Differential Regulation of p21\textsuperscript{ras} Activation in Neurons by Nerve Growth Factor and Brain-derived Neurotrophic Factor*

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Neurotrophins activate the Trk tyrosine kinase receptors, which subsequently initiate signaling pathways that have yet to be fully resolved, resulting in neuronal survival and differentiation. The ability of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) to activate GTP binding to p21\textsuperscript{ras} was investigated using cultured embryonic chick neurons. In both sympathetic and sensory neurons, the addition of NGF markedly increased the formation of Ras-GTP. The magnitude of the effect was found to depend upon the developmental stage, peaking at embryonic day 11 in sympathetic neurons and at embryonic day 9 in sensory neurons, times when large numbers of neurons depend on NGF for survival. Surprisingly, following the addition of BDNF, no formation of Ras-GTP could be observed in neurons cultured with BDNF. When sensory neurons were cultured with NGF alone, both NGF and BDNF stimulated GTP binding to Ras. In rat cerebellar granule cells, while the acute exposure of these cells to BDNF resulted in the formation of Ras-GTP, no response was observed following previous exposure of the cells to BDNF, as was observed with sensory neurons. However, this desensitization was not observed in a transformed cell line expressing TrkB. In neurons, the mechanism underlying the loss of the BDNF response appeared to involve a dramatic loss of binding to cell-surface receptors, as determined by cross-linking with radiolabeled BDNF. Receptor degradation could not account for the desensitization since cell lysates from neurons pretreated with BDNF revealed that the levels of TrkB were comparable to those in untreated cells. These results indicate that in neurons, the pathways activated by NGF and BDNF are differentially regulated and that prolonged exposure to BDNF results in the inability of TrkB to bind its ligand.

The formation of the vertebrate nervous system is regulated by the availability of a variety of soluble factors promoting the survival and differentiation of neurons. Among these factors is a family of related proteins referred to as neurotrophins that includes nerve growth factor (NGF),\textsuperscript{1} brain-derived neurotrophic factor (BDNF), and neurotrophins 3–6 (NT-3, NT-4/5, and NT-6). Gene knockout and antibody-mediated deprivation experiments have established the crucial role played by several of these proteins in the formation of the nervous system (for review, see Ref. 1). The involvement of the Trk subgroup of receptor tyrosine kinases in mediating the biological function of the neurotrophins has been demonstrated by the observation that loss-of-function mutations introduced in the genes coding for each of the three Trk receptors cause defects in the nervous system that are analogous to those observed in mice lacking the corresponding receptor ligand (for review, see Ref. 2). While these results do not indicate that the Trk proteins are the only receptor components necessary for mediating a neurotrophin response in neurons, they do show that this subgroup of tyrosine kinases plays a necessary role in mediating neuronal responses to neurotrophins.

Binding of neurotrophins to their respective Trk receptors results in the stimulation of tyrosine kinase activity, which initiates a signaling cascade involving the downstream targets of many growth factor receptors, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, phospholipase C, and, in particular, p21\textsuperscript{ras} (for review, see Ref. 3). The importance of the Ras pathway in neurotrophin signaling was first revealed using the rat pheochromocytoma PC12 cell line. The introduction of Ras antibodies or of dominant negative Ras mutants into these tumor cells suppressed NGF-induced fiber outgrowth (4). While much less work has been done using post-mitotic neurons, similar results were obtained with embryonic neurons: the introduction of a constitutively active mutant of Ras into chick sensory neurons led to neurotrophin-independent survival and fiber outgrowth (5). Similarly, Fab fragments blocking the activity of Ras prevented NGF-induced survival in these neurons (6). However, neurotrophin signaling appears to be differentially regulated depending on the neuronal context: in this same study, the introduction of anti-Ras Fab fragments failed to block the survival effects of NGF in chick (unlike rat (7)) sympathetic neurons, and constitutively active Ras was unable to support survival of these neurons. Taken together, these findings indicate that although neurotrophins act like many other growth factors, there appears to be differential regulation of the signal transduction pathways in different neurons.

We were interested in investigating the involvement and regulation of the Ras pathway by measuring the formation of Ras-GTP following stimulation by NGF and BDNF of different populations of neurons at various embryonic ages. In confirmation of a recent report, we found that BDNF and NGF were both able to stimulate GTP binding to Ras in chick sensory neurons (8). NGF was also found to activate Ras in sympathetic neurons. Surprisingly, in contrast to the results obtained with NGF, cultured neurons became completely desensitized to BDNF when exposed for prolonged periods of time to BDNF.

EXPERIMENTAL PROCEDURES

Neuropotrophins—Recombinant neurotrophins were produced using the vaccinia virus system (BDNF and NT-3 (9)) or Chinese hamster...
ovary cells generously provided by Genentech (BDNF and NGF). NGF purified from the submandibular gland of an adult male mouse as described (10) was also used. Identical results were obtained with neurotrophins from each source.

Cell Culture—Dorsal root ganglia (DRG) were isolated from chick embryos at E6, E9, and E12, dissociated with trypsin, and plated as described (11), with minor modifications. Briefly, the ganglia were treated with 0.1% trypsin for 15 min at 37°C and then dissociated in the medium used to culture the neurons, which was F14 containing 10% heat-inactivated horse serum and 5% fetal calf serum (culture medium), by trituration through a siliconized, fire-polished Pasteur pipette. Following a 3-h preplating to remove most of the non-neuronal cells, the neuronal cells were plated on a substrate coated with polylysine and medium conditioned by RN22 schwannoma cells or laminin.

Sympathetic neuronal cultures from embryos at E7, E11, or E15, as indicated, were prepared as described (11). Briefly, lumbosacral chains were isolated, were treated with 0.1% trypsin for 15 min at 37°C and then dissociated in a 1% solution of trypsin in phosphate-buffered saline. Equal amounts of sample were separated on a polyethyleneimine-cellulose TLC plate (Merck), and the ratio between GDP and GTP was determined by phosphorimager.

Analysis of GDP and GTP Bound to p21ras—Neurons were plated on 6-cm dishes at a density of 0.5 × 10^6 cells/plate (see above), washed twice with a physiological buffer (120 mM NaCl, 5 mM KCl, 0.84 mM Na,HPO4, 0.22 mM KH,PO4, 2.6 mM NaHCO3, 5 mM glucose, 3 mM MgCl2, pH 7.4), and incubated for 1 h at 37°C, followed by a 30-min wash at 37°C in phosphate-free KRH buffer (25 mM HEPES, pH 7.3, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 3 mM MgCl2, 5 mM Mg,bovine serum albumin, 0.1 mM cytochrome c). The cells were then loaded for 4 h at 37°C with 0.25 μCi of [32P]-ATP (Amersham Corp.) in 1.5 ml of KRH buffer. After incubation with or without the indicated neurotrophin for 2 h, the cells were rinsed twice (2 min each), reloaded with [32P]-labeled ATP, and dissociated in ice-cold Tris-buffered saline, pH 7.4, and lysed with 0.5 ml of lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM PMSF, 4 μg/ml leupeptin, 1 mM orthovanadate). The plates were scraped, and the lysate was rocked for 20 min on ice before the cell debris was separated by a 10-min spin at 14,000 × g. Triton X-100 (4%, v/v) was added, and incubation was continued for 20 min. Unreacted reagents were washed with ice-cold Tris-buffered saline, and pelleted at 10,000 × g for 1 h. The cell lysates were immunoprecipitated with the anti-Ras monoclonal antibody Y13-259. The lysates were washed as described above, removed by scraping in phosphate-buffered saline, and pelleted at 10,000 × g for 5 min. The cell debris was then disrupted in phosphate-buffered saline with the addition of 1 mM PMSF and 4 μg/ml leupeptin using a glass-Teflon tissue homogenizer for 1 min at 1500 rpm (B. Braun Biotech International), followed by sonication for 10 s on ice (Bronson sonifier). After removal of the nuclei by centrifugation at 1000 × g for 5 min, the membranes remaining in solution were pelleted at 100,000 × g for 1 h. The supernatants were then resuspended in KRH buffer, and radiolabeled BDNF was added and cross-linked as described for intact cells. The membranes were then repelleted at 100,000 × g for 1 h to separate them from the free ligand, and TrkB was immunoprecipitated as described above.

Western Blotting—20 × 10^6 cerebellar granule neurons were plated for 2 days with or without BDNF (20 ng/ml) on 10-cm dishes. After washing once with phosphate-buffered saline, they were collected in 500 μl of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM NaF, 250 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 0.1 mM ATP). Lysates were centrifuged, and the supernatants were immunoprecipitated with wheat germ agglutinin-Sepharose 6MB (Sigma). The immunoprecipitates were washed, run on 6% SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp.), and incubated with anti-TrkB serum that was raised to a peptide corresponding to the C terminus of the tyrosine kinase domain of human TrkB that also immunoprecipitates rat TrkB and TrkC (a kind gift from Dr. Alfredo Rodrı´guez-Te´bar). The immune complexes were precipitated with protein A-Sepharose for at least 1 h and then washed three times with lysis buffer and twice with Tris-buffered saline. Equal amounts of sample were separated on 6.5% SDS-polyacrylamide gels, which were then dried and analyzed with a Phosphorimager.

RESULTS
Neurotrophin Activation of p21ras in Sympathetic Neurons—Constitutively activated Ras introduced into chick sympathetic neurons does not promote their survival, and antibodies to p21ras do not influence NGF-mediated survival (5, 6). However, the presence of an activable Ras pathway has not been determined in these neurons. To address this question, E11 chick
sympathetic neurons were cultured for 2 days with 5 ng/ml NGF. After removal of the factor, the acute addition of NGF induced a robust response to this neurotrophin with an EC₅₀ of 60 ± 1.2 pM (or 1.6 ng/ml) and a maximum stimulation of 52 ± 2% (Fig. 1, lower panel). The response to NGF was found to peak rapidly, followed by a slow decline to a level twice that of control after 60 min (Fig. 1, upper panel). A similar response was also observed for sensory neurons (8) and PC12 cells (15).

Developmental Regulation of Neurotrophin Activation of p21ras in Sensory and Sympathetic Neurons—To determine if neurotrophin activation of the Ras signaling pathway is regulated during development, neurons were isolated from dorsal root ganglia at E6, E9, and E12. Isolated neurons were cultured with a mixture of 5 ng/ml each NGF, BDNF, and NT-3 to maximize survival of all types of neurons. Similarly, sympathetic neurons were prepared from embryos at days 7, 11, and 15, corresponding to stages during which neuronal numbers increase in these ganglia, in which there is extensive neuronal loss, and when cell number has plateaued, respectively (16). The cells were cultured in 5 ng/ml NGF, except for those of E7, which were cultured without factor as they can survive several days in culture in the absence of neurotrophin (17). Identical responses were observed if the E7 neurons were cultured with 5 ng/ml NT-3 (data not shown).

After 2 days in culture, the medium containing the factor(s) was removed, and acute stimulation of Ras was measured. A maximally effective concentration of NGF (1 nM or 26 ng/ml) was able to stimulate GTP binding to Ras in both sensory and sympathetic neurons at all stages examined. The maximal response to NGF varied during development with a peak at E9 in DRG and at E11 in sympathetic ganglia. However, even at the earliest time point, significant stimulation by this neurotrophin was observed (Fig. 2). With sympathetic neurons at E7, NT-3 weakly activated Ras at a stage when these neurons are known to show a survival response to this factor (18). Marginal but significant activation was also observed at E11, a time point at which NT-3, at low concentrations, no longer supports these cells in culture (18). However, by E15, there was no significant effect of NT-3 on Ras (Fig. 2).

Unlike with NGF and NT-3, BDNF was found not to stimulate the formation of Ras-GTP above control levels in sensory neurons at any stage examined. The reasons for this unexpected observation were then explored.

Desensitization of BDNF but Not NGF Stimulation of p21ras—Sensory neurons were cultured for 2 days with 5 ng/ml BDNF alone to select for neurons responsive to this factor. Nevertheless, after washing away the culture medium, acutely
Survival during the first 3 days of long-term cultures, these cells do not depend on BDNF for their survival. BDNF promotes the survival of a proportion of these cells in rats, and the number of BDNF-binding sites (33,000) per cell (19). Additionally, while readily be obtained in large numbers and have large numbers of neurons, they represent an attractive alternative as these neurons can be observed following a 24-h exposure of neurons to BDNF. This result suggested that at least some sensory neurons selected by BDNF must contain functional NGF receptors, a possibility that was directly examined (see below). Conversely, DRG neurons cultured with 0.1 ng/ml NGF showed a small but significant response to BDNF (Fig. 3), in agreement with previous results of Ng and Shooter (8).

TrkB mRNA in NGF-selected sensory neurons—Since the formation of Ras-GTP following exposure of neurons to neurotrophins is most likely due to the activation of the corresponding receptor tyrosine kinase, the presence of TrkB mRNA in neurons dependent on NGF for survival was investigated by reverse transcription-PCR using tyrosine kinase-specific primers. Neurons selected with NGF for 24, or even 60 h, were found to contain TrkB mRNA, suggesting a colocalization of TrKA and TrkB (Fig. 4). The presence of TrkB mRNA was also observed following a 24-h exposure of neurons to BDNF. This indicates that the lack of BDNF response is not a consequence of the disappearance of TrkB mRNA.

Desensitization and receptor inactivation in rat cerebellar granule cells—Functional, high affinity BDNF receptors are only present in small numbers in primary sensory neurons of the chick (14), which renders further biochemical analyses difficult. In view of this, cultured rat cerebellar granule cells were selected by culturing with either 5 ng/ml BDNF (left panel) or 0.1 ng/ml NGF (right panel). After removing the factor used for culturing, the cells were then acutely exposed to NGF (solid bars), BDNF (dotted bars), or no factor (open bars) and lysed, and the percent of GTP bound to p21ras was assessed as described under “Experimental Procedures.” Shown are the means ± S.E. of three or more experiments. The asterisks indicate the degree of significance relative to that in the absence of factor based on Student’s t test (p ≤ 0.01). CON, control.

When BDNF was added to granule cells that had not been cultured with BDNF, the formation of Ras-GTP could readily be observed (Fig. 5). However, exposure of the cells to BDNF for 24 h led to the disappearance of the response to acutely added BDNF (Fig. 5). This result indicates that the desensitization to BDNF observed with chick sensory neurons can also be observed with other TrkB-expressing neurons. Interestingly, this loss of responsiveness is not observed when TrkB is expressed in a non-neuronal context. Incubation of the cell line A293 expressing chick TrkB (14) for 24 h with 20 ng/ml BDNF did not lead to any measurable desensitization when compared with cells acutely exposed to BDNF (Fig. 6).

To further investigate the mechanisms underlying the loss of BDNF signaling through Ras, changes in cell-surface receptor availability were assessed in granule cells using radiolabeled BDNF. After cross-linking and immunoprecipitation with a Trk-specific antibody, a radioactive band at ~150 kDa was detected (Fig. 7, lanes A and B), which was absent when incubation was performed in the presence of 100-fold excess unlabeled BDNF (Fig. 7, lane D). Immunoprecipitates from cells treated for 24 h with BDNF revealed a dramatic decrease in receptor availability, suggesting a loss of receptor binding capability in BDNF-pretreated neurons (Fig. 7, lane C).

The cross-linking experiments were repeated using cell membranes isolated by high speed centrifugation to probe for...
then thoroughly rinsed to remove the culture medium, and intact cells or in the presence of 20 ng/ml BDNF (CON) or in the presence of 20 ng/ml BDNF. Lysates were prepared from the cells, and equal amounts of protein were separated by 6.5% SDS-PAGE and transferred to Immobilon-P membranes, and TrkB was detected with an antibody raised to a specific extracellular sequence of rat TrkB as described under “Experimental Procedures.”

This study shows that cultured sensory and cerebellar neurons respond to the addition of neurotrophins by the formation of Ras-GTP. This response is also observed in sympathetic neurons, where the activation of Ras has been previously shown to be neither sufficient nor necessary for neuronal survival. Unexpectedly, the formation of Ras-GTP in response to BDNF can only be seen when neurons are acutely exposed to BDNF; culturing them in the presence of BDNF leads to the complete disappearance of the response. This phenomenon is not observed with NGF, nor is it observed when the receptor tyrosine kinase for BDNF, TrkB, is expressed in non-neuronal cells.

Induction of Ras-GTP Formation by NGF in Sympathetic Neurons—The NGF response seen in sympathetic neurons is very similar to that reported for the PC12 cell line (8, 20, 21). It required essentially identical NGF concentrations, which are in the range of the high affinity binding constant (22), and was very rapid, peaking at 2 min, followed by a quick decline that plateaued to a level above that measured in nonstimulated cells. A long-lasting activation of Ras has been suggested to be crucial for promoting neural differentiation (15). However, the fact that NGF could activate Ras in these neurons was unex-
expected since it has previously been shown that inactivating this pathway in chick sympathetic neurons did not block NGF-induced survival or neurite outgrowth (6). Furthermore, the introduction of a constitutively active Ras mutant into these neurons, unlike with sensory neurons, did not promote their survival (6). However, not only is the Ras pathway activated in sympathetic neurons, but the maximum effect of NGF in sympathetic neurons, like in sensory neurons, was observed at a time when programmed cell death eliminates large numbers of neurons in these ganglia. Therefore, although not required or sufficient for survival, NGF signaling via Ras does occur in sympathetic neurons and in a developmentally regulated manner. It is conceivable that in chick sympathetic neurons, unlike sensory neurons, pathways other than Ras are involved in cell survival and that the Ras pathway is required for aspects of differentiation that are not reflected by cell survival or neurite outgrowth, including, for example, regulation of neurotransmitter uptake and synthesis (23).

Induction of Ras-GTP Formation by BDNF in Cultured Neurons—The stimulation of Ras by BDNF in sensory neurons could only be observed under conditions in which the neurons were not pre-exposed to BDNF. Thus, neurons were cultured with NGF, which is expected to select for NGF-dependent neurons. However, the effects seen after BDNF addition suggest that at least some of these neurons must express TrkB in addition to TrkA. In fact, coexpression of TrkA and TrkB in adult rat has recently been demonstrated in vivo in dorsal root ganglia (24). During development, however, in situ hybridization studies suggest a largely nonoverlapping pattern of expression, although this technique cannot exclude low but functionally relevant expression of TrkB in neurons expressing predominantly TrkA. In the present study, the finding that NGF-selected cells respond to BDNF by the formation of Ras-GTP (and vice versa) indicates that TrkA and TrkB could both be present in some individual neurons. This was further supported by PCR analysis, which documented the presence of TrkB mRNA in neurons selected with NGF for as long as 60 h after the start of the cultures. However, the possibility that TrkA-expressing neurons secrete TrkB, thereby allowing some TrkB-expressing neurons to survive, cannot be ruled out, especially in view of the cell density used.

Coexpression of neurotrophin receptors in at least some neurons is also likely in view of the results obtained with sensory neurons demonstrating that many NGF neurons first depend on NT-3 or BDNF for survival (25). A shift from NT-3 to NGF dependence has also been suggested by results obtained in vivo in antibody deprivation experiments (26).

The formation of Ras-GTP in response to BDNF was not only observed in sensory neurons, but also in granule cells isolated from the postnatal rat cerebellum. I just like with sensory neurons, a striking feature of this response is that it was completely abolished by preincubation of the neurons with BDNF. Unlike with peripheral neurons, cultured neurons from the central nervous system are much less dependent on the addition of exogenous neurotrophin for their survival (19). This, together with the availability of large numbers of cells displaying 10 times more BDNF receptors than sensory neurons, made them the ideal object for studying the unexpected phenomenon of desensitization.

To investigate the mechanisms involved in the loss of response to BDNF, we looked for changes at the level of the receptor. The experiments with the granule cells indicate that in cells treated with BDNF, receptor availability decreases dramatically, as indicated by cross-linking experiments. This is not likely due to a massive receptor degradation since cell lysates obtained from treated and untreated cells seemed to contain comparable levels of full-length TrkB protein in Western blot experiments, in contrast with the drastic loss in binding capacity. Internalized intact receptors are also unlikely to account for the loss in binding since BDNF could not be cross-linked to membranes isolated from BDNF-treated cells. What biochemical mechanism underlies the receptor inactivation is currently under investigation.

Interestingly, the phenomenon of desensitization is not observed when similar experiments are performed with TrkB expressed in a cell line able to internalize BDNF at a rate such that ~30% of surface-bound BDNF is internalized within 30 min (27). The conclusion that neuronal receptors for BDNF, while not degraded, become incapable of binding BDNF might also explain recent observations made with embryonic rat cortical and hippocampal neurons (28). A loss of phosphatidylinositol signaling was observed after pre-exposure to BDNF or NT-3. While the physiological significance of BDNF desensitization and receptor inactivation in these neurons is not clear, it is possible that such mechanisms are also observed in vivo. Indeed, in a recent study, it was observed that during rat development, the phosphorylation of TrkB through BDNF in isolated brain tissues becomes increasingly attenuated to become only marginal in adult animals (29). This could reflect prolonged exposure of neurons to BDNF in vivo since during development, BDNF levels increase from very low levels in early embryonic tissue to substantial levels in the adult brain (see, for example, Refs. 30 and 31). Interestingly, in the study by Knüsel et al. (29), the phosphorylation of TrkB by NGF was shown to still be possible with tissue from adult brain, suggesting that also in vivo, the phenomenon of desensitization to NGF does not occur.

It is interesting to consider the ability of the BDNF receptor to undergo desensitization in the context of recent evidence indicating that BDNF is able to rapidly enhance spontaneous synaptic activity (32). Receptor inactivation provides an efficient means of protecting neurons exposed for prolonged periods to BDNF. In addition, it has been reported that cells expressing truncated TrkB receptors can internalize BDNF (27), thus preventing accumulation of BDNF and avoiding desensitization. Of note in this context is the observation that no truncated variants of the NGF receptor TrkA have been found. Finding the mechanisms by which BDNF receptors lose the ability to bind their ligand will be important in order to understand the conditions under which BDNF could be best applied to adult neurons in vivo.

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Regulation of Ras Activation by Neurotrophins

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