The BRN-3A Transcription Factor Protects Sensory but Not Sympathetic Neurons from Programmed Cell Death/Apoptosis*

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Inactivation of the gene encoding the POU domain transcription factor BRN-3A results in the absence of specific neurons in knockout mice. Here we demonstrate for the first time a direct effect of BRN-3A on the survival of neuronal cells. Specifically, overexpression of BRN-3A in cultured trigeminal ganglion or dorsal root ganglion sensory neurons enhanced their survival following the withdrawal of nerve growth factor. Moreover, reduction of BRN-3A levels impaired the survival of these neurons. The survival of sympathetic neurons was not affected by either approach. Similarly, overexpression of BRN-3A activated the endogenous Bel-2 gene in trigeminal neurons, but not in sympathetic neurons. The protective effect of BRN-3A on trigeminal neuron survival following nerve growth factor withdrawal significantly increased during embryonic development. In contrast, overexpression of the related factor BRN-3B enhanced survival of trigeminal neurons only at an early stage of embryonic development. Thus, BRN-3A (and in some circumstances, BRN-3B) can promote the survival of nerve growth factor-dependent sensory but not sympathetic neurons, allowing it to play a direct role in the survival of some (but not all) neuronal populations in the developing and adult nervous systems.

The BRN-3 transcription factors are members of the POU family of transcription factors, which was originally defined on the basis of a common 150–160-amino acid DNA-binding domain found in the mammalian transcription factors Pit-1, Oct-1, and Oct-2 and the nematode regulatory protein Unc-86 (for review, see Refs. 1 and 2). All the original founder members of the POU family are expressed in neuronal cells, with Pit-1 playing a key role in the development of the pituitary gland (3), whereas Unc-86 is essential for the development of specific neuronal cell types, particularly sensory neurons in the nematode (4, 5).

Three BRN-3 factors have been defined in mammalian cells, where they constitute the most closely related mammalian factors to Unc-86, with each factor being encoded by a distinct gene (6). The three BRN-3 factors are BRN-3A (also known as BRN-3 or BRN-3.0) (7–9), BRN-3B (also known as BRN-3.2) (9, 11), and BRN-3C (also known as BRN-3.1) (7, 12). These three closely related factors are expressed in distinct but overlapping groups of neurons in the developing and adult nervous systems (7–9, 11, 12), with BRN-3A, for example, defining the earliest post-mitotic neurons to appear in the developing central nervous system (13).

The expression patterns of Brn-3a, Brn-3b, and Brn-3c as well as their relationship to Unc-86, suggest that they may play key roles in the development of specific neuronal cell types in mammals. In agreement with this, inactivation of the gene encoding BRN-3C in knockout mice results in defective inner ear cell function due to the failure to produce sensory hair cells (14), and mutation in the human gene encoding BRN-3C has recently been reported to be the cause of progressive deafness in an Israeli family (15). Similarly, inactivation of the gene encoding BRN-3B results in the loss of ~70% of retinal ganglion cells, with little effect on other neurons (14, 16).

In contrast to the relatively organ-specific effects of inactivation of Brn-3b or Brn-3c, inactivation of BRN-3A causes widespread losses of specific populations of motor neurons and of proprioceptive, mechanoreceptive, and nociceptive sensory neurons. This results in mice with uncoordinated limb movement and impaired suckling that die shortly after birth (17, 18). This widespread role for BRN-3A is paralleled by its ability to activate the expression of a number of different genes expressed in neuronal cells such as those encoding pro-opiomelanocortin (7), SNAP-25 (19), α-internexin (20), and the neurofilaments (21). In contrast, none of these genes are significantly activated by BRN-3B (19–21).

Interestingly, the losses of neuronal cells in Brn-3a knockout mice have previously been suggested to be due to a loss of expression of specific neurotrophic factors or their receptors (17). In turn, this would evidently disrupt neurotrophin signaling, resulting in a loss of their survival-promoting effects and consequently leading to the observed neuronal cell death. However, no direct effect of BRN-3A on the promoters of genes encoding neurotrophins or their receptors has been reported. We have recently reported that BRN-3A overexpression can activate the promoter of the Bel-2 proto-oncogene ~50-fold in cotransfection assays (22, 23). Similarly, BRN-3A overexpression results in ~15-fold overexpression of BCL-2 protein derived from the endogenous Bel-2 gene in a transfected neuronal cell line (22, 23). These findings raise the possibility that BRN-3A may have a direct effect on the survival of neuronal cells. We have therefore investigated the effect of increasing or decreasing the levels of BRN-3A in specific neuronal cell types on their survival in the presence or absence of neurotrophins.

MATERIALS AND METHODS

Plasmid Constructs—Full-length Brn-3a and Brn-3b cDNA expression vectors have been described elsewhere (22, 23). The BRN-3A-specific antisense construct was generated by polymerase chain reaction (PCR)* using primers GGATCCGCTGCAGAGCAACCTCTTC and GTGCGAGGCGGCGCCAGAGATC, where boldface and under-
lined sequences indicate *Bmor* and *SaIl* restriction enzyme recognition sites, respectively. The 240-base pair PCR product was ligated into the pGEM-T vector (Promega) and subcloned as a *Bmor*/*SaIl* fragment into the mammalian expression vector pJF7.

**Culture of Primary Neurons—**Dorsal root, trigeminal, and superior cervical ganglia were dissected from 7-day-old Sprague-Dawley rat pups at postnatal day 1 or from staged C57 mouse embryos, where the day of finding the vaginal plug was designated embryonic day (E) 0.5. Following dissection, ganglia were incubated for between 5 and 15 min at 37 °C with 0.05% trypsin (Worthington; in calcium- and magnesium-free Hanks’ balanced salt solution). The precise period of incubation in trypsin was dependent on the age of the ganglia. After 2 min of incubation, trypsin was decanted, and the pelleted cells were washed with complete Ham’s F-14 medium supplemented with 2 mM glutamine, 0.35% bovine serum albumin, and 60 mg/ml penicillin, and 100 mg/ml streptomycin. Neurons were plated at a density of 500–1000 neurons in a 50-μl droplet on a 13-mm coverslip in a 24-well plate. Coverslips were precoated with poly-lys-ornithine (0.5 mg/ml overnight) and laminin (20 μg/ml over night). The cells were then left between 5 h and overnight to adhere and were cultured for 24 h. The neurons were incubated at 37.5 °C in a humidified 3.5% CO2 incubator in a defined medium consisting of growth medium and spun again at 15 × g for 5 min at room temperature. Neurons were collected into a pellet, whereas the non-neuronal cells (∼95%) remained in the supernatant. The supernatant was decanted, and the pelleted cells were washed with complete growth medium and spun again at 15 × g for 5 min.

Neurons were cultured as described (25, 26). The purified neurons were plated at a density of 500–1000 neurons in a 50-μl droplet on a 13-mm coverslip in a 24-well plate. Coverslips were precoated with poly-lys-ornithine (0.5 mg/ml overnight) and laminin (20 μg/ml overnight). The cells were then left between 5 h and overnight to adhere and were cultured for 24 h. The neurons were incubated at 37.5 °C in a humidified 3.5% CO2 incubator in a defined medium consisting of Ham’s F-14 medium supplemented with 2 μg/ml growth hormone, 0.35% bovine serum albumin (Pathocyte-4, ICN), 60 mg/ml progesterone, 16 μg/ml putrescine, 400 ng/ml l-thyroxine, 38 μg/ml sodium selenite, 340 ng/ml triiodothyronine, 80 mg/ml penicillin, and 100 μg/ml streptomycin. Neurons were recognized by their bipolar morphology under phase-contrast optics (25, 26).

Prior to transfection, cells were cultured for 24 h in medium supplemented with recombinant nerve growth factor (NGF, Life Technologies, Inc.) at a final concentration of 20 ng/ml. In control experiments, this 24-h incubation was sufficient to select for NGF-dependent neurons. Thus, the resulting cultures showed no enhanced survival when other neurotrophic factors were added together with NGF compared with NGF alone. The following day, 1 μg of pCi expression vector containing the murine long form of cholesterol was formulated into cationic liposomes with dioleoyl-L-α-phosphatidylethanolamine (27). This combination ensured amplification of only the exogenous mRNA from the transfected plasmid (see Fig. 3) were forward primer 5′-CGGGTTGACTGGCAAAA3′ and reverse primer 5′-GGTGGCTTCGGCGTTGC3′. Primers to specifically amplify the exogenous *Brn-3a* mRNA (see Fig. 7) consisted of a forward primer derived from the untranslated region of the expression vector 5′-CTCTGACCTTGAACCTGCT-3′ and a reverse primer specific for a reporter RNA 5′-TGAAGTGCTTGGTCCT-3′. This combination ensured only the exogenous *Brn-3a* mRNA derived from the transfected expression vector, producing a 380-base pair product. The primers for the endogenous *Icl-2* gene were 5′-AGAATCACAGGAGTCTTGCTTCA3′ and 5′-GCTTGAAGTCACCTGGAATA-3′, which produce a 430-base pair product. *Annexin V Labeling—*Cells were fixed in 4% paraformaldehyde for 20 min, washed three times with phosphate-buffered saline, and then placed in annexin buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). 10 μl of a *Annexin V* phycoerythrin (30 μg/ml; R&D Systems) in annexin buffer and 10 units of annexin buffer at a final concentration of 300 μl added to 15 μl of annexin buffer. Cells were then washed twice with phosphate-buffered saline and viewed under a fluorescence microscope. The number of apoptotic nuclei was counted and expressed as a percentage of total nuclei.

**Reverse Transcription-PCR Assay—**The reverse transcription-PCR assay was carried out as described previously using conditions that have been shown to generate a linear relationship between the input mRNA and the signal obtained. The total *Brn-3a* mRNA (amplifying both endogenous *mRNA* and exogenous *mRNA* from the transfected plasmid) (see Fig. 3) were forward primer 5′-CGGGTTGACTGGCAAAA3′ and reverse primer 5′-GGTGGCTTCGGCGTTGC3′. Primers to specifically amplify the exogenous *Brn-3a* mRNA (see Fig. 7) consisted of a forward primer derived from the untranslated region of the expression vector 5′-CTCTGACCTTGAACCTGCT-3′ and a reverse primer specific for a reporter RNA 5′-TGAAGTGCTTGGTCCT-3′. This combination ensured amplification of only the exogenous *Brn-3a* mRNA derived from the transfected expression vector, producing a 380-base pair product. The primers for the endogenous *Icl-2* gene were 5′-AGAATCACAGGAGTCTTGCTTCA3′ and 5′-GCTTGAAGTCACCTGGAATA-3′, which produce a 430-base pair product. *Annexin V Labeling—*Cells were fixed in 4% paraformaldehyde for 20 min, washed three times with phosphate-buffered saline, and then placed in annexin buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). 10 μl of *Annexin V* phycoerythrin (30 μg/ml; R&D Systems) in annexin buffer and 10 units of annexin buffer at a final concentration of 300 μl added to 15 μl of annexin buffer. Cells were then washed twice with phosphate-buffered saline and viewed under a fluorescence microscope. The number of apoptotic nuclei was counted and expressed as a percentage of total nuclei.

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**Statistics—**Single factor ANOVA was used to analyze differences for each group of treatments, with differences among means within each group of treatments being compared using Student’s *t* test. Significance was accepted at the 0.05 level (p < 0.05) in each group of treatments. As a result of multiple comparisons, the Bonferroni method was applied manually to the results of the ANOVA tests. In Bonferroni’s test, to achieve significance, each comparison must show significance well beyond the 0.05 (95% confidence) level. The level of significance required is determined by the following equation: \[ C = k/(2k - 2) \] where *k* is the number of treatments and \( 1 \) is the factorial of the preceding number. The corrected level of significance is determined by dividing 0.05 by *C*.
BRN-3A has previously been shown to be expressed at high levels in trigeminal ganglion neurons (7, 25), and significant losses of such neurons are observed in Brn-3a knockout mice, with the size of the ganglion at E20 being approximately half that in wild-type or heterozygous mice and the dorsal division of the anteromedial lobe of the ganglion being completely absent at postnatal day 0.5 (17, 18). We therefore investigated the effect of overexpressing BRN-3A on the ability of these neurons to survive both in the presence and absence of NGF, whose removal results in the death of significant numbers of neurons by programmed cell death or apoptosis.

Trigeminal ganglion neurons obtained from rat pups at postnatal day 1 were separated from non-neuronal cells on a metrizamide density gradient. The resulting cultures contained >95% neurons on the basis of their typical bipolar morphology and extensive neurites after 2 days in culture. The phase-contrast images of the cultures are very similar to those reported previously using this method (33, 34). In addition, we also confirmed the identity of these cells by their positive staining for α-internexin, a type IV intermediate filament that is a specific marker for neurons of the peripheral nervous system (Fig. 1) (35, 36). Neurons were cultured for 24 h in medium supplemented with NGF, and plasmid DNA was then introduced by liposome-mediated transfection. In control experiments in which a GFP-expressing reporter construct was transfected into these cultures by this method, transfection efficiencies of >70% of the neurons were routinely observed (Fig. 2).

Parallel samples of neurons were transfected with either empty expression vector lacking any insert or the same expression vector containing full-length cDNA for Brn-3a cloned downstream of the constitutive cytomegalovirus gene promoter. Each transfection also included a β-galactosidase expression vector, allowing successfully transfected cells to be visualized by staining for β-galactosidase. Cell survival was then calculated for each sample on the basis of the number of surviving transfected neurons in each case. To confirm that Brn-3a expression was elevated in the transfected cells, we used a reverse transcription-PCR assay, which we have previously used to measure Brn-3a mRNA levels in small amounts of material under conditions in which the signal obtained is linearly related to the amount of mRNA in the sample (25, 29–32). The primers used were selected to amplify both the endogenous Brn-3a mRNA and the exogenous Brn-3a mRNA derived from the expression vector, thereby allowing total Brn-3a expression to be measured. As expected, clear overexpression of BRN-3A was observed in the neurons transfected with the Brn-3a expression vector compared with those transfected with the empty expression vector (Fig. 3).

Moreover, a clear enhancement of survival in the cells transfected with Brn-3a was observed compared with those transfected with vector even in the presence of NGF (Fig. 4A). In the absence of NGF, this difference became even larger, with almost 3-fold the number of surviving neurons being observed in the cultures transfected with Brn-3a compared with the control cultures transfected with vector alone (p < 0.05 in Student’s t test when survival in the absence of NGF of cells transfected with vector versus Brn-3a is compared and p < 0.0001 in a single ANOVA test; Bonferroni’s test confirmed significance at 95% confidence, p < 0.0084). Hence, BRN-3A overexpression can protect trigeminal neurons from cell death both in the presence and absence of NGF.

Interestingly, this protective effect was paralleled by enhanced expression of the endogenous Bcl-2 gene in the cells transfected with Brn-3a (Fig. 3). These findings represent the first demonstration that BRN-3A can induce expression of the endogenous Bcl-2 gene in primary neuronal cells (as opposed to a neuronal cell line) and suggest that Brn-3a may mediate its effect on neuronal survival, at least in part, via activation of Bcl-2.

Similar results were also observed in experiments carried out using postnatal dorsal root ganglion neurons, which also express Brn-3A (7, 10) and are lost in the knockout mice (17, 18). Thus, as shown in Fig. 4B, enhanced survival was observed for the Brn-3a-transfected DRG neurons both in the presence of NGF.
and absence of NGF, although the effect was less dramatic than in the case of the trigeminal neurons, possibly because more DRG neurons survive in the absence of NGF ($p < 0.0005$ in a single ANOVA test). These data thus indicate that BRN-3A overexpression can enhance the survival of both trigeminal and DRG neurons in the presence and absence of a specific neurotrophic factor.

To determine whether endogenous BRN-3A expression also plays a role in the survival of these neurons, we utilized an antisense approach. Thus, we generated a specific antisense construct in which a 240-base pair region derived from the N terminus of BRN-3A that cannot cross-hybridize to either Brn-3b or Brn-3c was expressed in an antisense orientation under the control of the CMV promoter. We have previously used a similar approach to successfully reduce the level of BRN-3A in an immortalized neuronal cell line (19). This construct was introduced into trigeminal and DRG neurons by liposome-mediated transfection as described above. As expected, reduced expression of endogenous BRN-3A was observed in the cells transfected with the antisense construct (Fig. 3).

In these experiments, both trigeminal (Fig. 4A) and DRG (Fig. 4B) neurons showed significantly reduced survival when transfected with the Brn-3a antisense construct compared with the transfection with empty expression vector. This reduced survival was observed both in the presence and absence of NGF, indicating that expression of BRN-3A is important for survival under both these conditions ($p < 0.001$ for trigeminal neurons and $p < 0.01$ for DRG neurons when survival of cells transfected with vector is compared with that of cells transfected with the Brn-3a antisense plasmid in Student’s $t$ test; Bonferroni’s test confirmed significance at 95% confidence, $p < 0.0084$). Interestingly, in this case, the higher survival of DRG neurons in the absence of NGF was paralleled by a greater reduction in their survival when transfected with the Brn-3a antisense construct ($p < 0.0001$ for trigeminal neurons and $p < 0.0005$ for DRG neurons in a single ANOVA test) (Fig. 4, compare A and B).

Interestingly, the level of endogenous Bcl-2 expression was reduced in the cells transfected with the antisense construct (Fig. 3). This indicates that the endogenous expression of Bcl-2 in sensory neurons is dependent upon Brn-3a and that the effect of reduced BRN-3A expression on neuronal survival may be mediated, at least partly, via reduced BCL-2 expression.
To investigate the nature of the death processes in neurons in which BRN-3A expression has been manipulated, we used TUNEL labeling to monitor apoptotic death in DRG neurons in which BRN-3A expression had been increased or decreased. As illustrated in Fig. 5, BRN-3A overexpression dramatically reduced the number of TUNEL-labeled cells when NGF was withdrawn. Similarly, reduced BRN-3A levels produced enhanced apoptosis even in the presence of NGF (p < 0.0001 in a single ANOVA test).

To confirm the protective effect of BRN-3A against apoptotic death, we used annexin V surface staining of the transfected cells, which measures the translocation of phosphatidylserine to the outer surface of the cell membrane, which occurs early in apoptotic cell death and is distinct from the later DNA degradation, which is measured by TUNEL labeling. As indicated in Table I, cells transfected with BRN-3A showed a greatly reduced proportion of annexin V-positive cells compared with cells transfected with expression vector alone, and this was statistically significant (p < 0.0005 in Student’s t test and p < 0.0001 in a single ANOVA test; Bonferroni’s test confirmed significance at 99% confidence, p < 0.017). Hence, BRN-3A appears to exert its effects on neuronal survival by modulating the extent of apoptotic cell death.

Although our data suggest that endogenous BRN-3A plays a key role in the survival of trigeminal and DRG neurons both in the presence and absence of NGF, it was evidently also possible that the effect of the antisense construct was due to some nonspecific toxic effect of this construct. We therefore tested the effect of introducing this construct into cultured sympathetic neurons derived from the superior cervical ganglia. Thus, these neurons do not express endogenous BRN-3A (25) and should not therefore be affected by antisense Brn-3a.

In these experiments (Fig. 6), no statistically significant difference in survival was observed for the SCG neurons in the presence of NGF, whether they were transfected with vector or the antisense construct. Similarly, extensive cell death was noted in the absence of NGF in both the cells transfected with the vector control and those transfected with the Brn-3a antisense construct, and there was no statistically significant difference between the two groups. Hence, the effect of the Brn-3a antisense construct is indeed specific to neurons that express BRN-3A, rather than representing a nonspecific toxic effect of this construct.

In these experiments, we also investigated the effect of introducing a Brn-3a expression vector into the SCG neurons. Most interestingly, the extensive cell death that was observed in these neurons upon NGF withdrawal was not reduced in any way in the cells transfected with the Brn-3a expression vector compared with those transfected with expression vector lacking any insert or the Brn-3a antisense construct, with no statistically significant difference in survival being observed in the three groups (Fig. 6).

To confirm that BRN-3A was expressed from the plasmid vector in SCG as well as trigeminal ganglion neurons, we used primers that will amplify only the exogenous Brn-3a mRNA derived from the expression vector in a reverse transcription-PCR assay. BRN-3A expression from the transfected construct was readily detectable in the SCG cells transfected with the Brn-3a expression vector, indicating that this effect was not due to a failure of the construct to express BRN-3A in this neuronal cell type (Fig. 7A). These findings indicate that the ability of overexpressed BRN-3A to protect neuronal cells from cell death upon neurotrophic factor
withdrawal is neuronal cell type-specific, with trigeminal and DRG neurons being protected, whereas SCG neurons are not protected by the overexpression of BRN-3A.

Interestingly, in previous experiments (22, 23), we have shown that BRN-3A can activate Bcl-2 promoter constructs in cotransfections carried out in DRG neurons or the ND7 neuronal cell line, but not in baby hamster kidney fibroblast cells. We therefore tested whether overexpression of BRN-3A in SCG neurons was able to enhance the expression of the endogenous Bcl-2 gene. As shown in Fig. 7B, no increase in Bcl-2 expression was observed in the SCG cells overexpressing BRN-3A. In contrast, overexpression of BRN-3A in trigeminal ganglion neurons clearly resulted in overexpression of Bcl-2 (Fig. 7C) in accordance with our previous results (Fig. 3). Hence, the ability of BRN-3A to protect trigeminal neurons from apoptosis is associated with its ability to induce Bcl-2 expression, whereas this does not occur in SCG neurons.

To extend these studies, we wished to investigate the ability of BRN-3A to protect trigeminal ganglion neurons at different stages of development. To do this, we initially utilized trigeminal cultures from E17 embryos as opposed to the postnatal cultures we had previously used. To efficiently introduce the Brn-3a gene into these cultures, we prepared a disabled herpes simplex virus vector expressing both BRN-3A and GFP. This vector lacks functional viral genes encoding the Vmw65 transactivator protein and the ICP34.5 neurovirulence factor, and we have previously shown that it is able to effectively deliver genes to neuronal cells both in vitro and in vivo without causing significant neuronal cell death (28).

The E17 cultures were therefore infected with this virus or with control virus expressing only a reporter gene. The effect on survival in the presence or absence of NGF was assessed as described above. In these experiments (Fig. 8), a clear increase in survival following NGF withdrawal was noted in the cells infected with the BRN-3A virus ($p < 0.0001$ in Student's $t$ test and $p < 0.0001$ in a single ANOVA test), confirming that this effect could also be observed in embryonic neurons and using a different method to deliver BRN-3A.

To extend the studies on the ability of BRN-3A to protect neurons from different stages of development, we carried out similar studies examining the effect of overexpressing BRN-3A by liposome-mediated transfection on trigeminal ganglion cultures prepared at different embryonic stages from E11 to E18. Because of the different proportions of NGF-dependent neurons at different embryonic stages, we cultured the cells in medium containing NGF for 24 h to remove the neurons that are dependent on other neurotrophins rather than on NGF (see “Materials and Methods”). We then either removed NGF or retained it and compared the protective effect of BRN-3A on these NGF-dependent neurons derived from different embryonic stages. In these experiments, BRN-3A overexpression had
of different stages of embryonic development (Fig. 9). BRN-3A can protect trigeminal ganglion neurons at a number of stages throughout development due either to a direct effect of BRN-3A on neuronal survival or to an indirect effect on the ability of neurons to respond to survival-promoting stimuli such as neurotrophins. Our data support a model in which BRN-3A has a direct effect, promoting neuronal survival, rather than an indirect one, regulating the responsiveness of neurons to specific neurotrophic stimuli. Thus, overexpression of BRN-3A in trigeminal or DRG neurons in culture can promote their survival particularly fol-

Fig. 9. Survival of trigeminal ganglion neurons obtained at the indicated stages of embryonic development, transfected with either empty expression vector (solid lines) or Brn-3a expression vector (dotted lines), and then cultured in the presence (A) or absence (B) of NGF. Values are the mean ± S.D. (error bars) of at least three independent experiments.

little effect on survival in the presence of NGF compared with the level of survival observed in cells treated with vector alone at the different stages of development examined, presumably because of the high levels of survival that were observed under these conditions (Fig. 9A). In contrast, in the absence of NGF, the cultures overexpressing BRN-3A showed clearly enhanced survival compared with the cultures treated with vector, which was observed at all time points (p < 0.0001 in Student’s t test for comparison of BRN-3A- or vector-treated cells at all time points tested; Bonferroni’s test confirmed significance at 99% confidence, p < 0.0024). This indicates that overexpression of BRN-3A can protect trigeminal ganglion neurons at a number of different stages of embryonic development (Fig. 9B).

As BRN-3A is expressed in trigeminal ganglia throughout this period of development (25), we also wished to determine whether antisense inhibition of BRN-3A expression would reduce the survival of trigeminal ganglion neurons at different developmental stages. As indicated in Fig. 10, antisense inhibition of BRN-3A expression led to reduced survival of the trigeminal ganglion neurons in the presence of NGF when these neurons were derived from the later stages of embryonic development, with highly significant differences in survival being observed between the vector control- and antisense Brn-3a-treated neurons in cultures prepared from E13 and E14 onward. In contrast, much smaller effects were observed at E12, with no effect at all at E11.

Hence, the endogenous BRN-3A that is expressed in neurons at all these stages (25) appears to play a more significant survival-promoting role as development proceeds. This is in agreement with the finding that the trigeminal ganglia of Brn-3a knockout mice appear to be of normal size early in embryonic development and exhibit significant differences in size from those of wild-type animals only later during embryonic development and postnatally (17, 18).

In view of the finding that antisense inhibition of BRN-3A expression did not affect the survival of early trigeminal ganglion cultures even though BRN-3A is expressed in such cultures (25), we examined the ability of the closely related POU factor BRN-3B to protect trigeminal ganglion cultures at different stages of embryonic development. BRN-3B was overexpressed in these cells using a plasmid expression vector in exactly the same manner as BRN-3A, and the survival of these cells was compared with that of cells treated with expression vector lacking any insert. In these experiments, no statistically significant alteration in survival was observed in the cells overexpressing BRN-3B compared with the vector controls in cultures maintained in the presence of NGF (Fig. 11A).

Similarly, late stage trigeminal ganglion cultures from E13 onward did not exhibit enhanced survival when they were engineered to overexpress BRN-3B and NGF was subsequently removed (Fig. 11B), paralleling the lack of effect of BRN-3B in postnatal cultures (data not shown). Most interestingly, however, trigeminal ganglion cultures from E11 and E12 did display significantly enhanced survival (p < 0.05) when engineered to overexpress BRN-3B and subsequently exposed to removal of NGF compared with the survival of similarly treated control cells transfected with empty expression vector alone (Fig. 11B). Hence, BRN-3B does appear to be able to substitute for BRN-3A in early stage trigeminal ganglion cultures, but not at any later stage. Hence, it is possible that following antisense inhibition of BRN-3A expression at early stages, the survival of the neurons is maintained by BRN-3B, which is also expressed in such early stage trigeminal ganglion neurons, albeit at low levels (25).

**DISCUSSION**

The BRN-3A POU family transcription factor clearly plays an essential role in the correct development of the nervous system, with significant losses of sensory and motor neurons being observed in knockout mice lacking a functional gene for this factor (17, 18). Evidently, these effects could occur because specific neurons fail to develop or because neurons die during development due either to a direct effect of BRN-3A on neuronal survival or to an indirect effect on the ability of neurons to respond to survival-promoting stimuli such as neurotrophins. Our data support a model in which BRN-3A has a direct effect, promoting neuronal survival, rather than an indirect one, regulating the responsiveness of neurons to specific neurotrophic stimuli. Thus, overexpression of BRN-3A in trigeminal or DRG neurons in culture can promote their survival particularly fol-
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Survival of trigeminal ganglion neurons obtained at the indicated stages of embryonic development, transfected with either empty expression vector (solid lines) or Brn-3b expression vector (dotted lines), and then cultured in the presence (A) or absence (b) of NGF. Values are the mean ± S.D. (error bars) of at least three independent experiments.

The effects of BRN-3A on neuronal survival are likely to be mediated by the ability of this transcription factor to up-regulate the expression of specific genes involved in neuronal survival. Thus, previous studies have reported that BRN-3A can act as an activator of expression of a wide variety of different genes expressed in neuronal cells (for review, see Refs. 8 and 37), and it is therefore likely that some of the Brn-3a target genes encode protein products that can promote neuronal survival. In particular, we have previously shown that overexpression of BRN-3A in both cultured dorsal root ganglion neurons and an immortalized neuronal cell line can activate the Bcl-2 promoter in cotransfection assays and also results in the activation of the endogenous Bcl-2 gene in a neuronal cell line (22, 23). Hence, we have extended these observations by showing that BRN-3A expression can activate the endogenous Bcl-2 gene in primary sensory neurons and that inhibition of BRN-3A expression reduces endogenous BCL-2 expression. Thus, BRN-3A may promote neuronal survival by activating the expression of the Bcl-2 proto-oncogene, which has a survival-promoting effect.

However, mice lacking BCL-2 show significant losses of neurons only after birth (38), whereas the Brn-3a knockout mice show extensive losses much earlier in development (17, 18). Hence, it is likely that BCL-2 is not the only target for activation by BRN-3A. Indeed, we have recently demonstrated that the gene encoding the related BCL-X protein is also activated by BRN-3A.2 This is of particular interest since the extensive neuronal cell losses observed in mice lacking BCL-X occur at a similar time and affect the same populations of neurons as those affected in the Brn-3a knockout mice (39). Interestingly, Huang et al. (40) have recently demonstrated that mice lacking BRN-3A do not produce trkC-expressing neurons rendering the gene encoding this neurotrophin receptor, another candidate for regulation by BRN-3A. Evidently, therefore, a number of potential candidate genes for regulation by BRN-3A exist, and further studies will be required to elucidate which of these are the most important in mediating its effects on neuronal survival.

Most interestingly, however, the ability of BRN-3A to promote survival and to up-regulate BCL-2 expression is dependent upon the type of neuron in which the effects of BRN-3A are tested. Thus, as expected, the use of the Brn-3a antisense construct did not result in reduced survival in cultures of sympathetic neurons obtained from superior cervical ganglia since these neurons do not express endogenous BRN-3A (25). More importantly, however, overexpression of BRN-3A was unable to promote the survival of these neurons upon withdrawal of NGF, in complete contrast to the results obtained with postnatal trigeminal or dorsal root ganglion neurons. This effect was paralleled by the inability of BRN-3A to activate the endogenous Bcl-2 gene in sympathetic neurons. These data therefore clearly indicate that the ability of BRN-3A to promote survival is dependent upon the type of neuron investigated.

Although the sympathetic neurons represent the most extreme example of this effect in the neuronal types that we have tested, similar effects were also observed within the sensory neuron system. Thus, a difference in responsiveness to BRN-3A was noted during the development of the trigeminal ganglion, with overexpression of BRN-3A having a relatively weak protective effect at the early stages of development compared with that observed at later stages. Similarly, antisense inhibition of BRN-3A had no or an insignificant effect on trigeminal ganglion neuron survival at early stages and a much more dramatic effect at later stages of development. It is of particular interest that we began to observe clear effects of reduced BRN-3A expression in cultured neurons derived from E13 and E14 onward. Thus, in a study of Brn-3a knockout mice, losses of trkA- and trkB-expressing neurons were observed at E12.5 (trkB) and E13.5 (trkA) onward (40). This suggests that the normal survival pattern of trkA- and trkB-expressing neurons may be dependent on the survival-promoting effects of BRN-3A. Hence, BRN-3A may be required both for the formation of trkC-expressing neurons (which do not form in the knockout mice) (40) possibly by directly stimulating the expression of the gene encoding the factor (see above) and also for maintaining the survival of appropriate numbers of trkA- and trkB-expressing neurons during the natural period of programmed cell death.

Interestingly, the relatively weak effect of BRN-3A in early trigeminal ganglion cultures contrasts with the clear protective effect of the overexpression of BRN-3B in such cultures, which is in contrast to the inability of BRN-3B to protect later stages of trigeminal ganglion development or adult trigeminal or dorsal root ganglion cultures. Hence, BRN-3B appears to be able to substitute for BRN-3A only at very precise developmental

2 M. D. Smith, E. Ensor, and D. S. Latchman, unpublished data.
It should be noted, however, that BRN-3A is expressed only at low levels in the early trigeminal ganglion (25). It is therefore clear that specific differences exist between different types of neurons in the nervous system both prior to and after birth in terms of their responsiveness to the survival-promoting effects of BRN-3A as well as their dependence on any endogenous BRN-3A for survival.

In this regard, it is of interest that we have demonstrated that the activation of the Bcl-2 promoter by BRN-3A can be observed only in cotransfections of specific neuronal cells and not following transfection into fibroblasts (23). Hence, it is possible that BRN-3A may only be able to activate the Bcl-2 promoter in a limited range of neuronal cells perhaps due to a requirement for a coactivator, which is only present in such cells.

It is clear, however, that BRN-3A overexpression can directly promote the survival of some (but not all) neuronal cell types, whereas its endogenous expression is essential for the survival of such neuronal cell types. These results thus establish BRN-3A as a novel survival-promoting transcription factor for some (but not all) neuronal cells.

REFERENCES
1. Ryan, A. K., and Rosenfeld, M. G. (1997) *Genes Dev.* 11, 1207–1225
2. Verrijzer, C. P., and van der Vliet, P. C. (1993) *Biochim. Biophys. Acta* 1173, 1–21
3. Anderson, B., and Rosenfeld, M. G. (1994) *J. Biol. Chem.* 269, 29335–29338
4. Desai, C., Garriga, G., McIntire, S. L., and Horvitz, H. R. (1988) *Nature* 336, 638–646
5. Finney, M., Ruvkin, G., and Horvitz, H. R. (1988) *Cell* 55, 757–769
6. Theil, T., McLean-Hunter, S., Zornig, M., and Moroy, T. (1995) *Nucleic Acids Res.* 23, 5921–5929
7. Gerrero, M. R., McEvilly, R. J., Tuner, E., Lin, C. R., O'Connell, S., Jenne, K. J., Hobbs, M. V., and Rosenfeld, M. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 10841–10845
8. Latchman, D. S. (1996) *Philos. Trans. R. Soc.* 351, 511–515
9. He, X., Tracey, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. S., and Rosenfeld, M. G. (1989) *Nature* 340, 35–42
10. Lilleyrop, K. A., Budhram-Mahadeo, V. S., Lakin, N. D., Terrenghi, G., Wood, J. N., Polak, J. M., and Latchman, D. S. (1992) *Nucleic Acids Res.* 20, 5093–5096
11. Turner, E. E., Jenne, K. J., and Rosenfeld, M. G. (1994) *Neuron* 12, 205–218
12. Ninkina, N. N., Stevens, G. E. M., Wood, J. N., and Richardson, W. D. (1993) *Nucleic Acids Res.* 21, 3175–3182
13. Fedtsova, N. G., and Turner, E. E. (1996) *Mech. Dev.* 53, 291–304
14. Erkman, L., McEvilly, J., Luo, L., Ryan, A. K., Hooshmand, F., O’Connell, S. M., Keithley, E. M., Rapaport, D. H., Ryan, A. F., and Rosenfeld, M. G. (1996) *Nature* 381, 603–606
15. Vahana, O., Morell, K., Lynch, E. D., Weiss, S., Kagen, M. F., Abitau, N., Morrow, J. E., Lee, M. K., Skvorak, A. B., Morton, C. C., Blumenfeld, F., Frydman, M., Friedman, T. B., King, M.-C., and Avraham, K. B. (1954) *Science* 270, 1950–1998
16. Gan, L., Xiang, M., Zhou, L., Wagner, D. S., Klein, W. H., and Nathans, J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 3920–3925
17. McEvilly, R. J., Erkman, L., Luo, L., Sawchenko, P. E., Ryan, A. F., and Rosenfeld, M. G. (1996) *Nature* 384, 574–577
18. Xiang, M., Gan, L., Zhou, L., Klein, W. H., and Nathans, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11950–11996
19. Lakin, N. D., Morris, P. J., Theil, T., Sato, T. N., Moroy, T., Wilson, M. C., and Latchman, D. S. (1995) *J. Biol. Chem.* 270, 15858–15863
20. Budhram-Mahadeo, V. S., Morris, P. J., Lakin, N. D., Theil, T., Ching, G. Y., Lilleyrop, K. A., Moroy, T., Liem, R. K. H., and Latchman, D. S. (1995) *J. Biol. Chem.* 270, 28523–28528
21. Smith, M. D., Morris, P. J., Dawson, S. J., Schwartz, M. L., Schlaepfer, W. W., Brett, P. M., Miller, A. D., and Gurling, H. M. D. (1997) *Neuroreport* 8, 1481–1484
22. Smith, M. D., Ensor, E. A., Coffin, R. S., Boxer, L. M., and Latchman, D. S. (1999) *J. Biol. Chem.* 274, 16715–16722
23. Smith, M. D., Dawson, S. J., Boxer, L. M., and Latchman, D. S. (1998) *Nucleic Acids Res.* 26, 4100–4107
24. Camu, W., and Henderson, C. E. (1992) *J. Neurosci. Methods* 44, 59–62
25. Wyatt, S., Ensor, E., Begbie, J., Ernoff, P., Reichardt, L. F., and Latchman, D. S. (1998) *Mol. Brain Res.* 55, 254–264
26. Davies, A. T. (1995) in *Neural Cell Culture: A Practical Approach* (Cohen, J., and Wilkin, G. P., eds) pp. 153–175, Oxford University Press, New York
27. McQuillin, A., Murray, K. D., Etheridge, C., J., Stewart, L., Cooper, R. G., Brett, P. M., Miller, A. D., and Gurling, H. M. D. (1997) *Neuroreport* 8, 1481–1484
28. Coffin, R. S., Maclean, A. R., Latchman, D. S., and Brown, S. M. (1996) *Gene Ther.* 3, 886–891
29. Budhram-Mahadeo, V. S., Ndiasang, D., Ward, T., Weber, B. L., and Latchman, D. S. (1999) *Oncogene* 18, 6684–6691
30. Ndiasang, D., Morris, P. J., Chapman, C., Ho, L., Singer, A., and Latchman, D. S. (1998) *J. Clin. Invest.* 101, 1687–1692
31. Smith, M. D., Dawson, S. J., and Latchman, D. S. (1997) *Mol. Cell. Biol.* 17, 345–354
32. Ndiasang, D., Budhram-Mahadeo, V., and Latchman, D. S. (1999) *J. Biol. Chem.* 274, 28521–28527
33. Vogel, K. S., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Parad, L. F. (1995) *Cell* 82, 733–742
34. Buchman, V. L., and Davies, A. M. (1993) *Development* 118, 989–1001
35. Atlahan, E. S., Sacher, M. G., and Mushynski, W. E. (1997) *J. Neurosci. Res.* 47, 390–310
36. Duprey, P., and Paulin, D. (1995) *Int. J. Dev. Biol.* 39, 443–457
37. Latchman, D. S. (1997) *Int. J. Oncol.* 10, 1133–1139
38. Michaelidis, T. M., Sendtner, M., Cooper, J. D., Airaksinen, M. S., Holmman, B., and Meyer, M. (1996) *Neuron* 17, 75–89
39. Mutoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K. I., Negishi, I., Senju, S., Zhang, Q., and Fujii, S. (1995) *Science* 267, 1506–1510
40. Huang, E. J., Zang, K., Schmidt, A., Saulys, A., Xiang, M., and Reichardt, L. F. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11950–11996
41. Motoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K. I., Negishi, I., Senju, S., Zhang, Q., and Fujii, S. (1995) *Science* 267, 1506–1510
42. Hoang, E. J., Zang, K., Schmidt, A., Saulys, A., Xiang, M., and Reichardt, L. F. (1999) *Development* 126, 2869–2882