicate cultures, $n = 5$.

other neuronal populations that are responsive to the neurotrophins, we did not observe any notable effects of BDNF, NT-3, NT-4, or NGF on the survival of E18 hippocampal neurons, even when cultures were established at low densities. However, BDNF, NT-3, and NT-4 very markedly increased the number of neurons expressing detectable levels of either calbindin or AChE. Together these two neuronal populations account for only a relatively small percentage of the hippocampal cells found to be neurotrophin responsive by induction of c-*fos*. We suspected that the c-*fos*–positive cells would include either glutamatergic or GABAergic neurons, the predominant phenotypes of hippocampal neurons. While staining for GABA-positive neurons and measurements of high-affinity GABA or glutamate uptake did not reveal any obvious effects of NGF, BDNF, or NT-3, it is quite possible that these neurotrophins mediate other effects on GABAergic or glutamatergic neurons not apparent by these assays. Alternatively, this may leave open the identity of a substantial cell population that responds to BDNF, NT-3, and NT-4. Whereas we believe molecular approaches may now provide us with a general method to reveal more easily the actions of potential growth factors that have important effects on neurons other than promoting their survival, a major hurdle still lies in identifying phenotypic markers that define specific cell types that respond to the neurotrophins. The very low percentage of non-neuronal cells in our cultures indicates that the as yet unidentified neurotrophin-responsive cells are neurons.

Consistent with the absence of the TrkA receptor in the adult hippocampus or in our cultures, NGF failed to elicit any functional responses when compared to the other neurotrophins. Although BDNF, NT-3, and NT-4 all acted to induce AChE and calbindin, it is of interest to note that there were differences in the time course and magnitude of the effect of each of these neurotrophins. BDNF and NT-4 yielded indistinguishable results in terms of the percentage of responding cells, as well as the magnitudes and dose dependencies of their effects on calbindin-containing cells, consistent with the fact that BDNF and NT-4 are both preferred ligands for the same receptor, TrkB. NT-3, on the other hand, clearly was distinct from either BDNF or NT-4 in terms of the responses it exerted, presumably due to the fact that it utilizes a different receptor, TrkC. Thus, although much of our evidence suggests that TrkB and TrkC were coexpressed by a substantial proportion of the hippocampal neurons, it is apparent that either there were some subpopulations of the hippocampal neurons expressing only one and not the other Trk receptor, or perhaps TrkB and TrkC are capable of mediating different responses in the cells in which they are coexpressed. Not only did NT-3 produce the largest increase in the number of calbindin-containing neurons, but it also acted at earlier times in culture than did BDNF. Such early actions of NT-3 are consistent with previous observations that NT-3 expression was most abundant in the hippocampus early in development, whereas peak BDNF expression did not occur until late in development (Maisonpierre et al., 1990b). In the light of high levels of expression of BDNF and NT-3 in the hippocampus and the fact that a substantial proportion of the neurons are responsive to the neurotrophins, it will be interesting to determine whether certain of the neurotrophins act not only as classic target-derived neurotrophic factors, but also as either paracrine or autocrine factors.

Taken together, the absence of a survival-promoting effect and the clear indication that a large percentage of hippocampal

neurons are functionally responsive to three of the neurotrophins strongly suggest that these neurotrophic factors may play other important roles in regulating the function of a number of different subpopulations of hippocampal neurons as they mature. Consistent with such a role is the recent data indicating that expression of the neurotrophins within the hippocampus is exquisitely susceptible to physiological stimuli. For example, a striking spatiotemporal pattern of increased neurotrophin expression in the hippocampus was observed following induction of seizure activity by electrical or chemical stimulations (Gall and Isackson, 1989; Zafra et al., 1990; Ernfors et al., 1991; Gall et al., 1991; Isackson et al., 1991; Dugich-Djordjevic et al., 1992). Given that the hippocampal formation is a major component of cognitive function, these findings raise the intriguing possibility that neurotrophins may have key effects on neuronal plasticity, associated with learning and memory.

The potential therapeutic use of neurotrophic growth factors has received a lot of recent attention, especially since it became clear from both *in vitro* and *in vivo* studies that NGF promotes the survival and maintenance of basal forebrain cholinergic neurons, one of the major neuronal populations that degenerate in Alzheimer's disease (Hefti and Weiner, 1986; Hefti et al., 1989; Phelps et al., 1989). NGF has been shown not only to rescue cholinergic neurons from experimental axotomy but also to improve the performance of age-impaired rats in various cognitive tasks (Fischer et al., 1987). Despite the potentially beneficial effects of NGF toward compromised cholinergic neurons, there are many other neuronal populations that are affected in Alzheimer's disease, including neurons of the cerebral cortex and hippocampus, that are probably not responsive to NGF. Indeed, there is currently very little information on the neurotrophic factor requirements of neurons in these brain regions, the lack of which has been one of the motivations for the present study. It has been reported that there are decreased levels of calbindin in the hippocampus from patients with various degenerative diseases, including Alzheimer's type dementia (Iacopino and Christakos, 1990; Sutherland et al., 1992), and in hippocampal slices from aged rats (Dutar et al., 1991). In addition, it has been suggested that the expression of calbindin, a calcium-binding protein widely expressed in the CNS (Baimbridge and Miller, 1982; Baimbridge et al., 1982; Sequier et al., 1990), may protect neurons from neuronal damage (Sloviter, 1989; Iacopino et al., 1992). In the present study, we have demonstrated that three of the neurotrophins, BDNF, NT-4, and to a larger extent NT-3, were able to act specifically to induce calbindin mRNA as well as protein in hippocampal neurons. This may be indicative of upregulation of other calcium-binding protein occurring in other cells. Such an increase in calcium-binding capacity may have significant physiological implications in that it may be important in preventing cell death in select populations of hippocampal neurons. In this context, it is interesting to note that a major decrease in BDNF level was identified in groups of Alzheimer patients (Phillips et al., 1991). It will be interesting to see whether this correlates closely with calbindin level in this region.

Note added in proof

While the manuscript was under review, Collazo et al. (Neuron 9:643–656) reported similar findings of NT-3 in hippocampal cultures.

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