Research report

NT-3 regulates expression of Brn3a but not Brn3b in developing mouse trigeminal sensory neurons

Sean Wyatt a,*, Liz Ensor a, Jo Begbie a, Patrik Ernfors c, Louis F. Reichardt b, David S. Latchman a

a Department of Molecular Pathology, University College London Medical School, The Windeyer Building, 46 Cleveland Street, London, W1P 6DB, UK
b Department of Physiology and Howard Hughes Medical Institute, University of California, San Francisco, CA 94143, USA
c Laboratory of Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, BZ Lab., Stockholm, S-171 77, Sweden

Accepted 23 December 1997

Abstract

We have used a quantitative RT-PCR approach to determine the levels of Brn3a and Brn3b POU domain transcription factor mRNAs in the developing mouse trigeminal ganglion from E10 to E18. Using low density neuronal cultures, we have shown that NT-3 can regulate the expression of Brn3a mRNA in trigeminal neurons during the periods that they are differentiating and innervating their peripheral and central targets. In contrast to Brn3a, Brn3b mRNA is expressed at extremely low levels in the early trigeminal ganglion. Trigeminal neurons from early ganglia express low levels of Brn3b mRNA in culture and do not up-regulate Brn3b mRNA in response to a number of growth factors and experimental conditions. However, at later ages, when in vivo levels of Brn3b mRNA are high, FGF2, TGFβ1 and retinoic acid all up-regulate Brn3b mRNA expression in cultured trigeminal neurons. Since NT-3 regulates the developmental expression of Brn3a, Brn3a may mediate some of the effects that NT-3 exerts on sensory neurons and their progenitors. Similarly, Brn3b may mediate some of the effects that FGF2, TGFβ1 and retinoic acid have on neurons. © 1998 Elsevier Science B.V.

Keywords: POU factor; Trigeminal; Development; mRNA expression; Neurotrophin

1. Introduction

The POU (Pit-Oct Unc) family of transcription factors were initially defined by virtue of a 150–160 amino acid DNA binding domain common to the mammalian transcription factors Pit-1, Oct-1 and Oct-2 and the C. elegans regulatory gene unc86. More recently, many additional members of this family have been characterised and the family has been subdivided into six distinct classes based on sequence homology [38]. POU factors play important roles in the determination of a number of cellular phenotypes. For example, Pit-1 is required for specification of three cell types in the anterior pituitary [22]. Brn2 has been shown to be necessary for the development and survival of neurosecretory neurons in the hypothalamus [31], and

unc86 is required for the commitment of several neuroblast lineages and the specification of various neuronal phenotypes [16].

The Brn3 subfamily of POU domain factors contains three members, Brn3a, Brn3b and Brn3c, that have also been termed Brn-3.0, Brn-3.2 and Brn-3.1, respectively [17,18,23,32,36,44]. Since the Brn3 family are the most closely related factors to unc86, they may be the mammalian homologues of this gene and therefore may play a role in the determination of neuronal phenotype. This hypothesis is supported by the observation that, with the exception of a small amount of Brn3a mRNA expression in cells of the neuro-endocrine and immune systems, the expression of Brn3 factors is exclusively restricted to the embryonic and adult nervous system [17,32,36,44,45]. Co-transfection of Brn3 expression cDNAs and promoter/reporter cDNA constructs into cell lines has identified a number of neuron specific genes that are potential targets for Brn3a and Brn3b transactivation or repression
[4,17,22,27–29]. In addition, data from experiments with the ND7 neuronal cell line has suggested a role for Brn3a in the acquisition of a neuronal phenotype and subsequent neurite outgrowth [21]. More recently, a transgenic approach has been used in a bid to determine the role that Brn3 factors play in specifying neuronal phenotypes. Mice that contain a null mutation for Brn3b possess 60–70% fewer retinal ganglion cells and exhibit subtle differences in the morphology of the superior colliculus. Loss of both Brn3c alleles in mice results in the loss of cochlear and vestibular hair cells, degeneration of spiral and vestibular ganglia and complete deafness [10]. Brn3a knockout mice show a loss in specific subsets of motor and sensory neurons that may be partly due to decreased expression of BDNF and neurotrophin receptors [26].

To further characterise the roles that Brn3a and Brn3b play in the development of primary sensory neurons we have determined the temporal expression of mRNAs for these proteins in the well characterised developing mouse trigeminal ganglion from embryonic day 10 (E10) to E18. In addition, we have used low density neuronal cultures to study the regulation of these two mRNAs by neurotrophic growth factors as a first step to investigating whether Brn3a or Brn3b mediate any of the effects of these proteins on sensory neurons. In particular, we have examined the effects of a variety of neurotrophins and neurotrophic growth factors that have previously been shown to be expressed in the target of trigeminal neurons, or in the ganglion itself, during the developmental period in question. We have also determined the effect of depolarization by KCl and treatment with retinoic acid on the expression of Brn3a and Brn3b mRNAs.

2. Results

2.1. Developmental expression of Brn3a and Brn3b mRNAs

To take account of the increase in size of trigeminal ganglia between E10 and E18, levels of Brn3a and Brn3b mRNAs were initially determined relative to the levels of mRNA for the ubiquitously expressed ‘housekeeping’ protein GAPDH (Fig. 1). Brn3a mRNA is expressed at high levels from E10 to E14. After E14, there is a sharp drop in
Fig. 3. Developmental expression of Brn3 mRNAs in the trigeminal ganglion. The calculated mean levels of Brn3a and Brn3b mRNAs/trigeminal neuron are shown in (a) and (b), respectively. The means ± S.E.M. of at least four separate measurements are shown for each age.

Fig. 4. Expression of Brn3a mRNA in E11 trigeminal neurons in culture. (a) is a bar chart showing the levels Brn3a mRNA in E11 trigeminal neurons cultured in unsupplemented culture medium (control) or with the addition of: 50 pg/ml BDNF (low BDNF), 5 ng/ml BDNF (high BDNF), 50 pg/ml NT-3 (low NT-3), 5 ng/ml NT-3 (high NT-3), 50 pg/ml BDNF + 50 pg/ml NT-3, 50 pg/ml BDNF + 1 ng/ml FGF2, 50 pg/ml BDNF + 1 μM retinoic acid (RA), 50 pg/ml BDNF + 35 mM KCl, 50 pg/ml BDNF + 1 ng/ml NGF, 50 pg/ml BDNF + 1 ng/ml TGFβ1 and 50 pg/ml BDNF + 1 ng/ml LIF. There is no significant difference in the expression of Brn3 mRNAs between 24 and 48 h in culture, therefore the data shown is an average from 24 and 48 h timepoints except for the control cultures which only survive for 24 h. The means ± S.E.M. of 3 experiments, each containing duplicate dishes at 24 and 48 h, are shown for each culture condition. (b) Levels of Brn3a mRNA in E11 trigeminal neurons cultured for 24 h with the addition of different concentrations of NT-3. (c) Time course of Brn3a mRNA expression in neurons grown either in unsupplemented culture medium (squares) or in medium supplemented with 1 ng/ml NT-3 (circles). The value shown at 0 h is the in vivo estimate of Brn3a mRNA levels in the E11 trigeminal ganglion. For (b) and (c), the mean ± S.E.M. of three separate experiments are shown.
pression of Brn3b mRNA relative to GAPDH mRNA is markedly different to that of Brn3a mRNA. Brn3b mRNA is expressed at very low levels in the early trigeminal ganglion. From E14 to E18, there is approximately a three-fold rise in the expression of Brn3b mRNA.

Between E10 and E18, the cellular composition of the developing trigeminal ganglion undergoes significant changes due to proliferation and differentiation of precursors, a period of cell death and expansion of the satellite cell population [1,7,40]. We have used low temperature differential sedimentation [6] to separate neurons and non-neuronal cells in the developing trigeminal ganglion at E14 and P2. RT-PCR of RNA extracted from these two cell types clearly shows that Brn3a and Brn3b mRNAs are predominantly expressed in neurons at both ages (Fig. 2).

The number of neurons within the developing embryonic trigeminal ganglion has previously been determined [7]. Since we have shown that Brn3a and Brn3b mRNAs are not expressed in satellite cells, and the data from work with neuronal cell lines suggest that neuronal precursors contain only low levels of Brn3a mRNA compared to post-mitotic neurons [21,33], data on the expression of Brn3a and Brn3b mRNAs can be presented in terms of fg/neuron at each embryonic age (Fig. 3). When the

![Fig. 5. Expression of Brn3a mRNA in E13 trigeminal neurons in culture. (a) is a bar chart indicating the levels of Brn3a mRNAs in E13 trigeminal neurons cultured with the addition of; 250 pg/ml NGF (low NGF), 10 ng/ml NGF (high NGF), 250 pg/ml NGF + 1 ng/ml BDNF, 250 pg/ml NGF + 1 ng/ml NT-3, 250 pg/ml NGF + 1 μM retinoic acid (RA), 250 pg/ml NGF + 35 mM KCl, 250 pg/ml NGF + 1 ng/ml FGF2, 250 pg/ml NGF + 1 ng/ml TGFβ1 and 250 pg/ml NGF + 1 ng/ml LIF. There is no significant difference in the expression of Brn3 mRNAs between 24 and 48 h in culture, therefore the data shown is an average from 24 and 48 h timepoints. The means ± S.E.M. of 3 experiments, each containing duplicate dishes at 24 and 48 h, are shown for each culture condition.(b) Levels of Brn3a mRNA in E13 trigeminal neurons cultured for 24 h in the presence of 2 ng/ml NGF with the addition of different concentrations of NT-3. (c) Graph showing the % survival of E13 trigeminal neurons cultured for 24 h with 2 ng/ml NGF and varying concentrations of NT-3. % survival is calculated relative to the number of neurons present after 3 h in culture. For (b) and (c) the mean ± S.E.M. of 2 separate experiments each containing duplicate dishes are shown.
expression of Brn3a and Brn3b mRNAs as expressed as fg/neuron, and not relative to GAPDH, changes in the neuronal expression of Brn3 mRNAs during development are no longer partly masked by the expression of GAPDH mRNA by an increasingly large complement of non-neuronal cells. As a result, the developmental pattern of Brn3 mRNA expression in Fig. 3 shows some notable changes compared to that shown in Fig. 1. For example, the drop in Brn3a mRNA levels that occurs between E14 and E15 is reduced from almost five-fold to three-fold, and there is now a three-fold rise in levels of Brn3a mRNA in trigeminal neurons from E14 and E18. Another notable difference is that the average amount of Brn3b mRNA expressed by E18 trigeminal neurons is over eight-fold higher than that expressed by E14 neurons. Figs. 1 and 3 clearly show that there is an almost ten-fold change in the ratio of Brn3a mRNA to Brn3b mRNA between E14 to E15 and that this ratio remains more or less constant until E18. The abrupt change in the ratio of Brn3a to Brn3b may have important consequences. These are discussed below.

2.2. The regulation of Brn3a mRNA expression in trigeminal neurons

Low density cultures (500–1000 cells/35 mm dish) were used to investigate factors that regulate the expression of Brn3a and Brn3b mRNAs in trigeminal neurons at different stages of development.

A total of 80% of E11 trigeminal neurons survive for 24 h in culture in the absence of added neurotrophins; however, NT-3 or BDNF is required for longer term neuronal survival [2]. Neither BDNF alone, or BDNF in combination with LIF, retinoic acid, NGF, 35 mM KCl, FGF2 or TGFβ1, increases Brn3a mRNA levels above those in neurons cultured for 24 h without added factors (Fig. 4a). In contrast, NT-3, either alone or in combination with BDNF, significantly increases the neuronal expression of Brn3a mRNA. The NT-3 induced increase of Brn3a mRNA expression after 24 h in culture is dose-dependent and saturates at 250 pg/ml NT-3 (Fig. 4b). Fig. 4c shows the time course of the induction of Brn3a mRNA expression by NT-3. In the absence of NT-3, Brn3a mRNA levels undergo a small rise soon after neurons are placed in cultures. However, after 9 h, there is a steady fall in Brn3a mRNA levels up until 24 h at which time levels are about 50% lower than those found in neurons in the intact ganglion. In the presence of NT-3, there is a greater than three-fold up-regulation of Brn3a mRNA expression between 3 and 9 h after placing the neurons in culture. This rapid up-regulation of Brn3a mRNA is transient so that by 24 h, neuronal levels of Brn3a mRNA are similar to those found in vivo, although this is still two-fold higher than that found in neurons cultured in the absence of NT-3.

At E13, the majority of trigeminal neurons survive in culture for 48 h in the presence of NGF. In contrast, only 25% of neurons survive for 24 h in the absence of neurotrophins and this number is reduced to less than 5% at 48 h [2]. As at E11, NT-3, but none of the other growth factors tested, increases neuronal expression of Brn3a mRNA (Fig. 5a). The addition of increasing amounts of NT-3 to neurons grown with NGF increases Brn3a mRNA expression in a dose-dependent manner, but does not increase neuronal survival (Fig. 5b and c). It therefore seems unlikely that NT-3 increases the expression of Brn3a...
mRNA by promoting the survival of an NGF-non-responsive, NT-3 dependent sub-population of neurons expressing elevated levels of Brn3a mRNA.

At E16, trigeminal neurons are critically dependent on NGF for survival in vitro. At this age, NT-3 does not appear to significantly up-regulate the amount of Brn3a mRNA expressed by neurons in low density cultures (Fig. 6). None of the other factors tested was able to significantly alter the neuronal expression of Brn3a mRNA at E16.

To find out whether NT-3 plays a role in regulating the neuronal expression of Brn3a mRNA in vivo as well as in vitro, we have measured the levels of Brn3a mRNA in trigeminal ganglia from embryos containing a null mutation in the NT-3 gene [12,14] and trigeminal ganglia from their wild type littermates at each age between E11 and E18. The loss of both NT-3 alleles leads to a 60% reduction in the number of trigeminal neurons by birth. To compensate for the loss of neurons in NT-3 knockout ganglia, we have measured the amount of mRNA for the ‘housekeeping’ protein GAPDH in wild type and knockout ganglia and expressed levels of Brn3a mRNA relative to GAPDH mRNA (Fig. 7). The relative levels of Brn3a mRNA in trigeminal ganglia from NT-3−/− embryos are not reduced compared to wild type ganglia from E11 to E14. However, the relative levels of Brn3a mRNA are significantly reduced in trigeminal ganglia from NT-3−/− embryos compared to ganglia from wild type em-

Fig. 8. Bar charts showing the amounts of Brn3b mRNA expressed by E11 (a), E13 (b), E16 (c) and E14 (d) trigeminal neurons cultured in media with a variety of supplements. In (a), culture conditions are the same as those described in the legend to Fig. 4 (a). In (b), culture conditions are the same as those described in the legend for Fig. 5 (a). In (c), culture conditions are the same as those described in the legend to Fig. 6. In (d), the levels of Brn3b mRNA are shown in E14 trigeminal neurons cultured in media supplemented with; 250 pg/ml NGF, 250 pg/ml NGF + 1 pg/ml TGFβ1, 250 pg/ml NGF + 1 ng/ml FGF2 or 250 ng/ml NGF + 1 μM retinoic acid. Since there is no significant difference between the levels of Brn3b mRNA expressed by trigeminal neurons after 24 or 48 h in culture, the data shows the average of 24 and 48 h timepoints. For (a), (b), (c) and (d), the mean ± S.E.M. of three separate experiments are shown, each containing duplicate dishes at 24 and 48 h.
The expression of Brn3a increases the length of these processes and over-differentiation of ND7 cells is paralleled by a substantial elaboration of neuritic processes that characterizes the expression of Brn3a mRNA in ND7 neuronal cell line [4, 21]. In addition, the proteins, SNAP-25 and alpha-internexin, are activated by factors that play a role in directing neurite outgrowth. In support of this hypothesis, the promoters of two such proteins are capable of up-regulating Brn3b mRNA expression. Since none of these factors promote neuronal survival at E16 (data not shown), they cannot be increasing the average level of Brn3b mRNA in E16 trigeminal neurons by promoting the survival of FGF2, TGFb1 or retinoic acid-dependent neuronal sub-populations expressing higher levels of Brn3b mRNA. Retinoic acid, but not TGFb1 or FGF2, appears to be capable of up-regulating Brn3b mRNA levels in neurons as young as E14 (Fig. 8d).

3. Discussion

3.1. Developmental expression of Brn3a and Brn3b mRNAs

We have shown that Brn3a and Brn3b mRNAs display distinct temporal patterns of expression in developing trigeminal sensory neurons. Brn3a mRNA is expressed at high levels throughout the period of peripheral and central target field innervation that takes place from E10 to E14 [7, 34]. Furthermore, from E14 to E15, there is a marked drop in the amount of Brn3a mRNA expressed by trigeminal neurons that coincides with the completion of target field innervation. It therefore seems reasonable to suggest that Brn3a may regulate the expression of some of the proteins that play a role in directing neurite outgrowth. In support of this hypothesis, the promoters of two such proteins, SNAP-25 and alpha-internexin, are activated by Brn3a in the ND7 neuronal cell line [4, 21]. In addition, the elaboration of neuritic processes that characterizes the differentiation of ND7 cells is paralleled by a substantial increase in the expression of Brn3a mRNA [21] and over-expression of Brn3a increases the length of these processes [33].

In contrast to Brn3a mRNA, Brn3b mRNA is expressed at extremely low levels from E10 to E14 in the trigeminal ganglion. It therefore seems unlikely that Brn3b plays a role in regulating any aspect of neuroblast proliferation, neuronal differentiation or target field innervation in trigeminal neurons. This is in contrast to the role that Brn3b appears to play in the development of retinal ganglion cells [10]. In this case, Brn3b is primarily expressed during the period of retinal ganglion cell differentiation. Interestingly, Brn3a expression does not commence until after the onset of Brn3b expression in the developing retinal ganglion, suggesting markedly different roles for Brn3a and Brn3b in the development of these two different neuronal cell types.

There is a ten-fold change in the ratio between the average amount of Brn3a and Brn3b mRNAs expressed in trigeminal neurons between E14 and E15. Brn3b protein has previously been observed to be concentrated in a distinct subset of neurons in adult trigeminal ganglia [45], suggesting that the large up-regulation of Brn3b mRNA in trigeminal neurons between E14 and E18 occurs in only a subset of neurons. Therefore, the change in the ratio of Brn3a to Brn3b between E14 and E15 may be much greater than ten-fold fold in some neurons, and much less in others. Since Brn3b has been shown to reduce the activity of a number of promoters that are activated by Brn3a [4, 28], a large shift in the ratio between Brn3a and Brn3b may provide a mechanism to rapidly down-regulate expression of specific target genes. A number of genes appear to be dramatically down-regulated in a subset of trigeminal neurons during this developmental period and may be potential targets for Brn3 proteins. These include tyrosine hydroxylase, somatostatin, a range of nicotinic acetylcholine receptor subunits and somatostatin receptors [13, 20, 25, 47].

3.2. Regulation of Brn3a mRNA expression in trigeminal neurons

Using low density neuronal cultures, we have shown that distinct factors regulate the expression of Brn3a and Brn3b mRNAs in developing trigeminal sensory neurons in vitro. Regulation of Brn3 mRNA expression by these factors is crucially dependent on the age of trigeminal neurons and suggests that these factors may be at least partially responsible for generating the developmental pattern of Brn3 mRNA expression observed in the trigeminal ganglion.

NT-3 increases the expression of Brn3a mRNA in a dose-dependent manner in cultures of E11 and E13 trigeminal neurons, but not E16 neurons. A rapid, greater than three-fold increase in the neuronal levels of Brn3a mRNA occurs over the first 9 h in culture at E11. This up-regulation is transient, so that by 18 h, Brn3a mRNA levels are similar to those in vivo. In addition to up-regulating Brn3a mRNA levels in the short term, NT-3 prevents a two-fold reduction in the expression of Brn3a mRNA that occurs in trigeminal neurons in culture. The data we present here raise the possibility that Brn3a may mediate some of the neuronal survivors.
effects that NT-3 has on the differentiation, maturation and survival of sensory neurons [2,15,40,41].

Analysis of the expression of Brn3a mRNA in trigeminal ganglia of NT-3 knockout embryos fails to demonstrate a reduction in Brn3a mRNA expression in the absence of NT-3 at ages between E11 an E14. The most parsimonious interpretation of this data is that NT-3 does not regulate the expression of Brn3a mRNA in developing sensory neurons in vivo. However, a discrepancy between the regulation of gene expression in vitro and in vivo has been reported previously for a number of genes. For example, although NGF regulates the expression of mRNA for the low affinity neurotrophin receptor, p75, in developing sensory neurons in culture [42], the developmental expression of p75 mRNA in the same sensory neurons is unaffected in NGF \( \text{--/--} \) embryos [8]. Moreover, NT-3 appears to play a role in inducing the rapid increase in the expression of TrkA mRNA that leads to the acquisition of NGF responsiveness by developing immature sympathetic neurons [37]. However, TrkA mRNA expression is regulated normally in sympathetic neurons of NT-3 \( \text{--/--} \) embryos [43]. The discrepancy between gene regulation in vitro and in vivo may be partly due to a built-in redundancy in the mechanisms that regulate gene expression.

The loss of NT-3 appears to alter the relative levels of neuronal precursors and differentiated neurons in the early development of at least some sensory ganglia [15]. In neuronal cell lines, differentiation of neurons from precursors is associated with a large up-regulation of Brn3a mRNA expression [21]. If this is also the case in vivo, an altered ratio of differentiated neurons relative to precursors in the developing trigeminal ganglia of NT-3 \( \text{--/--} \) embryos may mask a reduction in the expression of Brn3a mRNA in differentiated neurons. At E15 and E16, there are no neuronal precursors in the trigeminal ganglia of wild type or NT-3 knockout embryos and this may explain why the loss of a functional NT-3 allele leads to reduced neuronal Brn3a mRNA expression at these ages.

Why were we not able to detect significant up-regulation of Brn3a mRNA expression by NT-3 in E16 neurons in vitro when loss of NT-3 in vivo reduces Brn3a mRNA expression at this age? In-situ hybridization studies in quail [46] and rat sensory ganglia [11] reveal that the number of neurons expressing mRNA for the high affinity NT-3 receptor, \( \text{trkC} \), is reduced as development proceeds. If the same situation exists in the developing mouse trigeminal ganglion, the number of neurons in which NT-3 can potentially up-regulate Brn3a mRNA expression in E16 cultures may be small. A two- to threefold up-regulation of Brn3a mRNA levels in a small number of neurons may be masked by the inability of the majority of neurons to respond to NT-3.

In support of the hypothesis that NT-3 regulates Brn3a mRNA expression in developing trigeminal neurons in vivo, NT-3 has been shown to be expressed at high levels in the maxillary target of trigeminal neurons from E11 to E14. Moreover, the reduction in neuronal Brn3a mRNA levels that occurs at the end of the period of target field innervation coincides with a marked drop in target field NT-3 expression [2].

3.3. Regulation of Brn3b mRNA expression in trigeminal neurons

The amount of Brn3b mRNA expressed by E11 and E13 trigeminal neurons in culture was found to be very close to the low levels expressed in vivo at these ages. None of the factors employed in our study are able to modulate the expression of Brn3b mRNA in neurons at these two ages. In contrast, retinoic acid, TGF\( \beta \)1 and FGF2 can all up-regulate Brn3b mRNA expression in E16 trigeminal neurons. Retinoic acid, but not TGF\( \beta \)1 or FGF2, can also increase Brn3b mRNA expression in neurons as young as E14. All of these factors may therefore be partly responsible for the eight-fold up-regulation of Brn3b mRNA expression that occurs in trigeminal neurons between E14 and E18, and consequently Brn3b may mediate the effects of these factors on late embryonic trigeminal neurons. FGF2, TGF\( \beta \)1 and retinoic acid are all present in the environment of sensory neurons from the earliest period of development [3,19,30]. The low levels of Brn3b mRNA in trigeminal neurons in vivo between E10 and E14 may therefore reflect a lack of expression of functional receptors for these factors during this period or the absence of appropriate intracellular messenger systems. Although early sensory neurons appear to express mRNAs for FGF receptors and some members of the RAR/RXR retinoid receptor families [9,24,39], it is possible that the correct combinations of receptor proteins, expressed at appropriate levels, are not present in trigeminal neurons until later in development.

4. Materials and methods

4.1. Neuronal methods

Mouse embryos were obtained from overnight matings of C57 mice. Pregnant females were killed by cervical dislocation and the age of the embryos was determined by the criteria of Theiler [35]. 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 (HBSS)). The precise period of incubation in trypsin was dependent on the age of the ganglia. After removal of the trypsin solution, the ganglia were washed twice with 10 ml of Hams F12 medium containing 10% HIHS and were gently triturated with a fire polished, siliconised Pasteur pipette to give a single cell suspension. The cells were plated at a density of 200–500 neurons/35 mm dish (Nunc). Dishes were pre-
coated with polyornithine (0.5 mg/ml overnight) and laminin (20 µg/ml for 4 h). Because of the large number of satellite cells present in the E16 trigeminal ganglion, E16 trigeminal neurons were purified by low temperature differential sedimentation before plating [6]. The neurons were incubated at 37°C in a humidified 3.5% CO₂ incubator in a defined medium consisting of Hams F14 supplemented with: 2 mM glutamine, 0.35% bovine serum albumin (pathocyte-4, ICN), 60 ng/ml progestosterone, 16 µg/ml putrescine, 400 ng/ml l-thyroxin, 38 ng/ml sodium selenite, 340 ng/ml tri-iodo-thyronine, 60 µg/ml penicillin and 100 µg/ml streptomycin. Neurons were recognised by their bipolar morphology under phase-contrast optics. The total number of neurons in each culture dish was estimated by counting the number of neurons in a 10 mm x 10 mm grid in the centre of the dish and multiplying this by the quotient of the total growth area of the dish and the grid area.

Neurotrophins and growth factors were added to the culture medium prior to plating. NGF, FGF2, TGFβ1, BDNF, LIF and NT-3 were purchased from Autogen. Since retinoic acid is light sensitive, a fresh batch was dissolved in the dark in a small volume of ethanol, before being added to the culture medium, for each experiment. Cultures were kept in the dark until neuron counting and RNA extraction. In experiments where FGF2 was added to neuronal cultures and in whole ganglia. The reverse transcription reaction was then amplified in a 50 µl PCR reaction containing: 1X Promega PCR buffer, 1.5 mM MgCl₂, 0.1 mM dNTPs, 1.5 units of Taq polymerase (Promega), 40 ng of P3 end-labelled primers specific for either Brn3a, Brn3b or GAPDH cDNA.

The forward assay primer for amplifying Brn3a cDNA was: 5’-TTGACCAGAGACACTTAC-3’ and the reverse primer was: 5’-TTTGGTGATGGTGATAG-3’. These hybridize 76 bp apart in mouse Brn3a cDNA and 88 bp apart in the Brn3a competitor cDNA. The forward primer for assaying Brn3b cDNA was 5’-AGCTGCTTGAGCAC-CTAAGC-3’ and the reverse primer was 5’-TTTG- GTGCATGGGTTGTCATG-3’. These hybridize 118 bp apart in mouse Brn3b cDNA and 126 bp apart in the Brn3b competitor cDNA. The forward assay primer for GAPDH cDNA was: 5’-TCCAGTATGACCCCACCTC-3’ and the reverse primer was: 5’-TCCTGAAGATGATGGT- GATGG-3’. These hybridize 128 bp apart in mouse GAPDH cDNA and 132 bp apart in the GAPDH competitor cDNA.

Brn3a and GAPDH cDNAs were amplified by cycling at 95°C for 1 min, followed by 1 min at 53°C, then by 90 s at 72°C. For Brn3b cDNA, cycling conditions were the same except the annealing step was 1 min at 58°C. The exact number of PCR cycles was dependent on the initial target cDNA concentration but was typically between 25 and 30 cycles.

The RT-PCR products of the native Brn3 and GAPDH mRNAs and the cRNA competitor species were separated on 8% non-denaturing polyacrylamide gels that were subsequently dried and autoradiographed. Reactions were set up so that the intensity of the autoradiographic signals for the native mRNA and competitor cRNA RT-PCR products were approximately the same to avoid inaccuracies encountered due to the narrow linear response range of X-ray film. The intensities of the autoradiograph signals from the RT-PCR products of the native mRNA and competitor cRNA were calculated using a laser densitometer and the appropriate attached software (Biorad). The amount of Brn3a, Brn3b or GAPDH mRNAs in each total RNA sample was calculated from these values.
References


[27] N.G.N. Milton, A. Bessis, J.P. Changeux, D.S. Latchman, The neuronal acetylcholine receptor alpha2 subunit gene promoter is activated by the Bm3b POU transcription factor and not by Bm3a or Bm3c, J. Biol. Chem. 270 (1995) 15143–15147.


[38] M. Wegner, D.W. Drolet, M.G. Rosenfeld, POU-domain proteins:


[41] E.M. Wright, K.S. Vogel, A.M. Davies, Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival, Neuron 9 (1992) 139–150.


