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The same cellular signaling pathways mediate survival in sensory neurons that switch their trophic requirements during development

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Abstract

A distinct subpopulation of rat dorsal root sensory (DRG) neurons, termed P-neurons, switch their trophic requirements for survival during development from nerve growth factor (NGF) at embryonic stages to basic fibroblast growth factor (bFGF) just after birth. We investigated in cultured P-neurons the intracellular signaling pathways mediating survival before and after this switch. The NGF-induced survival was completely blocked by either wortmannin (100 nM) or PD98059 (25–50 nM), which selectively inhibit the phosphatidylinositol 3-kinase-AKT (PI3 kinase-AKT) and mitogen-activated kinase kinase extracellular regulated kinase (MEK-ERKs) pathways, respectively. NGF activated AKT and ERKs in single embryonic P-neurons, as assayed by immunofluorescence of phorphorylated proteins. In concordance with the survival assays, wortmannin and PD98059 blocked AKT and ERKs activation, respectively. Following the trophic switch, bFGF used the same signaling pathways to promote survival of post-natal P-neurons, as either wortmannin or PD98059 blocked its effect. Also, bFGF activated AKT and ERKs in single P-neurons, and this activation was blocked by the same inhibitors. These results strongly suggest that both pathways concurrently mediate the action of NGF and bFGF during embryonic and post-natal periods, respectively. Thus, we report the novel result that the switch in trophic requirements occurs with conservation of the signaling pathways mediating survival.

Keywords: dorsal root ganglion, MEK-ERKs, PD98059, PI3K-AKT, survival, wortmannin.

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During development, the survival of sensory neurons is absolutely dependent upon a constant exposure to growth factors, without which they die via apoptosis (Kaplan and Miller 2000). It has become increasingly evident that some types of sensory (Buchman and Davies 1993; Molliver *et al*. 1997; Enokido *et al*. 1999; Baudet *et al*. 2000; Acosta *et al*. 2001), sympathetic (Birren *et al*. 1993; Davey *et al*. 2000; Enomoto *et al*. 2000; Andres *et al*. 2001), and other neurons (D'Mello *et al*. 1993; Kubo *et al*. 1995; Bhave *et al*. 1999; Klocker *et al*. 2000; Linseman *et al*. 2002) require more than one trophic factor either sequentially or simultaneously. The need of more than one trophic factor has been shown both *in vitro* and *in vivo*, and also implied by works in null mutants for neurotrophins or their receptors (Davies 1994; White *et al*. 1996; Liebl *et al*. 1997; Forgie *et al*. 2000; Huang and Reichardt 2001).

We are interested in analyzing possible patterns of cellular signaling mediating survival in neurons that switch their requirement from one specific factor to another during development. This question is important to understand the complex interplay of elements involved in the interaction of developing neurons with survival-inducing clues available

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Abbreviations used: anti-p-Akt, antiphospho-Akt antibodies; anti-p-ERK, antiphospho-ERK antibodies; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; ERK, extracellular-regulated kinase; FITC, fluorescein isothyocianate; FRS2α, fibroblast growth factor receptor substrate; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; MEM 10, Eagle minimal essential medium supplemented with 10% fetal bovine serum; NGF, nerve growth factor; PD98059, 2-(2′-amino-3′-methoxyphenyl) oxanaphthalen-4-one; PI3-kinase, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulfate; SIT, silicon intensified target; TBST, Tris-buffered saline–Tween.

either freely (general extracellular space) or from restricted domains (e.g. innervation targets). Among the possibilities, neurons may simply change the membrane receptors sensing the trophic molecules, keeping unaltered the intracellular signaling machinery, or the switch may also involve important changes downstream the receptors. To explore these two broad possibilities, we took advantage of a well characterized subpopulation of rat sensory neurons that switch their trophic requirements at birth. These neurons, termed P-neurons, sequentially require nerve growth factor (NGF) at embryonic stages and basic fibroblast growth factor (bFGF) at early post-natal stages (Acosta *et al*. 2001). Furthermore, they express the NGF (TrkA) but not the bGFG (flg) receptor at embryonic stages whereas the opposite occurs early after birth. It is presently unknown, however, what signaling pathways mediate the NGF and bFGF trophic effects and whether the intervening pathways change after the trophic switch.

Two signaling pathways which mediate neuronal survival through TrkA or flg in several cell types have been investigated here, namely the phosphatidylinositol 3 kinase-AKT (PI3 kinase-AKT) and the mitogen-activated kinase kinase extracellular regulated kinase (MEK-ERKs) pathways (Kaplan and Miller 2000). Which of these pathways is involved depends on the apoptotic stimulus, and experimental conditions (Yao and Cooper 1995; Crowder and Freeman 1998; Anderson and Tolkovsky 1999; Desire *et al*. 2000). By using *in vitro* survival experiments and single-cell immunofluorescence in the presence of selective pharmacological inhibitors, we found that NGF as well as bFGF use both pathways at embryonic and early post-natal stages, respectively. Thus, this report shows for the first time a pattern of signaling mediating survival in which two different trophic factors use the same pathways in neurons that switch their trophic requirements during development.

Materials and methods

All procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* of the Society for Neuroscience.

Reagents and antibodies

Rat tail collagen type I was from Biomedical Technologies (Stoughton, MA, USA) and the enzymes used for tissue dissociation were from Worthington (Lakewood, NJ, USA). 2′-Amino-3′-methoxyflavone (PD98059), and wortmannin were obtained from Calbiochem (La Jolla, CA, USA). LY294002 and U0126 were obtained from Cell Signaling Technology (Beverly, MA, USA). Minimum essential medium Eagle and Hanks balanced salts and poly-D-lysine were obtained from Sigma (St Louis, MO, USA). Fetal bovine serum was from Natocord (Cordoba, Argentina). Mouse NGF7S and human bFGF were from Alomone Laboratories (Jerusalem, Israel). Stock solution of NGF (20 ng/μL) and bFGF (5 ng/μL) were prepared in sterile water; aliquots were maintained at − 70°C. Anti-pS^{473} AKT pAb was obtained from Promega (Madison, WI, USA) and Cell Signaling. Anti-phospho ERKs Santa Cruz (Santa Cruz, CA, USA). Anti-α-tubulin Sigma. ExtrAvidin Peroxidase Staining Kit Extra-2,Sigma.

Cell culture

The embryonic day 18 and post-natal day 5 were abbreviated as E18, and P5, respectively. Sensory neurons from rat DRGs were isolated as described previously (Acosta and Lopez 1999). Briefly, embryonic or post-natal DRGs were enzymatically dissociated by incubating the tissue for 15–30 min at 37°C with 0.1% trypsin and 0.02% collagenase, or 0.1% trypsin and 0.1% collagenase, respectively. The enzymatic activity was halted by adding 1 mL of Eagle minimal essential medium supplemented with 10% fetal bovine serum (MEM10). After centrifugation at 326 *g* (5 min), the pellet was resuspended in MEM10 containing NGF (50 ng/mL) or bFGF (20 ng/mL). A final step of cell dissociation was performed mechanically by passing the material through Pasteur pipettes of increasingly smaller tip diameters. Approximately, 70 μL of the cell suspension was plated on coverslips coated with 0.25% collagen and 0.05% poly-D-lysine. E18 cultures were grown on poly-D-lysine alone (1 mg/mL, approx. 300 ng/mm^2) because the neurons showed some tendency to detach from the mixed substrate. No differences were found in the survival of post-natal neurons grown in any of those substrates. Plating cell density was standardized, using a Neubauer chamber, to approximately 10^4 cells/mL. The coverslips were placed in an incubator (36°C, 5% CO_2) for 1–2 h to allow for cell adhesion. The cells were kept in different media, depending on the particular experiment, as specified in the Results section.

The effect of NGF and bFGF on cell survival, and the signal transduction pathways involved, were examined by adding to the culture dishes MEM10 alone or supplemented with trophic factors in presence or absence of specific inhibitors of PI3 kinase-AKT and MEK-ERKs pathways, until reaching a volume of approximately 2 mL. After keeping the cultures in those conditions for 24 h to permit the stabilization of neuronal number and morphological phenotypes, we performed the first neuronal counting, which was defined as the initial condition in all survival assays. Immediately after the first counting, the MEM10 was completely replaced by defined media N2 alone (control groups) or supplemented with NGF for E18 or bFGF for P5 cells, with or without the pharmacological inhibitors of the MEK (PD98059, at a final concentration of 25 μM for E18 and 50 μM for P5) and/or the PI3-kinase (wortmannin, 100 nM). The concentration of PD98059 used in embryonic neurons was 25 μM because the dimethyl sulfoxide (DMSO) concentration required to dissolve PD98059 at a 50-μM concentration tended to damage the neurons. This range of concentrations (25–50 μM) is within values that selectively inhibited the phosphorylation of the target pathways in different cell types (Klesse and Parada 1998; Vaillant *et al*. 1999; Tsui-Pierchala *et al*. 2000). Half of the media were replaced every 24 h, and a fresh aliquot of trophic factors was added daily to the media. A 75-mM stock of PD98059, and a 200-μM stock of wortmannin in DMSO were diluted into culture medium immediately before use. Because of its reported liability (Kimura *et al*. 1994; Anderson and Tolkovsky 1999) fresh inhibitors were added every 12 h by replacing the appropriate dish volume. Similarly, a 50-mM stock of LY294002 and a 10-mM stock of U0126 in DMSO were diluted into culture medium immediately before use. These inhibitors were first added when plating the cells,

and then a fresh dose added daily. The stock of the inhibitors were stored in light-proof containers at − 20°C until used. DMSO alone, at the concentration used with the inhibitors, was added to control cultures run along with the pharmacological tests in order to control for an eventual effect of the vehicle. For example, when test cultures were treated with 50 μM PD98059 dissolved in 1.5% DMSO, the control cultures were treated with 1.5% DMSO. The cultures consisted of a mixed population of neuronal and non-neuronal cells. With every change of media, β-arabinocytofuranoside was added (5 and 10 μM, for E18 and P5 cultures, respectively) to eliminate dividing fibroblasts. Penicillin–streptomycin (150 U/150 μg/mL, respectively) was always included in the media. In the case of DRGs P5 cultures, the dissociated ganglia were passed throughout a 20% Percoll gradient by centrifugation at 400 *g* (6–8 min) to reduce the fibroblast population.

Evaluation of survival

Neuronal survival was assessed in cultures grown on etched grid coverslips from Bellco Glass Co. (Vineland, NJ, USA), whose alphanumeric co-ordinates allowed counting the cells within an identified region of the culture. A minimum of 400 neurons were counted in each replication of the experiments. Taking advantage of their relative small fractional contribution to the culture population, the survival of each individual P-neuron was followed and recorded. This method has been successfully used in a previous work to accurately assess the survival of this subpopulation of neurons (Acosta *et al*. 2001). Survival was estimated as the average percentage (± SEM) of cells remaining alive, relative to their number at the initial cell counting. The neurons were counted daily during 3–4 days *in vitro* (DIV). Dying cell were recognized on the basis of signs such as pyknosis, shrinkage and fragmentation, membrane disruption, loss of adhesion to the substratum or complete lysis. Because, as noted above, the morphological integrity of each individual P-neuron was recorded, the chances of mistakenly count a live cell as dead, or vice versa, were negligible. P-neurons never became round over the entire duration of the experiment. Very few neurons identified as round at the time of the first counting became pear-shaped by the time of the second counting (48 h after plating). Those cells were counted as P-neurons, and might have caused, at the most, an overestimation of survival of 10%. The number of replications of the experiments is stated in the Results section or figure legends. The results were analyzed statistically using a two-way repeated measures ANOVA test (one factor repetition, treatment × DIV). The percentage of P-neuron survival was the dependent variable, and the repeated measures were provided by the survival percentage at each consecutive DIV. The interaction between factors was evaluated with multiple comparison tests (post-hoc analysis with the Fisher test; $p = 0.05$). For E18 and P5, the interaction values were $F_{4,9} = 6.53$, $p = 0.01$ and $F_{3,17} = 6.14$, $p = 0.006$, respectively.

Inmunocytochemistry

The activation of PI3 kinase-AKT and MEK-ERKs transduction pathways by NGF or bFGF was evaluated in single P-neurons by imunocytochemistry. The protocol is described in Figs 3 and 5. Briefly, after a period of trophic support deprivation, the cells were treated with NGF (E18 neurons) or bFGF (P5 neurons), and subsequently fixed at different times post trophic treatment. This protocol was carried out in the absence and presence of the pharmacological inhibitors. The cells were washed for 5 min with phosphate-buffered saline (PBS) at 37°C, and then fixed with 4% paraformaldehyde-sucrose for 30 min at room temperature, and washed again for 5 min with PBS. Subsequently, the cells were permeabilized and non-specific binding sites were blocked with blocking-solution [Triton X-100 0,03%; bovine serum albumin (BSA) 1%; bovine fetal serum 10%] during 1 h at room temperature. The coverslips were covered with primary antibodies in blocking buffer for approximately 18 h at 4°C. We used primary antibodies that selectively labeled the phosphorylated forms (catalytically activated) of AKT (antibody p-AKT) and ERKs (antibody p-ERKs), at a 1 : 50 dilution. This dilution yielded the same number of labeled neurons as lower dilutions, with a minor loss of fluorescence intensity. The primary antibodies were washed three times with PBS and the cells incubated at room temperature for 1 h with the corresponding secondary antibodies diluted in 1% BSA, conjugated with fluorescein isothyocianate (FITC). Finally, the cells were washed three times, and the coverslips mounted on glasses with FluorSave (Calbiochem, La Jolla, CA, USA). Controls included cell cultures incubated with the secondary antibody, but in the absence of primary antibody, to check for unspecific labeling with the former. These cells displayed significantly less immunofluorescence than those incubated with the primary antibody.

Images were acquired using a epifluorescence microscope (Olympus BX System Attachment Model BX-FLA, Shibuya-Ku, Tokyo, Japan) by means of a silicon intensified target (SIT) camera (Hamamatsu, Middlesex, NJ, USA), set for manual sensitivity, gain, and black level. They were digitized directly into a miroVIDEO DC30 plus control (Pinnacle Systems, Mountain View, CA, USA) controlled by a host computer. Fluorescence intensity measurements were performed pixel by pixel on identified neurons. Using these data, we then calculated the average fluorescence intensity within the cell body by Mocha™ (Jandel Scientific, San Rafael, CA, USA). In each experiment, the fluorescence data were normalized to the basal control fluorescence of the same experiment. An arbitrary value of 1 was assigned to the media of fluorescence intensity of control condition cells. The results were analyzed statistically using Kruskal–Wallis one-way analysis of variance on ranks test. The difference between the average fluorescence intensity in the various treatment and control groups was evaluated with multiple comparison tests (Dunn's method; $p < 0.05$).

Western blot analysis

The specific blockade of the activity of PI3 kinase-AKT and MEK-ERKs by the pharmacological inhibitors was confirmed with inmunoblots. Cultures used for western analysis had higher density to allow biochemical analysis. The P5 DRGs cells of three 35-mm tissue culture dish were washed with cold PBS, and lysed in 70 μL ice-cold lysis buffer RIPA [150 mM NaCl, 50 mM Tris, pH 8.0, deoxycholic acid 0.5%, sodium dodecyl sulfate (SDS) 0.1% and Triton X-100 1%] containing 5 mM EGTA, 5 mM EDTA, 1 mM fenantrolin, 40 μM benzamidine, protease [2 μg/mL aproptin, 1 μg/mL pepstatin, 2 μg/mL leupeptin, 100 μg/mL phenylmethylsulphonyl fluoride (PMSF)] and phosphatase inhibitors (50 mM NaF, 1 mM sodium orthovanadate). Samples were centrifuged at 8165 *g* (15 min), the supernatant recovered and kept at − 20°C. Cell extracts were resolved on 10% polyacrylamide–SDS gels before

electroblotting onto nitrocellulose membrane. Blots were washed in Tris-buffered saline–Tween (TBST), blocked with BSA 5% for 1 h at room temperature, and incubated overnight at 4°C with primary antibodies dissolved in TBST (p-ERKs 1 : 200; pS^{473} AKT 1 : 750; Anti-α-tubulin 1 : 750). After incubation with ExtrAvidin Peroxidase Staining Kit, inmunoreactive bands were visualized by chemiluminescence and exposure to Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA).

Results

Pharmacological specificity of AKT and ERKs inhibitors

We tested whether the PI3 kinase-AKT and MEK-ERKs signaling pathways mediated the survival promoting effects of NGF or bFGF on P-neurons by examining if the inhibition of those pathways prevented the effect of the trophic factors. To inhibit the PI3 kinase-AKT and MEK-ERKs pathways, we used wortmannin and PD98059, respectively. Whereas such agents proved to be efficient and selective inhibitors (Davies *et al*. 2000), those properties were confirmed here prior to running the survival experiments. Because phosphorylation of AKT and ERKs reflect the activation of the PI3 kinase-AKT and MEK-ERKs pathways, respectively (Sturgill *et al*. 1988; Payne *et al*. 1991; Alessi *et al*. 1996), our test consisted of measuring how potently and selectively wortmannin (100 nM) and PD98059 (50 μM) inhibited, in DRG cultures, the phosphorylation of AKT and ERKs induced by a strong trophic stimulus intended to maximally activate the enzymes (Fig. 1). Such stimulus consisted of a combination of NGF (50 ng/mL), bFGF (20 ng/mL), and 10% serum (NGF/bFGF/serum). As P-neurons represent a modest fraction of the *in vitro* DRG population (5%), the assay was carried out on the entire DRG neuronal population. Proteins were analyzed by western blot using antybodies that selectively labeled the phosphorylated forms (catalytically activated) of ERKs and AKT.

DRG neurons from P5 animals, kept in defined medium (MEM) for 24 h in the absence (control) or presence of one or the other inhibitor, were subsequently challenged with the trophic stimulus for 1 h. In the absence of trophic stimulation (MEM throughout, first column in Fig. 1), AKT phosphorylation was very low, probably reflecting a weak basal activity of the PI3 kinase-AKT pathway in these conditions, and no phosphorylation of ERKs was detectable. A similar AKT basal activity was observed by others in different cell type (Hetman *et al*. 1999; Han and Holtzman 2000). The trophic stimulus induced a marked increase in the expression of phosphorylated AKT and ERKs (Fig. 1, lane 2). wortmannin strongly inhibited the phosphorylation of AKT without affecting the level of ERKs phosphorylation (Fig. 1, lane 3). In contrast, PD98059 inhibited the ERKs, but not AKT, phosphorylation (Fig. 1, lane 4). The lack of complete inhibition of ERKs phosphorylation by PD98059 might be related to the decrease in its effectiveness in the presence of serum (Virdee and Tolkovsky 1996). These data showed that the effects of wortmannin and PD98059 were specific for the PI3 kinase-AKT and the MEK-ERKs pathways, respectively.

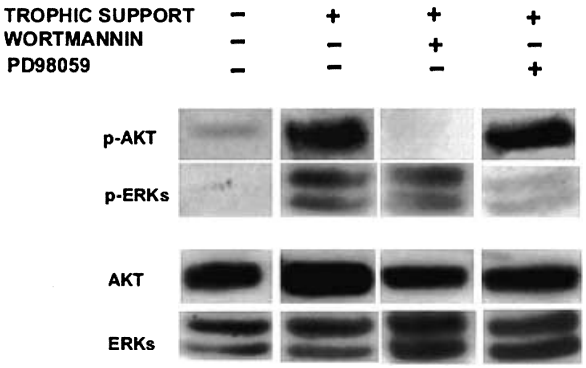

Fig. 1 Wortmannin and PD98059 specifically inhibited the phosphorylation of AKT and ERKs in DRG neurons. Western blot analysis of the expression levels of phosphorylated AKT and ERKs in lysates from cultured DRG neurons in response to a strong trophic stimulus (50 ng/mL NGF, 20 ng/mL bFGF, 10% serum). The levels of phosphorylation were estimated using antibodies that specifically recognize the phosphorylated forms of the enzymes (p-AKT and p-ERKs). As a control level the same membrane was stripped and reprobed with anti-Akt and anti ERKs. Each lane had a similar amount of protein, as indicated by the expression levels of total AKT and ERKs (bottom row). Lane 1 (control, defined media), minor and total lack of p-AKT and p-ERKs expression, respectively; lane 2, the trophic support increased p-AKT expression and induced a dramatic enhancement of p-ERKs expression; lane 3, 100 nM wortmannin completely blocked the trophic-induced increase in p-AKT but had no effect on p-ERKs expression; lane 4, PD98059 greatly blocked the increase in p-ERKs expression induced by the trophic stimulus, withouth changing the p-AKT expression levels. Similar results were obtained in two separate experiments.

PI3-kinase-AKT and MEK-ERKs mediate the NGF-induced survival of embryonic P-neurons

These experiments examined the effect of wortmannin and PD98059 on the NGF supported survival of E18 P-neurons *in vitro*. Cultures were plated and kept in MEM10 plus NGF for 24 h; then this media was replaced with defined media alone (control) or supplemented as follows: (i) NGF (50 ng/mL) alone; (ii) NGF plus wortmannin (100 nM); (iii) NGF plus PD98059 (25 μM); (iv) NGF plus wortmannin and PD98059. Survival was followed for 3 days, during which the media containing the inhibitors was replaced every 12 h to ensure a sustained suppression of PI3- and MEK kinase activities (Anderson and Tolkovsky 1999; Bhave *et al*. 1999). The unmistakable morphology of P-neurons, together with the alphanumeric-coded grid of the coverslips onto which the cells were plated, allowed us to follow each single P-neuron in the culture during the experiment. These experiments were run

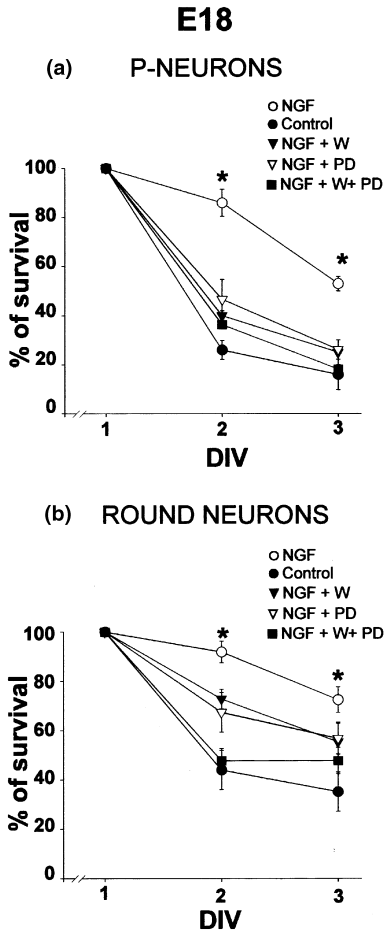

Fig. 2 Wortmannin or PD98059 completely inhibited the survival promoting effect of NGF on E18 P-neurons. Average percentage (± SEM) of surviving P-neurons (a) or round neurons (b) as a function of DIV, relative to their number at 1 DIV (taken as 100%), in cultures established from rats of ages E18. The cultures were maintained in defined media alone (●) or defined media supplemented with 50 ng/mL NGF (○), NGF + Wortmannin (100 nM; ▼), NGF + PD98059 (25 μM; ▽), and NGF + wortmannin + PD98059 (■). (a) Either wortmannin alone or PD98059 alone completely inhibited the NGF-induced survival of E18 P-neurons. The combined application of both inhibitors (wortmannin + PD98059) caused a similar effect as that obtained with either agent alone. Each plot contains data from two experiments, and about 20–100 neurons were counted in each experimental condition. (b) Each inhibitor alone only modestly affected the survival of round neurons, but together significantly blocked the NGF effect at 2 DIV. Each plot contains data from two experiments, with about 450 neurons counted in each experimental condition. Analysis of the data at 2–3 DIV (ANOVA, and Fisher LSD *post-hoc* test; $p < 0.01$ for P-neurons, and $p < 0.05$ for round neurons) revealed statistically significant differences (*) between the NGF-treated cells and the others treatments in P-neurons, and between the NGF-treated cells and NGF + wortmannin + PD98059 or control (at DIV 2), and only the control condition (at DIV 3) in round neurons.

on different dishes from the same culture to allow a reliable comparison of the results across conditions.

As shown in Fig. 2(a), the survival of E18 P-neurons was greatly enhanced by NGF (50 ng/mL), confirming previous observations (Acosta *et al*. 2001). In the presence of NGF, the survival of P-neurons at 2 and 3 DIV were 88.5 ± 0.5% and 55 + 0.8, respectively, and significantly lower in the absence of the factor. Either wortmannin alone or PD98059 alone completely inhibited the effect of NGF on survival, which did not differ from control with any of those agents. The effect of wortmannin and PD98059 combined did not exceed that of either agent alone. The difference between the survival observed with NGF and any of the other treatments (control or NGF plus inhibitors) was statistically significant over the entire period assayed (2–3 DIV). The fact that wortmannin alone or PD98059 alone completely inhibited the NGF effect suggested that neither pathway alone is sufficient to fully support the effect of NGF on the survival of embryonic P-neurons. This finding appears to further differentiate P-neurons from the round neurons, considered as a whole. Wortmannin alone or PD98059 alone only partially reduced the survival promoting effect of NGF in round neurons (Fig. 2b). Total blockade of survival was attained only by applying both inhibitors. Thus, in round neurons, the individual signaling pathways were able to support to some extent the NGF effect. Most importantly, these differential effects on P- and round neurons rule out an indiscriminate citotoxic effect of the inhibitors. The effects of wortmannin on the trophic-induced survival of P-neurons were replicated with LY294002 (10–50 μM), another inhibitor of the PI3 kinase-AKT pathway. Likewise, the PD98059 effect was replicated with the inhibitor U0126 (10–50 μM), an inhibitor of the MEK-ERKs pathway. In these experiments, survival at 2 and 3 DIV were 33 ± 5% and 31 ± 2% and NGF enhanced survival to 77 ± 6% and 56 ± 2%, whereas survival in the presence of NGF + LY294002 was 39 ± 2% and 25 ± 2%, and in the presence of NGF + PD98059 was 54 ± 2% and 14 ± 1%.

NGF activates AKT and ERKs in individual E18 P-neurons: selective inhibition by wortmannin or PD98059

The experiments described above are consistent with an involvement of the PI3 kinase-AKT and MEK-ERKs signaling pathways in the NGF-induced survival of P-neurons, in agreement with data in other cell types (Anderson and Tolkovsky 1999; Wert and Palfrey 2000). To test this possibility further, we tested whether NGF was able to activate those pathways in single P-neurons and, if so, whether wortmannin or PD98059 could inhibit that effect. Activation of the PI3 kinase-AKT and MEK-ERKs pathways was evaluated with antibodies that recognized the phosphorylated forms of AKT or ERKs (Tsui-Pierchala *et al*. 2000).

The experimental scheme was as follows: after an initial period (5 h) of incubation of embryonic DRG neurons in

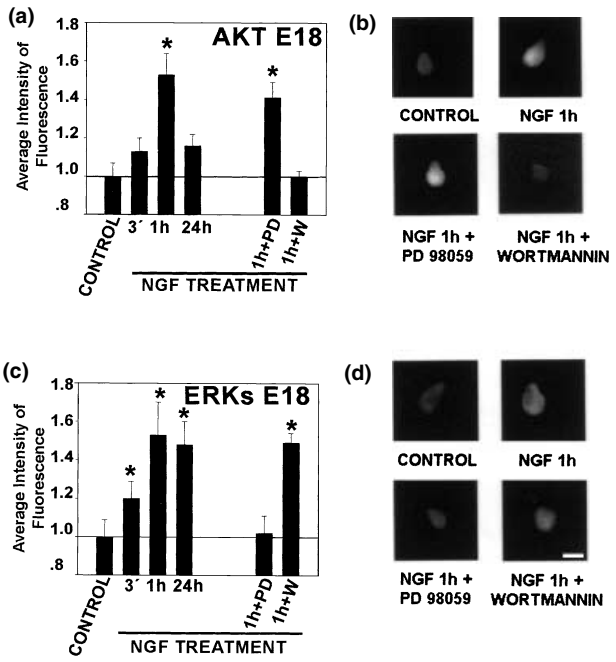

Fig. 3 AKT and ERKs activation by NGF in individual E18 P-neurons. Selective inhibition by wortmannin and PD98059. (a) Fluorescence intensity in individual P-neurons reflecting binding of antibodies that specifically react with the phosphorylated (activated) form of AKT. The bar plot shows the average fluorescence intensity at different treatment (± SEM). The number of experiments and neurons examined for each experimental condition were 2–3 and 10–35, respectively. The average fluorescence intensity clearly increased after 1 h of NGF treatment. Wortmannin (100 nM), but not PD98059 (25 μM), completely blocked the effect of NGF (rightmost bars). (b) Representative examples of individual P-neurons from the experiments shown in (a). (c) Fluorescence intensity in individual P-neurons reflecting binding of antibodies that specifically react with the phosphorylated (activated) form of ERKs. An increase in fluorescence intensity was detected 3 min after NGF treatment, and continued for 24 h. PD98059 (25 μM), but not wortmannin (100 nM), fully blocked the effect of NGF. The number of experiments and neurons examined for each experimental condition were 3 and 10–45, respectively. (d) Representative examples of individual P-neurons from the experiments shown in (c). Analysis of the data at different treatment (Kruskal–Wallis and Dunn's *post-hoc* test; $p < 0.05$) indicated significant differences (*) between each treatment respect to the control condition. The inhibitors were present throughout the experiment. Scale bar indicates 12 μm.

MEM10 plus NGF, the cultures were deprived from trophic support for 19 h, and subsequently treated with NGF for various times. The first period (plating and attachment of cells to the substrate) included trophic support to prevent an excessive cell death before running the rest of the experimental protocol. The period of trophic deprivation minimized the basal AKT and ERKs levels of phosphorylation, thus setting a non-stimulated background level against which the NGF effect could be contrasted. Work by others showed that ERKs activity fell to very low levels after a deprivation of NGF for 6 h in PC12 cells (Xia *et al*. 1995). A single dose of NGF (50 ng/mL) in serum-free media was added, and the cells fixed at 3 min, 1 h or 24 h. NGF induced a statistically significant increase in AKT phosphorylation 1 h after its addition (Fig. 3a). The photograph in Fig. 3(b) shows an example of the NGF effect in a single embryonic P-neuron. No increase in fluorescence was found in cells which continued deprived of NGF (control). These results indicate that NGF activated the PI3 kinase-AKT pathway in embryonic P-neurons. Wortmannin completely blocked the AKT activation induced by NGF, whereas PD98059 had no effect. These results further support the specificity of the pharmacological agents. NGF also induced a statistically significant increase in ERKs phosphorylation (Fig. 3c) that reached a maximum at 1 h and remained stable up to 24 h. The increase in ERKs phosphorylation was fully blocked by PD98059 and unaffected by wortmannin, again showing the selective action of those inhibitors. Figure 3(d) shows examples of single embryonic P-neurons displaying NGF-induced ERK phosphorylation and its selective inhibition by PD98059.

These findings clearly show that (i) NGF is capable of activating AKT and ERKs in single embryonic P-neurons, and (ii) the pharmacological inhibitors that blocked the NGF-induced survival in embryonic P-neurons were also capable of inhibiting the activation of the PI3 kinase-AKT and MEK-ERKs signaling pathways by the neurotrophin. These correlations suggest that those pathways could be involved in the NGF-induced survival of P-neurons during embryonic stages.

PI3-kinase-AKT and MEK-ERKs mediate the bFGF-induced survival of post-natal P-neurons

Post-natal P-neurons required bFGF to survive *in vitro*. In the presence of bFGF (20 ng/mL), 68 ± 8.2% of P5 P-neurons survived at 3 DIV, while only about 31% of the cells survived without the factor (Fig. 4a). This series of experiments examined the effect of wortmannin and PD98059 on the bFGF-supported survival of P5 P-neurons *in vitro*. Cultures were plated and kept in MEM10 for 24 h; then this media was replaced with defined media alone (control) or supplemented as follows: (i) bFGF (20 ng/mL) alone; (ii) bFGF plus wortmannin (100 nM); (iii) bFGF plus PD98059 (50 μM); (iv) bFGF plus wortmannin and PD98059. Either wortmannin or PD98059 blocked this survival-promoting effect of bFGF, indicating that the PI3 kinase-AKT and MEK-ERKs signaling could be involved. In control conditions (no trophic support), the rate of death of post-natal P-neuron was slower than of embryonic P-neurons: at 2 DIV, about 60% of the former survived and only about 20% of the latter. As a result, the effect of the inhibitors became statistically significant at 3 DIV. The survival of P-neurons treated with wortmannin or PD98059 in the absence of bFGF was similar to control (data not shown). Similarly to embryonic neurons, the

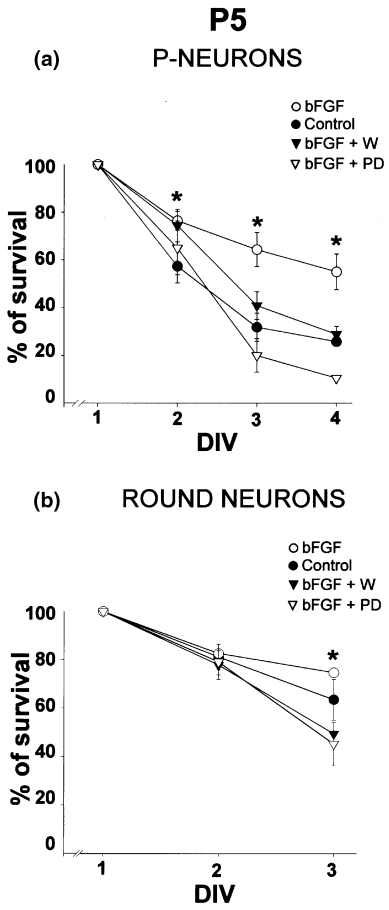

Fig. 4 Wortmannin and PD98059 inhibit the survival of P5 P-neurons induced by bFGF. Average percentage (± SEM) of surviving P-neurons (a) or round neurons (b) as a function of DIV, relative to their number at 1 DIV (taken as 100%), in cultures established from rats of ages P5. The cultures were maintained in defined media alone (●) or defined media supplemented with 20 ng/mL bFGF (○), bFGF + Wortmannin (100 nM; ▼), bFGF + PD98059 (50 μM; ▽). (a) Either wortmannin alone or PD98059 alone completely inhibited the bFGF-induced survival of P5 P-neurons. Each plot contains data from three experiments, and about 50–100 neurons were counted for each experimental condition. Analysis of the data at 2–4 DIV (ANOVA, and Fisher LSD *post-hoc* test; $p < 0.04$) indicated significant statistical differences (*) between the bFGF treatment and control at DIV 2; between bFGF treatment and control, or bFGF + wortmannin, or bFGF + PD98059 at DIV 3; and between bFGF treatment and bFGF + PD98059 at DIV 4. (b) Similar experiment as in (a) with P5 round sensory neurons, whose survival was barely affected by bFGF. A modest effect of wortmannin and PD98059 was observed at 3 DIV. Each plot contains data from three experiments, and about 300 neurons were counted for each experimental condition. The asterisk at DIV 3 indicate significant statistical differences between the bFGF or the control treatment versus bFGF + wortmannin or bFGF + PD98059.

effects of wortmannin and PD98059 were replicated with LY294002 (50 μM) and U0126 (50 μM), inhibitors of the PI3 kinase-AKT and MEK-ERKs pathways, respectively. In these experiments, survival at 2 and 3 DIV were 46 + 3% and 38 + 2%, and bFGF enhanced survival to 75 + 3% and 51 + 4%, whereas survival in the presence of bFGF + LY294002 was 68 + 4% and 22 + 2%, and in the presence of bFGF + PD98059 was 48 + 3% and 0.0%. In agreement with previous data (Acosta *et al*. 2001), post-natal round neurons did not require bFGF for survival (Fig. 4b). However, treatment with wortmannin or PD98059 reduced the survival of round neurons, suggesting that other signals affecting the survival of post-natal round neurons might use the PI3 kinase-AKT and MEK-ERKs pathways.

BFGF activates AKT and ERKs in individual E18 P-neurons: selective inhibition by wortmannin or PD98059

The inhibition of bFGF-induced P-neuron survival by wortmannin and PD98059, suggested that bFGF could activate the PI3 kinase-AKT and MEK-ERKs pathways. This expectation was confirmed on single P-neurons in experiments analogous to those described in Fig. 3. Addition of bFGF to DRG cultures caused a clear activation of AKT and ERKs in single P5 P-neurons (Figs 5a–c). The increase in fluorescence immunolabeling for phosphorylated AKT or ERKs began at about 3 min after the addition of bFGF (Figs 5a and c). The results were statistically significant at all the time points evaluated for AKT, and at 1 h for ERKs. The increase in AKT phosphorylation induced by bFGF was fully blocked by wortmannin but not by PD98059 (Fig. 5a, rightmost bars), whereas the increase in ERKs phosphorylation was blocked by PD98059 but not wortmannin (Fig. 5c, rightmost bars). The photographs in Figs 5(b and d) show representative single P-neurons corresponding to each of the treatment groups.

These data indicate that (i) bFGF activates AKT and ERKs in single post-natal P-neurons, and (ii) the pharmacological inhibitors that blocked the bFGF-induced survival in post-natal P-neurons selectively inhibited the activation of the PI3 kinase-AKT and MEK-ERKs signaling pathways induced by bFGF. Therefore, and similarly to embryonic P-neurons, those pathways could be involved in the bFGF-induced survival of P-neurons during the early post-natal stage.

Lack of AKT or ERKs activation by NGF or bFGF at times at which these factors do not promote survival

Embryonic P-neurons required NGF but not bFGF to survive, whereas post-natal P-neurons required bFGF but not NGF (data of this work and Acosta *et al*. 2001). In concordance with those results, bFGF failed to induce an increase in AKT or ERKs phosphorylation in embryonic P-neurons (Fig. 6a). Analogously, NGF did not activate AKT and ERKs post-natal P-neurons (Fig. 6b). Although such lack of effects was actually expected as embryonic P-neurons express NGF but not bFGF receptors while post-natal P-neurons express bFGF but not NGF receptors (Acosta *et al*. 2001), it was important to run the experiments to

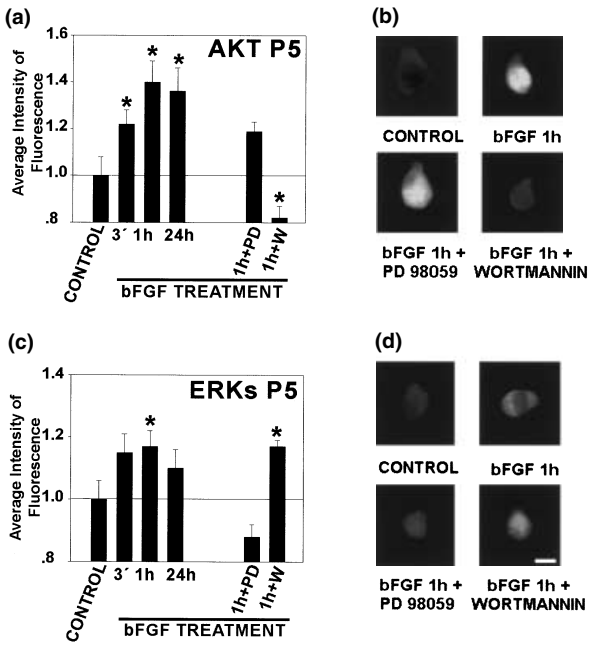

Fig. 5 AKT and ERKs activation by bFGF in individual P5 P-neurons. Selective inhibition by wortmannin and PD98059. (a) Fluorescence intensity in individual P-neurons reflecting binding of antibodies that specifically react with the phosphorylated (activated) form of AKT. The average fluorescence intensity increased as early as 3 min after bFGF treatment. Wortmannin (100 nM), but not PD98059 (50 μM), completely blocked the effect of bFGF (rightmost bars). The number of experiments and neurons examined were 2–5 and 11–54, respectively. (b) Representative examples of individual P-neurons from the experiments shown in (a). (c) Fluorescence intensity in individual P-neurons reflecting binding of antibodies that specifically react with the phosphorylated (activated) form of ERKs. Fluorescence intensity increase after 3 min of bFGF treatment, and stayed elevated for several hours. PD98059 (50 μM), but not wortmannin (100 nM), fully blocked the effect of bFGF (rightmost bars). The number of experiments and neurons examined were 2–5 and 10–47, respectively. (d) Representative examples of individual P-neurons from the experiments shown in (c). Analysis of the data at different treatment (Kruskal–Wallis and Dunn's *post-hoc* test; $p < 0.05$) indicated significant differences (*) between each treatment respect to the control condition. Scale bar indicates 11 μm.

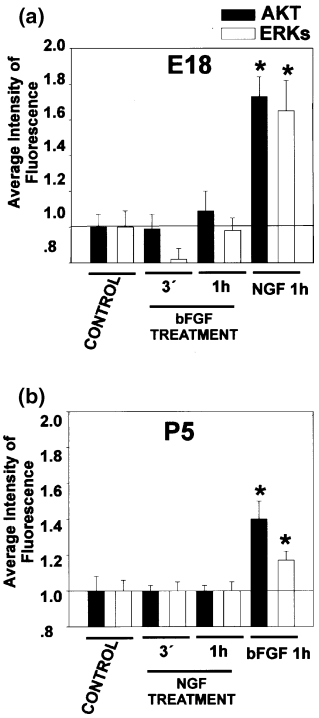

Fig. 6 NGF or bFGF failed to activate AKT (■) or ERKs (□) in P-neurons at ages in which those factors did not promote survival, and coincided with lack of expression of TrkA and flg. (a) Treatment with bFGF for up to 1 h failed to produce AKT or ERKs phosphorylation. Average data from individual E18 P-neurons ($n = 28$–31, 2–3 experiments. In comparison, NGF induced a clear activation of both enzymes in P-neurons of the same cultures ($n = 35$, three experiments). (b) Treatment with NGF for up to 1 h failed to produce AKT or ERKs phosphorylation. Average data from individual P5 P-neurons ($n = 19$–47, 2–3 experiments). In comparison, bFGF induced a clear activation of both enzymes in P-neurons of the same cultures ($n = 12$). Analysis of the data at different treatment (Kruskal–Wallis and Dunn's *post-hoc* test; $p < 0.05$) indicated significant differences (*) between each treatment respect to the control condition.

further control for the specific activation of AKT and ERKs by each trophic factor at the appropriate developmental stages. Moreover, we determined in the same batch of cultures that NGF and bFGF were indeed capable of inducing phosphorylation of AKT and ERKs when applied to embryonic and post-natal P-neurons, respectively (rightmost bars in each plot of Fig. 6), thus confirming the results previously shown in Figs 3 and 5.

Discussion

In this work we have studied the signaling pathways that mediate the survival promoting effects of NGF and bFGF in a defined subpopulation of sensory neurons (termed P-neurons) that sequentially require those factors during development. The most important findings of this study is that either NGF pre-natally or bFGF post-natally use both the PI3 kinase-AKT and MEK-ERKs pathways to induce survival of P-neurons. The data are consistent with the need of a concurrent activation of those pathways so that neither pathway alone can support survival in the presence of any of the factors. As the same pathways participate before and after the switch of trophic dependence, our results reveal a pattern of signaling in which the same cellular machinery is used by two different receptors sensing the survival environmental signals. As far as we are aware, this is the first report of two trophic factors using the same pathways to sequentially support the survival of a single class of sensory neurons during development.

The unequivocal identification of P-neurons in culture permitted both assessing the switch in survival dependence and examine the signaling pathways in individually identified single cells belonging to a distinct subpopulation of sensory neurons. Thus, the observations could be rigorously assigned to the neuronal group that undergoes the trophic switch. The survival of P-neurons and round neurons in the same culture was affected in different degrees by wortmannin and PD98059, indicating that those inhibitors did not prevent survival effects of the trophic factors in an indiscriminate way. For example, wortmannin alone or PD98059 alone do not significantly inhibit the survival promoting effect of NGF on embryonic round neurons, whereas either inhibitor by itself completely blocks the NGF effect on the P-neuron survival.

As for the signaling pattern observed in our case of trophic switch, the data indicate that any signaling mechanism accounting for this switch in P-neurons must satisfy the following: (i) a single receptor type (TrkA pre-natally or flg post-natally) concurrently activates the PI3 kinase-AKT and MEK-ERKs pathways; (ii) the concurrent activation of those pathways is required to support survival; (iii) inhibition of one of the pathways does not prevent the activation of the other. Several signaling schemes are compatible with those observations. We consider two simple possibilities. TrkA and flg may interact with a common protein coupling those receptors to the PI3 kinase-AKT and MEK-ERKs pathways. In support of this view, recent reports demonstrate that TrkA and flg interact at the membrane level with fibroblast growth factor receptor substrate (FRS2α), a protein that leads to concurrent activation of PI3 kinase-AKT and MEK-ERKs via membrane bound multidocking protein complexes (Ong *et al*. 2000, 2001; Hadari *et al*. 2001). Alternatively, the PI3 kinase-AKT and MEK-ERKs pathways may be activated separately (Obermeier *et al*. 1993; Crowder and Freeman 1998; Qian *et al*. 1998; Wert and Palfrey 2000; Chaudhary and Hruska 2001). In either case, the concurrently activated pathways would eventually converge on some downstream effector in order to induce survival. For example, the activation of the PI3 kinase-AKT and MEK-ERKs pathways by ligands that induce survival of cerebellar granule cells leads to phosphorylation of the protein BAD at serine 136 and 112, respectively, thereby inhibiting BAD-induced apoptosis (Datta *et al*. 1997; Bonni *et al*. 1999; Brunet *et al*. 2001). That protein has also been shown to be a downstream effector for NGF or bFGF in several cell types (Miho *et al*. 1999; Roberts *et al*. 2000; Pardo *et al*. 2002).

Our findings are in agreement with recent studies showing that both the PI3 kinase-AKT and MEK-ERKs must be concurrently active to support the antiapoptotic effects of trophic factors, such as brain-derived neurotrophic factor (BNDF) in cerebellar neurons (Bonni *et al*. 1999), tumor necrosis factor-α in osteoclasts (Lee *et al*. 2001), and amyloid precursor protein in hippocampal neurons (Cheng *et al*. 2002). However, P-neurons differ from those examples in that none of them involves a switch in trophic dependence of the type shown here. Furthermore, and most interestingly, in P-neurons two different receptors, expressed at different developmental stages, mediate survival concurrently using both pathways. This pattern of use of the PI3 kinase-AKT and MEK-ERKs pathways adds up to their capacity to individually sustain survival of various cell types (D'Mello *et al*. 1997; Dudek *et al*. 1997; Kauffmann-Zeh *et al*. 1997; Kennedy *et al*. 1997; Crowder and Freeman 1998; Wert and Palfrey 2000). Our conclusion relies on the observation that the inhibition of one of the pathways does not affect the other, as shown in Figs 1, 3 and 5. However, it should be noted that our claim of the concurrent need of both pathways encompasses any possible cross-talk between them (Rommel *et al*. 1999; Mograbi *et al*. 2001; van Rossum *et al*. 2001). Each pathway could have sustained survival to some extent in the presence of some cross-talk input from the other. In this case, such cross-talk input should originate downstream the site of action of the inhibitors.

Following standard procedures in many other reports (Virdee and Tolkovsky 1996; Abe and Saito 2000; Tsui-Pierchala *et al*. 2000), the results with wortmannin and PD98059 on neuronal survival were confirmed with LY294002 and U0126, inhibitors of the PI3 kinase-AKT and MEK-ERKs pathways, respectively. The potentially unspecific targets of each pair of inhibitors differ (Davies *et al*. 2000). Therefore, the fact that the same results were obtained with both inhibitors intended for each signaling pathway, strengthen the conclusion that the PI3 kinase-AKT and MEK-ERKs pathways mediated the NGF- and bFGF-induced survival in P-neurons. It was recently reported that ERK5, a novel member of the ERKs family (Kamakura *et al*. 1999; Cavanaugh *et al*. 2001), is involved in the survival-promoting effect of NGF on sensory neurons when the factor is applied to distal axons, but not to the somas, and that PD98059 blocks that effect (Miller and Kaplan 2001; Watson *et al*. 2001). In our experiments, NGF was required from the time of cell plating, before any axonal processes were present, and PD98059 impaired survival within 2 DIV, time at which there was minimal axonal development, much earlier than the experiments noting NGF action on distal axons of sensory (4 DIV; Watson *et al*. 2001) or sympathetic (12–14 DIV; Tsui-Pierchala and Ginty 1999) neurons. Thus, although a participation of the ERK5 cascade in the trophic dependent survival of P-neurons cannot be excluded, we think that most of the effect involves ERKs 1/2.

Our data suggest a global picture of the signaling machinery supporting the trophic factor-induced survival of P-neurons during early development. A key element of this machinery is the conserved signaling circuitry across pre-natal and early post-natal stages in which two different trophic factors sequentially rescue these sensory neurons from dying. Another important element suggested by our

data is that the exchange of the tyrosine kinase membrane receptors mediating survival could provide an efficient way of sensing two different environmental signals (NGF and bFGF) leading to the same final effect (survival). In this view, the temporal pattern of expression of TrkA and flg will determine which of those trophic factors will be capable of promoting survival using a common signaling module. Previous data indicate that receptor exchange occurs at the time of birth, at which both trophic factors induce similar survival (Acosta *et al*. 2001). It will be interesting to examine whether this novel perspective on the signaling pattern mediating a trophic switch is unique to P-neuron subpopulation of sensory neurons or also occurs in other cells that switch their trophic requirements during development (Birren *et al*. 1993; Davies 1994; Molliver *et al*. 1997; Enokido *et al*. 1999; Baudet *et al*. 2000; Enomoto *et al*. 2000).

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