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A role for the POU-III transcription factor Brn-4 in the regulation of striatal neuron precursor differentiation

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Both insulin-like growth factor-I (IGF-I) and brain-derived neurotrophic factor (BDNF) induce the differentiation of post-mitotic neuronal precursors, derived from embryonic day 14 (E14) mouse striatal multipotent stem cells. Here we ask whether this differentiation is mediated by a member of the POU-III class of neural transcription factors. Exposure of stem cell progeny to either IGF-I or BDNF resulted in a rapid upregulation of Brn-4 mRNA and protein. Indirect immunocytochemistry with Brn-4 antiserum showed that the protein was expressed in newly generated neurons. Other POU-III genes, such as Brn-1 and Brn-2, did not exhibit this upregulation. Basic FGF, a mitogen for these neuronal precursors, did not stimulate Brn-4 expression. In the E14 mouse striatum, Brn-4-immunoreactive cells formed a boundary between the nestin-immunoreactive cells of the ventricular zone and the β-tubulin-immunoreactive neurons migrating into the mantle zone. Loss of Brn-4 function during the differentiation of stem cell-derived or primary E14 striatal neuron precursors, by inclusion of antisense oligonucleotides, caused a reduction in the number of β-tubulin-immunoreactive neurons. These findings suggest that Brn-4 mediates, at least in part, the actions of epigenetic signals that induce striatal neuron-precursor differentiation.

Keywords: Brn-4/differentiation/neurogenesis/stem cells/striatum

Introduction

Development of the mammalian central nervous system (CNS) can be broadly divided into regional specification (Lumsden and Krumlauf, 1996; Rubenstein and Shimamura, 1997) followed by unique regional production of neuronal and glial cells. In both cases an interplay between environmental signals and intrinsic genetic programs underlies phenotype specification. In the basal forebrain, the Dlx family members of the homeobox gene family are expressed in a manner suggesting a role in both regional specification and neuronal production (reviewed in Rubenstein and Shimamura, 1997). In fact, deletion of both Dlx-1 and Dlx-2 resulted in disruption of the subventricular zone and differentiation of a late-born (perinatal) population of striatal neurons (Anderson *et al.*, 1997). Given that striatal neurogenesis begins at embryonic day 11 (E11) (Angevine and McConnel, 1974) and that differentiated neurons appear soon after E11 (Menezes and Luskin, 1994), it seems likely that other genes and environmental cues play a role in neuronal differentiation throughout striatal histogenesis.

We have isolated a self-renewing stem cell from the embryonic and adult mouse striatum that proliferates in response to epidermal growth factor (EGF) and produces progenitor cells that can differentiate into neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992, 1996; Reynolds *et al*., 1992; Weiss *et al*., 1996). Despite extensive characterization of the *in vitro* properties of these stem cells, the steps through which new neurons are produced are understood only partially. We have found that two types of stem cell-derived neuronal precursors, bipotent (neuron/astrocyte producing) and unipotent (neuron producing only), are stimulated to divide by basic fibroblast growth factor (bFGF; Vescovi *et al*., 1993). Subsequently, both brain-derived neurotrophic factor (BDNF; Ahmed *et al*., 1995) and insulin-like growth factor-I (IGF-I; Arsenijevic and Weiss, 1998) are able to enhance the differentiation of the post-mitotic neuronal precursors (without affecting neuronal survival). Two important observations from the latter study fueled our present investigation. First, in the absence of either BDNF or IGF-I, little or no neuronal differentiation was observed. Secondly, an exposure of neuronal precursors to IGF-I for as little as 2 h was sufficient to induce complete neuronal differentiation. This suggested to us that IGF-I initiated a genetic program that proceeded to drive post-mitotic neuronal precursors to terminal differentiation, such as the acquisition of the neuronal phenotype.

POU homeodomain-containing genes are a subclass of homeobox genes that comprise six or more families (POU-I to -VI; Ryan and Rosenfeld, 1997). The POU genes were identified originally as transcription factors that regulate cell type-specific gene expression in mammals (Bodner *et al*., 1988; Clerc *et al*., 1988; Ingraham *et al*., 1988; Ko *et al*., 1988; Scheidereit *et al*., 1988) and act as cell-fate determinant genes in nematodes (Finney *et al*., 1988). Null mutation and loss-of-function studies have provided some insights as to the functions of various POU families. For example, mutations in the POU-I gene Pit-1 resulted in the loss of three pituitary cell lineages (Li *et al*., 1990). As regards neural tissue, null mutations of the POU IV genes Brn-3.0/3/3a, Brn-3.1/3c and Brn-

3.2/3b, which are normally expressed in the developing mammalian CNS and peripheral nervous system (PNS), resulted in the loss of specific neuronal subsets through defects in terminal differentiation or survival (Erkman *et al*., 1996; Gan *et al*., 1996; McEvilly *et al*., 1996; Xiang *et al*., 1996; Xiang, 1998). In support of such conclusions, inhibition of Brn-3.0/3/3a expression was found to attenuate neurite outgrowth of cultured neuroblastoma cells (Lakin *et al*., 1995). Within the POU families, the POU-III class of transcription factors (Brn-1, Brn-2, Brn-4/RHS2 and Oct-6/SCIP/Tst-1) may be of particular interest for neuronal development, given that they are all expressed in the CNS during the peak periods of neurogenesis (Alvarez-Bolado *et al*., 1995). In this respect, Brn-2 disruption resulted in the loss of thyrotropin-releasing hormone-, vasopressin- and oxytocin-containing neurons in the developing paraventricular and supraoptic nuclei (Nakai *et al*., 1995; Schonemann *et al*., 1995) and block of Brn-2 function prevented pluripotential P19 cells from generating a neuronal lineage (Fujii and Hamada, 1993). Nevertheless, the specific roles of POU-III genes in mammalian CNS neurogenesis remain to be determined.

Given the expression patterns of POU-III transcription factors during mammalian CNS neurogenesis (Alvarez-Bolado *et al*., 1995), we examined whether members of this class of transcription factors participate in post-mitotic striatal neuron precursor differentiation. We found an upregulation of Brn-4 in E14 striatal stem cell-derived neuronal precursors by both IGF-I and BDNF. In addition, the differentiation of both stem cell-derived neuronal precursors and those from primary dissociates of the E14 striatum could be attenuated by Brn-4 antisense oligonucleotides. *In vivo*, Brn-4-immunoreactive cells formed a boundary between the nestin-immunoreactive cells of the E14 striatal ventricular zone and the β-tubulin-immunoreactive cells migrating into the mantle zone. These data suggest that Brn-4 plays an important role in neuronal precursor differentiation, perhaps as a downstream effector of epigenetic signals.

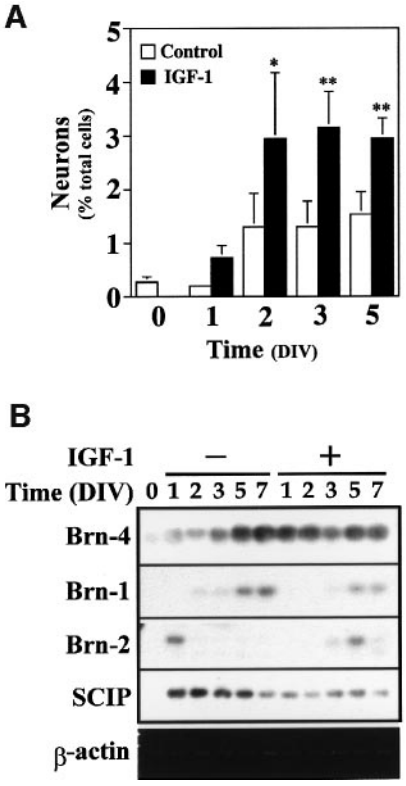

Fig. 1. Time course of IGF-I-induced neuronal differentiation and POU-III gene expression in EGF-generated CNS stem cell progeny. Undifferentiated EGF-generated spheres were dissociated and plated onto poly-L-ornithine-coated coverslips for immunocytochemistry at 10^5 cells/ml or in plastic dishes at 10^6 cells/ml for PCR-Southern analysis, in the absence or presence of 10 nM IGF-1 for 1–7 DIV. (**A**) An enhanced number of neurons (β-tubulin-immunoreactive cells) is observed after 2 DIV with IGF-I. The bars indicate means and the error bars the standard error of the mean (SEM). The number of neurons were counted for thirty 40× fields. $*p < 0.05$, $**p < 0.01$ (Mann–Whitney *U* test) versus both controls at 0 days and each time point ($n = 6$ independent cultures). (**B**) IGF-I enhances Brn-4 expression during the first 2 DIV. At each time point, PCR-Southern analysis was performed to measure gene expression. The RT–PCR of each POU-III gene was normalized by the amplified β-actin signal in the same reaction (internal control) to obtain quantitative results. An example is illustrated at the bottom of the figure.

Results

Brn-4 is an IGF-I-responsive gene whose expression correlates with the differentiation of post-mitotic neuronal precursors

We have shown previously that single E14 striatal EGF-responsive neural stem cells produce precursors to neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1996). Differentiation of the neuronal precursors, but not the glia, is enhanced dramatically by either BDNF or IGF-I (Ahmed *et al*., 1995; Arsenijevic and Weiss, 1998). We found that IGF-I induces a rapid differentiation of neuronal precursors (as little as 2 h exposure is sufficient), but does not act upon proliferation or survival. In order to determine whether any of the POU-III transcription factors play a role in IGF-I-stimulated neuronal-precursor differentiation, we examined the gene expression patterns of Brn-1, Brn-2, Brn-4 and SCIP using RT–PCR Southern blotting. Populations of EGF-generated precursors were plated in the absence or presence of 10 nM IGF-I, for varying periods, and the resultant cultures were analyzed for neuronal numbers and gene expression. (Figure 1). As we have previously observed (Arsenijevic and Weiss, 1998), IGF-I induced a 2- to 4-fold increase in neuronal numbers (type III β-tubulin-immunoreactive cells with neuronal morphology) which was apparent after 2 days *in vitro* (DIV; Figure 1A). Of the four POU-III genes examined in sister cultures, only Brn-4 expression was enhanced significantly by IGF-I (Figure 1B). The increase in Brn-4 expression due to IGF-I was detected as early as 1 DIV prior to the enhancement of neuronal differentiation. The relative difference in Brn-4 expression in the control versus IGF-I was most apparent at 1 and 2 DIV when IGF-I-enhanced neuronal differentiation was complete. Nevertheless, even in the absence of IGF-I, Brn-4 expression increased and, at later stages, reached levels similar to those seen in the presence of IGF-I. Neuronal differentiation also increased in the absence of IGF-I, albeit to a very limited extent. These data suggest that the early actions of IGF-I are associated with enhanced Brn-4 expression, while at later stages Brn-4 expression is IGF-I independent. The identical pattern of Brn-4 expression was observed in three inde-

pendent culture preparations. Other than Brn-4, only Brn-2 expression increased in the presence of IGF-I; however, this increase was transient and occurred after IGF-I enhancement of neuronal differentiation. In contrast, the expression of both Brn-1 and SCIP decreased in the presence of IGF-I. These results indicate that Brn-4 is a candidate molecule amongst the POU-III family of genes, for mediating growth factor-induced neuronal differentiation.

Both IGF-I and BDNF induce the rapid upregulation of Brn-4 mRNA and protein expression in post-mitotic neuronal precursors. In previous work we have shown that IGF-I-dependent neuronal differentiation could be maximally induced with as little as 2–24 h of exposure to the factor, without any influence on glial cell differentiation (Arsenijevic and Weiss, 1998). Moreover, both through bromodeoxyuridine incorporation studies and by waiting 48 h after plating, by which time all intrinsic mitotic activity stopped, we found that IGF-I's differentiating actions were restricted to post-mitotic neuronal precursors. Thus, in the next series of experiments, EGF-generated stem cell progeny were plated in the absence of added growth factors for 48 h. Subsequently, we examined the expression of Brn-4 and neuronal differentiation at several intervals within the first 24 h after exposure to 10 nM IGF-I. Within 2 h of exposure to IGF-I, a 30- to 100-fold increase in Brn-4 gene expression could be detected in stem cell progeny (Figure 2A). Furthermore, Brn-4 protein expression, detected with an antiserum to Brn-4, was increased after 6 h of IGF-I stimulation (Figure 2B; representative of three independent experiments). In sister cultures, a 3-fold enhancement of neuronal numbers (β-tubulin-immunoreactive cells) was detected 6 or 24 h after IGF-I stimulation (Figure 2D). These data suggest that IGF-I-induced increases in Brn-4 gene and protein expression immediately precede enhanced neuronal differentiation.

The presence of two bands in the Western blot (Figure 2B) prompted us to confirm Brn-4 antiserum specificity, in particular before proceeding to the use of indirect immunocytochemistry. Thus, we transfected NIH 3T3 cells with the full-length Brn-4 cDNA ligated into a CMV promoter-driven expression vector (pCI). Western blots of whole-cell extracts of the transfected cells and E14 whole brain showed two bands (Figure 2C), identical to those seen in stem cell progeny (Figure 2B).

These findings suggest a tight coupling between IGF-I actions on Brn-4 mRNA and protein expression and neuronal precursor differentiation. To confirm that Brn-4 expression indeed correlates with neuronal differentiation, we performed indirect immunocytochemistry. Forty-eight hours after plating, stem cell progeny were exposed to IGF-I for 6 h, fixed with paraformaldehyde and processed for dual-label immunocytochemistry for Brn-4 and type III β-tubulin (Figure 3). β-tubulin-immunoreactive neurons (Figure 3A) were indeed immunoreactive for Brn-4 (Figure 3B). However, Brn-4 immunoreactivity was not restricted to β-tubulin-immunoreactive cells, but was also detected largely in undifferentiated cells with neuron-like morphology and to a minor extent in some presumptive glial cells (Figure 3; data not shown). We quantified the numbers of β-tubulin-immunoreactive cells that expressed Brn-4, either in control cultures or those exposed to IGF-I

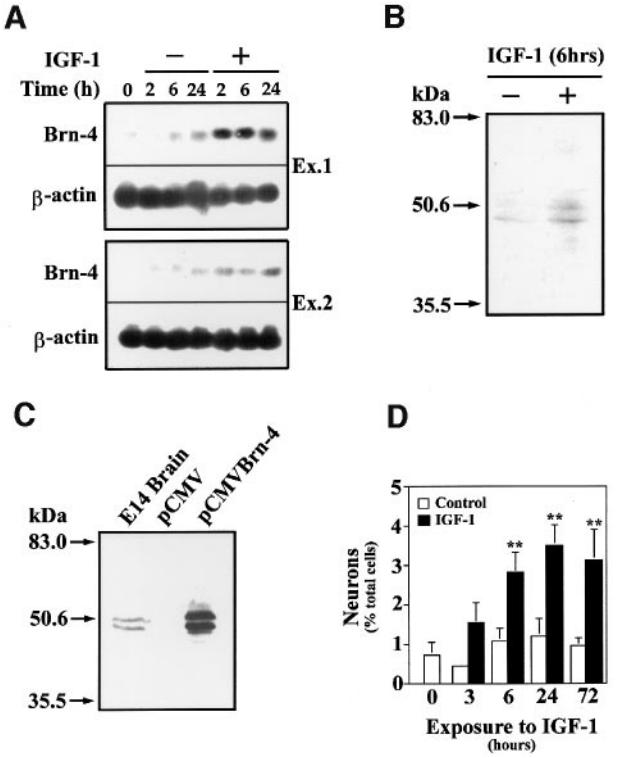

Fig. 2. A rapid increase in Brn-4 gene and protein expression due to IGF-I is associated with enhanced neuronal differentiation. EGF-generated stem cell progeny dissociation and plating and the RT–PCR Southern blotting were carried out as described in the legend for Figure 1. In these experiments, however, the cells were allowed to equilibrate (cessation of proliferation) without added growth factors for 2 DIV after plating. (**A**) Significant increases in Brn-4 mRNA expression were observed 2 h after administration of 10 nM IGF-I. The results of two independent culture experiments are illustrated. (**B**) Western blot analysis (see Materials and methods for details) shows a significant increase in two different sizes of Brn-4 protein expression after 6 h exposure to IGF-I. (**C**) The two forms of Brn-4 protein detected in stem cell progeny (Figure 2B) are also found in E14 brain, and can be produced by Brn-4 expression vector (pCMVBrn-4) transfected into NIH 3T3 cells but not in the cells transfected by pCMV control vector plasmids. (**D**) IGF-I increases the numbers of β-tubulin-positive cells rapidly. Stem cell progeny were processed for β-tubulin immunocytochemistry after incubation in the absence or presence of 10 nM IGF-I for 3, 6, 24 and 72 h. The number of neurons were counted as described in the legend to Figure 1. $^{**}p < 0.01$ (Mann–Whitney *U* test) versus both controls at 0 h and each time point ($n = 3$ independent cultures).

(10 nM) or BDNF (50 ng/ml) for 6 h (Table I). Virtually all β-tubulin-immunoreactive cells also expressed Brn-4, the number of double-labeled cells increased in the presence of IGF-I, and BDNF-treated cultures behaved identically to those that had been treated with IGF-I. The total number of Brn-4-immunoreactive cells exceeded the numbers of differentiating neuronal precursors, although many of the immunopositive cells exhibited weak staining. The IGF-I- or BDNF-induced increase of Brn-4-immunoreactive cells was in ~10% of total cells, while 2–3% of the total cells became differentiated neurons. Increases in, and the most intense immunoreactivities appeared selectively in the immature neurons and undifferentiated cells with clear neuron-like morphology. These data suggest that both factors, known to induce differentiation of neuronal precursors, induce a similar increase in β-tubulin-/Brn-4-immunoreactive cells.

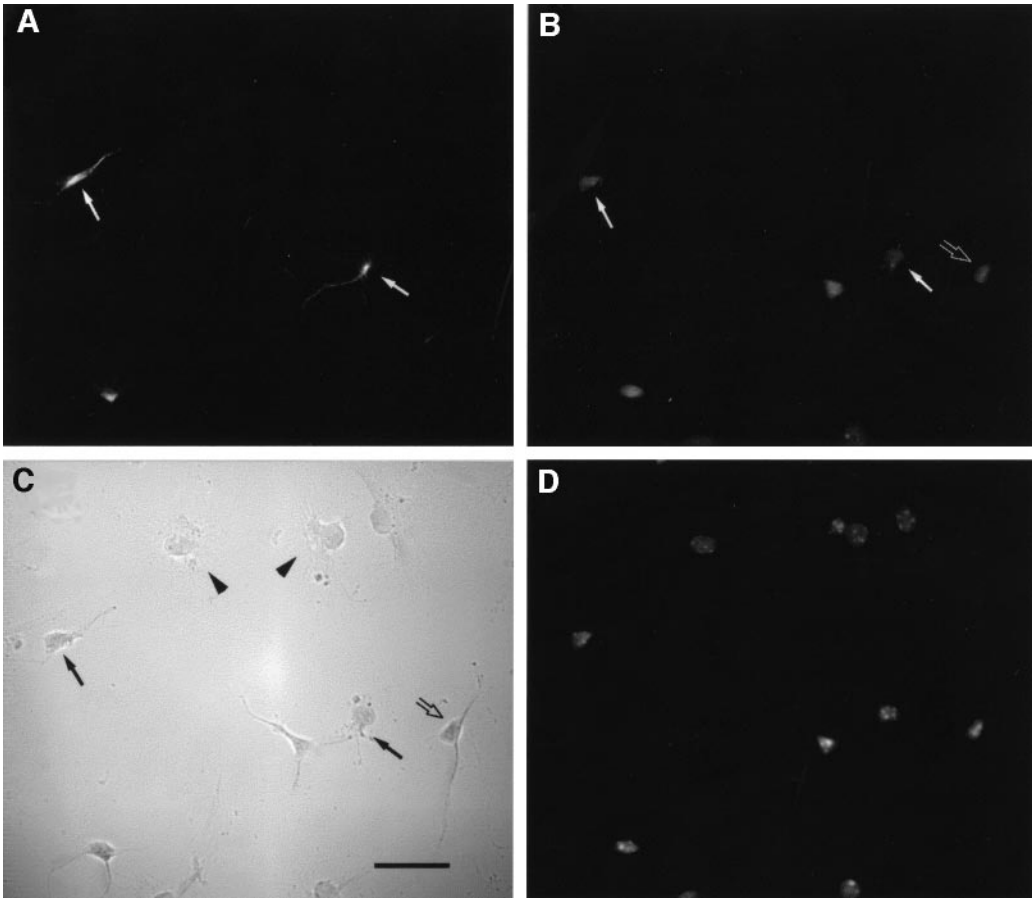

Fig. 3. Brn-4 is expressed in β-tubulin-immunoreactive neurons. After dissociation and equilibration for 2 DIV, EGF-generated stem cell progeny were exposed to 10 nM IGF-I for 6 h. Cells were immediately fixed and processed for immunocytochemistry. Dual-label, indirect immunocytochemistry found cells (see arrows) that were immunoreactive for the neuronal marker β-tubulin (**A**) and for Brn-4 (**B**). A cell with presumptive neuronal morphology (open arrow in B and C) was also Brn-4 immunoreactive. In this field, non-neuronal cells, arrowheads in (**C**), were not immunoreactive for Brn-4. (C) and (B) are phase contrast and Hoechst nuclear stains, respectively, of the same field. Scale bar, 25 μm.

Table I. The numbers of Brn-4-immunoreactive neurons increase in response to IGF-I and BDNF

Experiment	Condition	No. β-tubulin immunoreactive cells (% control)	No. β-tubulin +Brn-4 immunoreactive cells (% β-tubulin)	No. Brn-4-immunoreactive cells (% total cells)
1	Control	132	114 (86)	4241 (24)
	IGF-I	483 (366)	448 (93)	6340 (38)
2	Control	246	195 (79)	5026 (33)
	IGF-I	629 (256)	563 (90)	6703 (41)
1′	Control	28	26 (93)	1560 (11)
	BDNF	69 (246)	64 (93)	2325 (17)
2′	Control	161	134 (83)	2141 (12)
	BDNF	260 (161)	224 (86)	3459 (21)

[a]Cells were processed as described in the legend to Figure 3. All of the cells on the coverslip were counted in each experiment. The immunofluorescence in each cell varied in intensity.

Next we examined the specificity of IGF-I and BDNF actions on Brn-4 gene expression, in comparison with other factors that influence neuronal precursor biology. Basic FGF, a mitogen for EGF-generated neuronal precursors (Vescovi *et al.*, 1993), had no effect on Brn-4 expression (Figure 4A). On the other hand, neurotrophin 3 (NT-3), which we have recently found to influence neurite outgrowth of stem cell-derived neuronal precursors (Y.Arsenijevic and S.Weiss, unpublished observations), induced a small but detectable increase in Brn-4 expression. While our previous study suggests that distinct populations of stem cell-derived neuronal precursors respond to IGF-I and BDNF (Arsenijevic and Weiss, 1998), the rapid upregulation seen by IGF-I (Figure 2A) is mimicked by BDNF (Figure 4B). These findings suggest that, regardless of the populations examined, epigenetic factors that enhance neuronal precursor differentiation specifically do so, at least in part, by upregulating Brn-4 expression.

The pattern of Brn-4 expression *in situ* or in primary

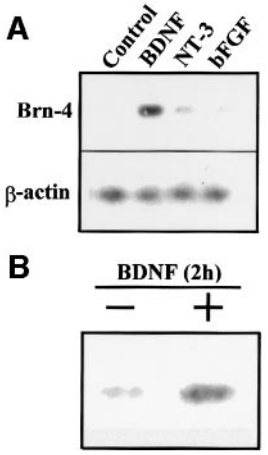

Fig. 4. BDNF mimics the actions of IGF-I on Brn-4 gene expression. Treatment of the cells and RT–PCR Southern blotting were carried out as described in the legends for Figures 1 and 2. (**A**) Actions of other growth factors on Brn-4 gene expression was analyzed using RT–PCR Southern blotting. Cells were exposed to the growth factors (BDNF, 50 ng/ml; bFGF, 20 ng/ml; NT-3, 100 ng/ml) for 6 h. (**B**) BDNF treatment upregulates Brn-4 mRNA as rapidly as IGF-I (2 h).

dissociates of the E14 striatum supports an *in vivo* role in neuronal differentiation. Although Brn-4 gene expression has been localized to the forebrain subventricular zone (SVZ) throughout development (Alvarez-Bolado *et al.*, 1995), neither its precise cellular phenotype nor its role in neurogenesis has been determined. In order to determine the Brn-4 cell-type specificity and consequently predict the function of Brn-4 in neurogenesis in the developing striatum, we performed dual-label immunohistochemistry with combinations of anti-Brn-4, anti-β-tubulin and anti-nestin (as a marker for proliferating progenitor cells; Dahlstrand *et al.*, 1995) antibodies. At E14, a peak period of neurogenesis in the striatum, intense Brn-4 immunoreactivities appear in a boundary between nestin-immunoreactive proliferating cells and the β-tubulin-immunoreactive area in the SVZ (Figure 5A and B). The front line of Brn-4-expressing cells, which were immediately adjacent to the nestin-immunoreactive zone, appeared to be devoid of β-tubulin immunoreactivity, suggesting that Brn-4 expression begins when the progenitors are exiting the cell cycle. A virtually identical pattern was observed in the thalamic primordia or epithalamus (Figure 5C). Next we examined Brn-4 expression in primary dissociates of the E14 striatum, which undergo virtually complete differentiation within 72 h of plating. At initial plating (2 h, Figure 5E and H), virtually all cells were Brn-4 immunoreactive. While both the cell number and the numbers of Brn-4-immunoreactive cells remained constant, by 6 and 24 h (Figure 5F, I and G, J, respectively) 30 and 50% of these cells became β-tubulin immunoreactive, respectively. Fewer than 1% of these cells incorporated bromodeoxyuridine after 72 h (data not shown), suggesting that the majority of Brn-4-expressing neuronal precursors are post-mitotic. On the other hand, Brn-4 immunoreactivity in some neurons that have strong β-tubulin immunoreactivity and more fully differentiated morphology tends to be diminished. Taken together these observations indicate that Brn-4 expression precedes and is associated with the early differentiation of neuronal precursors *in vivo*.

Next we asked whether IGF-I could enhance Brn-4 expression and neuronal differentiation in primary cultures of the E14 striatum. Surprisingly, exposure of E14 striatal cells to 10 nM IGF-I did not enhance either Brn-4 expression or neuronal differentiation as robustly as was observed in cultures of *in vitro*-generated EGF-responsive striatal stem cell progeny. Addition of IGF-I increased neuronal differentiation by only 15% (Figure 6A) while Brn-4 expression increased substantially in control cultures (without added IGF-I) within 2 h of plating (Figure 6B). This prompted us to ask whether auto/paracrine production and action of IGF-I enhances neuronal differentiation in the primary E14 striatal dissociates. RT–PCR analysis showed expression of both IGF-I and its receptor in the primary E14 striatal cultures (2 h after plating) (Figure 6C). Thus, we proceeded to examine whether IGF-I might be secreted into the medium and act on neuronal differentiation through increases in Brn-4 expression. This was done in two ways and the results are shown in Table II and Figure 7. First, culturing the primary E14 striatal dissociates at a 10-fold lower density (10^4 cells/ml) largely prevented the increase in neuronal differentiation that occurred at high density (10^5 cells/ml) in the absence of IGF-I (Table II). In these low-density cultures, further addition of IGF-I significantly increased neuronal differentiation. Secondly, a saturating concentration of anti-IGF-I-neutralizing antibodies (30 μg/ml) completely and specifically blocked the autonomous increase in neuron number (Table II) and Brn-4 expression (Figure 7A) in the higher-density cultures. Indirect immunocytochemistry showed that the decrease in Brn-4 expression was due to a reduction in protein levels in each neuronal precursor with no significant changes in the number of Brn-4-immunoreactive cells (Figure 7B). These results suggest that an autocrine/paracrine action of IGF-I acts to increase Brn-4 expression and promote neuronal differentiation in primary precursors of the embryonic striatum.

Neutralization of Brn-4 synthesis attenuates neuronal differentiation

In order to establish the link between Brn-4 production and neuronal differentiation, we sought to disrupt mRNA function with the use of antisense oligonucleotides. Phosphorothionate-modified antisense oligonucleotides complementary to the proximal translation initiation site (position –9 to +10) of Brn-4 mRNA were synthesized. In the first series of experiments, 48 h after plating of stem cell progeny, cultures were exposed to 10 nM IGF-I for 72 h in the absence or presence of antisense or sense oligonucleotides, fixed and processed for β-tubulin immunocytochemistry. The inclusion of antisense oligonucleotides to Brn-4 resulted in a >50% reduction in neuron numbers (Figure 8A). On the other hand, sense oligonucleotides were entirely without effect (see the absolute numbers of cells in the legend for Figure 8). In these experiments, when antisense oligonucleotides were added 24 h after IGF-I (delayed), there was no reduction in neuron number. The latter result both confirms the specific effect of the antisense oligonucleotides on the neuronal precursor population and suggests that Brn-4 expression is not required for neuronal survival, but rather for the early events associated with growth factor-stimulated neuronal differentiation. In addition, inclusion

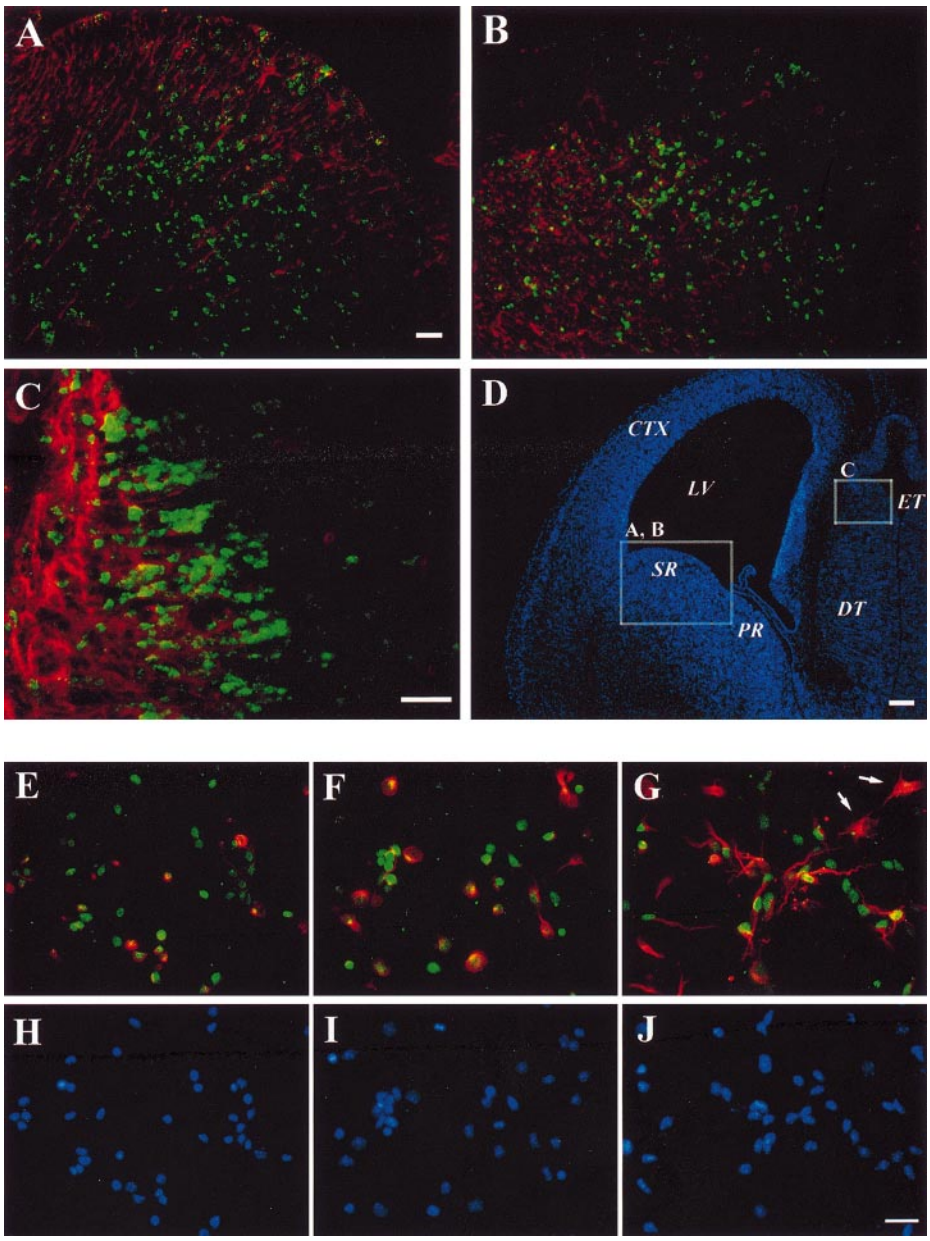

Fig. 5. Brn-4 expression precedes neuronal differentiation *in vivo* and *in vitro.* Indirect double immunofluorescence micrographs of anti-Brn-4 (green) and either anti-nestin or anti-β-tubulin (both red) in coronal sections of the E14 forebrain (A–D) and primary cultures of E14 striatum (E–J) are illustrated. (**A**) Most of Brn-4-immunoreactive cells (green) in the SVZ of the E14 striatum are not co-localized with nestin (red). (**B** and **C**) In both the striatum (B) and epithalamus (C), the Brn-4-immunoreactive cells (green) form bands that are immediately adjacent to newly-emerging β-tubulin-immunoreactive neurons (red). (**D**) A Hoechst staining of a forebrain section used for the immunostaining. CTX, cortex; DT, dorsal thalamus; ET, epithalamus; PR, pallidal ridge; SR, striatal ridge. Open squares show areas for higher magnification micrographs (A, B and C). (**E**–**J**) Dissociated striatal cells from E14 mouse embryo were cultured as described in the Materials and methods. Virtually all cells (all β-tubulin-immunoreactive cells and 70% of undifferentiated cells) are Brn-4 immunoreactive at 2 h after plating in the presence of 10 nM IGF-1 (E). After 6 (F) and 24 (G) h, double-labeled (for Brn-4 and β-tubulin) cell numbers increase in a time-dependent manner. Some neurons showing strong β-tubulin immunoreactivity were not Brn-4 immunoreactive (arrows). (H–J) are Hoechst nuclear stains for (E–G), respectively. Scale bars, 25 μm (A–C and E–J); 100 μm (D).

of antisense oligonucleotides in primary E14 striatal cultures 2 h after plating (allowing for cell attachment) and for 48 h thereafter resulted in a significant reduction both of neurons (50% of newly differentiated neurons) and total cell number (same as reduced number of neurons). There was no significant reduction of neuronal differentiation when either sense or missense oligonucleotides (Figure 8B) were added to these cultures. The missense oligonucleotides differed from the antisense construct by only two nucleotides, further confirming the specificity of the antisense actions. To demonstrate that administration of antisense oligonucleotides indeed results in a decreased level of functional Brn-4, Western blot analyses were performed. Primary striatal cultures were stimulated with 10 nM IGF-I for 24 h, in the absence or presence of sense, missense or antisense oligonucleotides. The inclusion of antisense oligonucleotides to Brn-4 mRNA caused a marked, selective reduction in the levels of

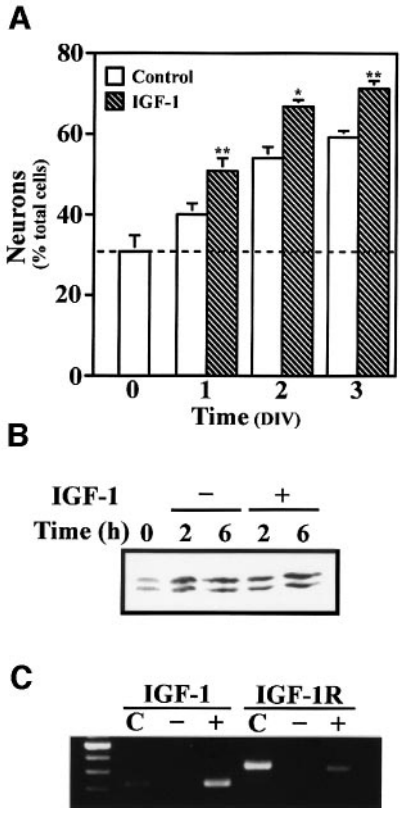

Fig. 6. Neuronal differentiation and Brn-4 expression in primary cultures of E14 striatum. Cells from mouse E14 striatum were cultured as described in Materials and methods. (**A**) Exogenous IGF-I slightly enhanced neuronal differentiation. At 2 h after plating (0 DIV), ~30% of cells are already differentiated neurons. The bars indicate means and the error bars the standard error of the mean (SEM). The number of neurons were counted for twenty 20× fields. *p <0.05, **p <0.01 (Mann–Whitney U test) versus both controls at 0 DIV and each time point. (n = 4 independent cultures). (**B**) Western blot analysis shows that Brn-4 protein expression increases rapidly even without the addition of IGF-I. (**C**) RT–PCR analysis shows that both IGF-I and IGF-I receptors are expressed in dissociated cultures of the striatum. C represents control tissue (E14 whole brain cDNA), and the '±' signs illustrate presence or absence of reverse transcription reaction.

Table II. IGF-I is a differentiation factor for neuronal precursors from the E14 striatum[a]

Treatment	Neurons (% of total cells ± SEM)	
	High density (10^5 cells/ml)	Low density (10^4 cells/ml)
At plating[b]	30.7 ± 3.9	31.6 ± 1.8
Control	59.0 ± 1.5	34.7 ± 2.5
IGF-1 (10 nM)	71.0 ± 1.9*	52.5 ± 5.3**
Mouse IgG (30 μg/ml)	54.9 ± 2.7	n.d.
Anti-IGF-I (30 μg/ml)	27.9 ± 3.7[c]	n.d.

[a]Cells derived from E14 striatum were cultured for 72 h and processed for immunocytochemistry. [b]Cells were fixed 2 h after plating to determine the number of differentiated neurons at plating. *p <0.05, **p <0.01 versus control, [c]p <0.01 versus mouse IgG (n=4). n.d., not determined.

Brn-4 protein (Figure 8C), whereas sense and missense oligonucleotides were without effect. Taken together, these data suggest that increased levels of intact Brn-4 are required, at least in part, for IGF-I-induced neuronal differentiation. Forced reduction of Brn-4 expression during differentiation may ultimately enhance neuronal cell death.

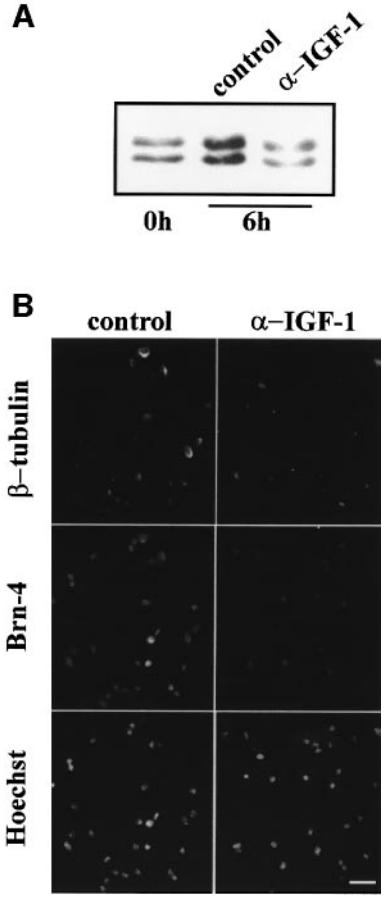

Fig. 7. Anti-IGF-I neutralizing antibodies inhibit up-regulation of Brn-4 expression in primary cultures of E14 striatum. E14 striatal cells were cultured as described in the legend to Figure 6. Thirty micrograms per milliliter of anti-IGF-I mouse monoclonal antibodies or control mouse IgG were administrated 2 h after plating. (**A**) The time-dependent increase of Brn-4 expression is blocked in the presence of the anti-IGF-I antibodies, as observed with Western blotting analysis. (**B**) Indirect immunocytochemistry shows that in the presence of anti-IGF-I antibodies, both β-tubulin and Brn-4 immunoreactivities were significantly reduced. Cells were fixed 6 h after the treatments. There was no significant change in the numbers of Brn-4-immunoreactive cells, rather there was a general reduction in the intensity of immunoreactivity per cell.

Discussion

These results lead us to conclude that the POU homeodomain-containing gene Brn-4 is involved in the growth factor-regulated differentiation of neuronal precursors, whether derived from striatal EGF-responsive CNS stem cells or directly from the E14 striatum. Two key observations support this conclusion. First, both IGF-I and BDNF, which are differentiation factors for post-mitotic neuronal precursors (Ahmed *et al*., 1995; Arsenijevic and Weiss, 1998), upregulate Brn-4 expression in a rapid fashion (within 2 h; a time previously shown to be sufficient to induce neuronal differentiation). Secondly, antisense oligonucleotides, which hybridize to the 5′-translation initiation site of Brn-4 mRNA, markedly blocked IGF-I-induced neuronal differentiation. Moreover, the expression pattern of Brn-4 protein in undifferentiated

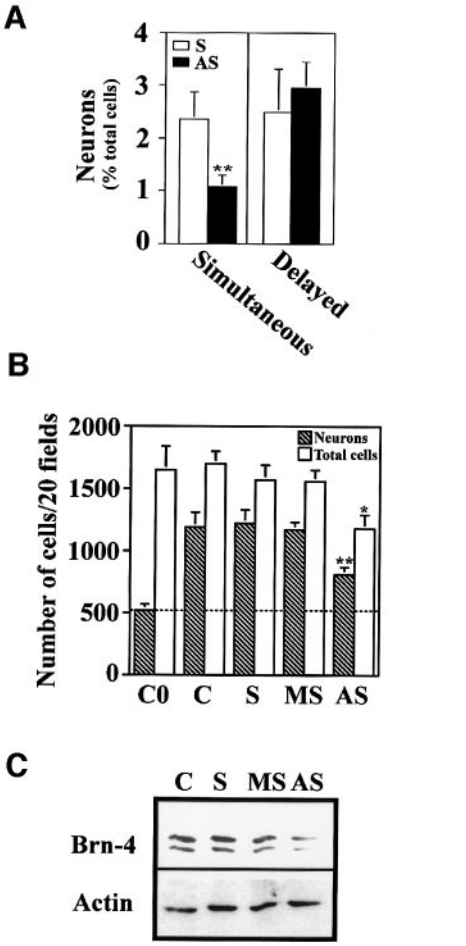

Fig. 8. Brn-4 antisense oligonucleotides attenuate IGF-I-induced neuronal differentiation and Brn-4 protein synthesis. (**A**) Antisense oligonucleotides attenuate neuronal differentiation but do not appear to influence neuronal survival. At 2 DIV, cells were stimulated for 72 h with 10 nM of IGF-I in the presence of Brn-4 sense (S) or antisense (AS) oligonucleotides (10 μM each). The total number of cells and those immunoreactive for the neuronal marker β-tubulin were compared with cells incubated with IGF-I in the absence of oligonucleotides (control). Average numbers of total cells and neurons in twenty 20× fields (mean ± SE) are: control, 841 ± 145 and 26 ± 5; sense, 975 ± 197 and 27 ± 6; antisense, 875 ± 191 and 12 ± 3**, respectively. $^{**}p < 0.01$ (Mann–Whitney *U* test) versus sense ($n = 4$ independent cultures). Delayed administration of oligonucleotides for 24 h after IGF-I stimulation resulted in no decrease in IGF-I-induced neuronal differentiation ($n = 4$). (**B**) A 48 h exposure of primary E14 striatal cultures to antisense oligonucleotides resulted in a reduction in both the number of neurons and total cells. Ten micromoles of each oligonucleotide (MS, missense) were co-administered with IGF-I at 2 h after plating. C0 is the number of neurons and total cells at time point 0 (2 h after plating), while C represents no added oligonucleotides for 48 h. $^{*}p < 0.05$, $^{**}p < 0.01$ ($n = 5$) versus C, S and MS. (**C**) Antisense oligonucleotides attenuate Brn-4 protein synthesis. Western blot of Brn-4 protein expression and a β-actin isoform following IGF-I exposure for 24 h, in the absence (C, control) or presence of either S, MS or AS oligonucleotides. Reduction in protein was seen only in the presence of AS oligonucleotides. Identical results were obtained using three independent batches of each oligonucleotides.

and recently differentiated neuronal precursors *in vitro* and *in vivo*, further supports a key role for Brn-4 in neuronal differentiation. A recent report of Dlx-1/2 null mutants, which resulted in disruption of the striatal SVZ and neuronal differentiation, also resulted in extinction of Brn-4 expression (Anderson *et al*., 1997). Taken together, these findings further suggest that Brn-4 is implicated in differentiation of neurons of the developing striatum.

We examined the expression of all the POU-III transcription factors during the differentiation of EGF-generated neuronal precursors. Only Brn-4 expression increased immediately (2 h) after the addition of IGF-I, preceding the onset of neuronal differentiation. BDNF stimulated Brn-4 expression in a similar fashion to that of IGF-I. The fact that BDNF-mediated signaling is restricted to the neuronal but not the glial lineage of EGF-generated stem cell progeny (Ahmed *et al*., 1995) reinforces the critical role for Brn-4 on neuronal precursors. These observations suggest that Brn-4 levels appear to correlate with the differentiation of neurons. Brn-4 expression levels increased somewhat even in the absence of added IGF-I or BDNF, suggesting that Brn-4 induction may also underlie neuronal differentiation that is independent of these factors or due to auto/paracrine stimulation as we observed in primary striatal cultures. Our previous studies have shown that IGF-I and BDNF can induce neuronal differentiation by EGF-responsive stem cell progeny even 5 days after plating (Ahmed *et al.*, 1995; Arsenijevic and Weiss, 1998), indicating that neuronal precursors can survive for this period without adding any known trophic molecules. In addition, BDNF and IGF-I act on distinct populations of neuronal precursors. Therefore, it is possible that an additional, as yet unknown, endogenous factor enhances Brn-4 expression in a population of neuronal precursors that are distinct from those responsive to BDNF and IGF-I. In that case, the level of Brn-4 expression itself may not be sufficient for the full differentiation of that population, perhaps requiring sustained or additional concentrations of a unique differentiation factor.

We also observed a later increase in Brn-4 gene expression that was IGF-I-independent and may be due to non-neuronal cell types. In fact, we have observed low levels of Brn-4 expression in some, but not all, astroglial cells (data not shown), suggesting an additional role for the transcription factor in CNS development. Such possible pleiotropic actions of Brn-4 may not be surprising given that other POU-III genes are found to be expressed in both neuronal and glial cells in the developing CNS (Ryan and Rosenfeld, 1997; Schreiber *et al*., 1997). Moreover, compelling evidence shows that Tst-1/Oct-6/SCIP is required for the terminal differentiation of Schwann cells and for the differentiation of specific neurons (Frantz *et al.*, 1994; Bermingham *et al.*, 1996; Jaegle *et al.*, 1996). Thus, it is possible that Brn-4 plays an as yet undetermined role in astroglial cell development as well as in neuronal differentiation. Of course, this in no way minimizes the critical role for Brn-4 in IGF-I- or BDNF-induced differentiation of EGF-generated neuronal precursors. Furthermore, the evidence for an *in vivo* role for Brn-4 during peak striatal neurogenesis (Figure 5), coupled with its function in primary embryonic striatal cultures (Figure 7 and Table II) in which virtually all of the cells become neurons, further supports our conclusion.

There have been very few reports of neurotrophic factor induction of developmentally regulated transcription factors, such as POU or Pax family members. Two distinct classes of POU factors, Oct-2 and Brn-3a/3.0, were found to be induced in sensory neurons by nerve growth factor (NGF; Kendall *et al.*, 1995) and NT-3 (Wyatt *et al.*, 1998),

respectively, while both BDNF and NGF induced a rapid increase in Pax-3 expression in cerebellar neurons in primary culture (Kioussi and Gruss, 1994). In none of these studies, however, was the role of the transcription factors elucidated. In fact, there is little information about downstream gene targets of neurotrophic factors, other than for immediate-early genes such as c-*fos* (reviewed in Segal and Greenberg, 1996). Thus, our results provide new insights in that the actions of both neurotrophins and IGF-I during neurogenesis may be mediated by developmentally regulated transcription factors. However, our results in primary striatal cultures, in which a neutralizing antibody to endogenous IGF-I inhibited neuronal differentiation and Brn-4 expression, but not the number of Brn-4-expressing cells, suggests that IGF-I signaling is required for the stage-specific and sufficient increase of Brn-4 levels required to facilitate neuronal differentiation. On the other hand, IGF-I is probably not required for the regionally specific expression of Brn-4, which may be regulated by other homeobox-containing genes such as Dlx 1 and 2 (Anderson *et al*., 1997). As both BDNF and IGF-I stimulate common signaling cascades such as the Ras-MAP kinase pathway and/or the PI3-kinase pathway (Marsh *et al*., 1993; Heumann, 1994; de Pablo and de la Rosa, 1995; Segal and Greenberg, 1996; Kaplan and Miller, 1997) to promote neuronal survival and maturation in both the CNS and PNS (Zirrgiebel *et al*., 1995; D'mello *et al*., 1997; Dudek *et al*., 1997; Parrizas *et al*., 1997), it is tempting to speculate that these pathways may be involved in enhancing Brn-4 gene expression. In preliminary experiments we have found that PD-098059 (a specific inhibitor for MEK-MAP kinase kinase; Alessi *et al*., 1993; Dudley *et al*., 1995), but not PI3-kinase inhibitors wortmannin (Arcaro and Wymann, 1993; Yano *et al*., 1993) and LY-294002 (Vlahos *et al*., 1994), inhibits IGF-I-mediated differentiation of neuronal precursors from the E14 striatum (T.Shimazaki and S.Weiss, unpublished results). We are currently analyzing signal transduction pathways that regulate neuronal differentiation, in relation to their putative regulation of Brn-4 expression.

Antisense oligonucleotides targeting specific mRNAs have been shown to inhibit gene expression *in vitro* and *in vivo* (Crooke, 1992; Stein and Narayanan, 1994; Crooke and Bennet, 1996; Stein, 1996). We elected to use antisense oligonucleotides to assess the functional properties of Brn-4. While simultaneous addition of antisense oligonucleotides to Brn-4 with IGF-I resulted in significant reduction in neuron numbers both in cultures of EGF-responsive stem cell progeny and primary E14 striatal cells, delays of 24 h (following IGF-I exposure) were without effect. This suggests that Brn-4 is required for neuronal differentiation. The restricted Brn-4 expression in the SVZ of the basal forebrain, but not the mantle zone, which is full of highly differentiated neurons, also implicates Brn-4 in neuronal differentiation rather than long-term survival. However, we cannot rule out that Brn-4 is important for survival or maintenance of immature neurons, as the experimental inhibition of Brn-4 production by antisense oligonucleotides and the resultant neuronal differentiation was only partial. Indeed, it is possible that a threshold level of Brn-4 operates in neuronal maintenance. In addition, the late onset of Brn-1 and Brn-2 expression seen 3 days after plating of EGF-responsible stem cell progeny may be important in neuronal survival and maintenance, and may also compensate for Brn-4 in such a role. In either case, this does not in any way detract from our conclusion, derived from the observation that reduced Brn-4 protein during neuronal differentiation results in reduced numbers of neurons, that Brn-4 is involved in neuronal differentiation. Interestingly, findings in a recent study of disruption of the class IV POU gene Brn-3b/Brn-3.2, whereby aborted differentiation of post-mitotic retinal ganglion precursors was followed by apoptotic cell death (Xiang, 1998), are quite similar to our results with E14 striatal dissociates. We observed reduction of both total cell and neuron numbers in primary E14 striatal cultures, by inclusion of antisense oligonucleotides targeting Brn-4 mRNA. This suggests that attenuation of Brn-4 expression during differentiation may cause death of neuronal precursors or immature neurons. Although we did not observe a significant reduction in total cell number in EGF-responsive stem cell progeny by inclusion of antisense oligonucleotides, we cannot rule out the possibility of neuronal cell death given that such a small decrease of cells could be obscured by variation in total cell number within each experiment. The precise relationship between disruption of early differentiation and programmed cell death is being investigated further.

The Brn-4 gene is expressed widely in the developing CNS and is subsequently restricted to the striatum and medial habenula, the paraventricular and supraoptic nuclei and the commissural organ (Alvarez-Bolado *et al*., 1995). Ectopic expression of XlPOU2, the *Xenopus* homologue of Brn-4, showed that the gene may play an early role in neuralizing the ectoderm (Witta *et al*., 1995). In the E14 striatum, Brn-4 is expressed predominantly in the SVZ (LeMoine and Young, 1992; Mathis *et al*., 1992; Alvarez-Bolado *et al*., 1995). Our immunohistochemical analyses of the E14 striatal SVZ, both *in vivo* and *in vitro*, find that the majority of Brn-4 expressing cells are neuronal precursors, as well as differentiating neurons. These results suggest that Brn-4 plays an important role in the initiation of a differentiation program *in vivo*. Brn-4 immunoreactivity was also detected in virtually all recently differentiated neurons derived from EGF-responsive stem cells. Thus, the Brn-4 expression pattern in stem cell progeny *in vitro* resembles closely that observed at their site of origin *in vivo*. Interestingly, we found a similar stage-specific expression pattern of Brn-4 in the epithalamus, suggesting Brn-4 plays a similar role in other forebrain structures. Moreover, given that BDNF, IGF-I and their receptors are widely expressed in neuronal cells differentiating from the SVZ (Baron-Van Evercooren *et al*., 1991; Kaisho *et al*., 1991; Bartlett *et al*., 1992; Bondy *et al*., 1992; Fryer *et al*., 1996; Jung and Bennet, 1996), it is reasonable to propose that a relationship between these factors, Brn-4 and neuronal differentiation operates during neurogenesis *in vivo*. This is further supported by our findings, whereby endogenous expression of IGF-I and its receptor regulates neuronal differentiation in primary E14 striatal cultures. We have summarized schematically the proposed relationship between Brn-4 and neuronal differentiation factors during neurogenesis in the developing striatum in Figure 9.

As regards the downstream targets of Brn-4, which are

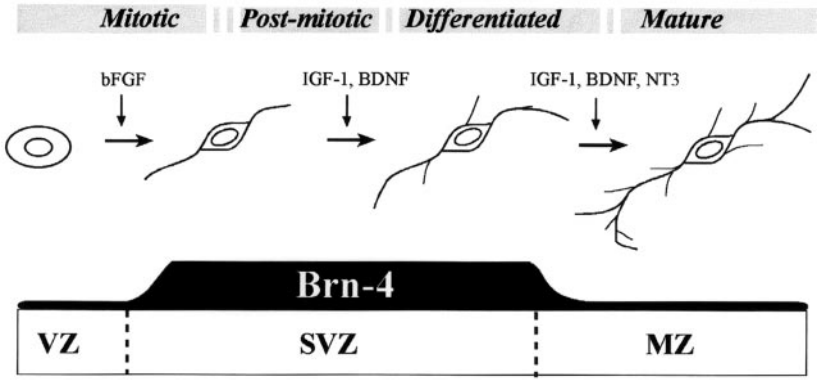

Fig. 9. Schematic representation of Brn-4 expression and neuronal differentiation in the developing striatum, and the putative factors regulating neurogenesis. In the embryonic striatum, Brn-4 is expressed in the SVZ throughout development. Virtually all the post-mitotic cells in the SVZ express Brn-4. Expression is maintained during the early differentiation of neurons and subsequently restricted to a small subset of neurons postnatally. Epigenetic signals which regulate neurogenesis are illustrated. bFGF is a mitogen (Vescovi *et al.*, 1993), IGF-I and BDNF are differentiation factors (Ahmed *et al.*, 1995; Arsenijevic and Weiss, 1998) for neuronal precursors. NT3 enhances neurite outgrowth (Y.Arsenijevic and S.Weiss, in preparation).

as yet unidentified, recent studies of Brn-3.0/3a (POU-IV family member) knockout mice may be instructive. In addition to descriptions of impaired and/or incomplete differentiation of specific subsets of PNS neurons (McEvilly *et al*., 1996; Xiang *et al*., 1996), a significant reduction in the expression of both BDNF and *trk*B was found (McEvilly *et al*., 1996) in the Brn-3.0/3a-deficient mice. Since the DNA-binding specificity of POU-III factors is similar to that of POU-IV factors (Li *et al*., 1993), it is reasonable to suggest that distinct POU factors may regulate the same genes in different cell types. In addition, it is noteworthy that a few potential auto-regulatory elements are present on the 5′ proximal region of the Brn-4 gene (Malik *et al*., 1996). Thus, it is tempting to speculate that a positive feedback loop of Brn-4 expression might operate in the regulation of BDNF and *trk*B expression, as part of the neuronal differentiation process. Moreover, the finding that Brn-3.0/3a can activate Bcl-2 gene expression directly (Smith *et al*., 1998) may explain a common phenotype in POU genes knock-out mice—the extinction of specific neurons. A surprising finding in this study was that Brn-4 mRNA produced two proteins of different size. Our Western blot analysis of point and deletion mutants suggest that the two forms are produced by protein modification such as phosphorylation and/or *O*-glycosylation in the N′-terminal transactivation domain (T.Shimazaki and S.Weiss, unpublished data). Since both phosphorylation and *O*-glycosylation can regulate the activity of transcription factors on their transactivation domain (Jackson and Tjian, 1988; Hunter and Karin, 1992), it is tempting to speculate that Brn-4 function is regulated at the post-translational level as well.

In addition to its role in CNS neurogenesis and evidence for its expression in some but not all astroglial cells, Brn-4 may play other roles. For example, de Kok *et al.* (1995) have shown that a small mutation in the human form of Brn-4 is a possible molecular defect which underlies deafness characterized by fixation of the stapes (DFN3), the most frequent X-linked form of hearing impairment. Moreover, some of the same patients exhibit mental retardation (de Kok *et al*., 1996). Whether those patients with complete deletions of the Brn-4 gene show specific anatomical abnormalities has yet to be determined. However, further basic studies of Brn-4 gene function through *in vitro* studies and targeted deletion *in vivo*, coupled with analysis of the disturbances in the human deletion phenotype, will lend further insights into the roles this transcription factor plays in neural cell genesis and function.

Materials and methods

Neural stem cell culture

Generation and differentiation of stem cell-derived progeny was carried out as described below. Striato-pallidum complexes were removed from CD-1 mouse fetuses at E14 and collected into phosphate-buffered saline (PBS) containing 0.6% glucose, penicillin (50 U/ml) and streptomycin (50 U/ml, both from Gibco-BRL). Tissue pieces were mechanically dissociated with a fire-polished Pasteur pipette. Cells were seeded at a density of 200 000 cells/ml into regular culture medium which contains Dulbecco's modified Eagle's medium (DMEM):F12 (1:1), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM) and HEPES buffer (5 mM), insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), selenium chloride (30 nM) (all from Sigma except glutamine from Gibco-BRL), in the presence of EGF (20 ng/ml, Chiron). Cells were grown for 7 DIV and formed floating cell clusters (spheres). These primary spheres were pelleted, dissociated and plated into culture flasks at 50 000 cells/ml in EGF-containing medium. After 7 DIV, the second generation of spheres were differentiated as follows. Spheres were rinsed free of EGF and insulin, dissociated and plated onto poly-L-ornithine-coated coverslips or 6-well culture dishes (Nunc) at a density of 100 000 or 1 million cells/ml with 1% fetal bovine serum (FBS) (UBI). Primary dissociates of the E14 striatum were differentiated directly in an identical manner. Human recombinant IGF-I (Chiron), human recombinant basic FGF (R&D Systems), human recombinant BDNF and human recombinant NT-3 (both from Pepro Tech Inc.) were added to the medium as described in the figure legends.

Immunocytochemistry

To observe neuronal differentiation and expression of Brn-4 *in vitro*, cells on coverslips were fixed with 4% paraformaldehyde and processed for indirect immunocytochemistry as described previously (Ahmed *et al*., 1995; Arsenijevic and Weiss, 1998). Primary antibodies used were mouse monoclonal anti-β-tubulin isotype III antibodies (1:1000; Sigma), rabbit polyclonal antibodies raised against N-terminal transactivation domain (amino acids 1–172) of rat Brn-4 (1:500; Shonemann *et al*., 1995). These were followed by incubation with fluorescein- or rhodamine-conjugated affinity-purified goat antibodies raised against mouse or rabbit IgG (Jackson Labs) as secondary antibodies. Hoechst 33258 (Sigma) was used for nuclear labeling to count total cells.

Dual-label immunocytochemistry on tissue sections was carried out using TSA™-Indirect kit (NEN) according to the manufacturer's protocol with minor modifications. Timed pregnant CD-1 mice were sacrificed

on gestational day 14 and embryos removed. Whole brains were dissected and fixed in 4% paraformaldehyde in PBS overnight at 4°C, and cryoprotected with 15% sucrose in PBS overnight at 4°C. Brains were embedded in O.C.T. Compound (Miles) and cut into serial coronal sections, 10 μm thick, and mounted on glass slides. Before immunolabeling, sections were hydrated for 5 min in PBS at room temperature (rt). After quenching, the sections were then blocked in TNB buffer (0.1 M Tris–HCl pH 7.5, 0.15 M NaCl and 0.5% NEN Blocking Reagent) for 30 min at rt. Anti-Brn-4, the first primary antiserum, was diluted 1:25 000 in TNB buffer and applied overnight at 4°C. After extensive washes and incubation in goat-anti-rabbit biotin diluted 1:200 in TNB for 1 h at rt, sections were incubated with streptavidin–HRP diluted 1:100 in TNB buffer followed by incubation for 10 min in biotinylated-tyramide diluted 1:50 in Amplification Diluent (NEN). The Brn-4 immunoreactivities were visualized by fluorescein-conjugated streptavidin. The second primary antibodies (undiluted anti-nestin mouse monoclonal antibodies: Rat 401 from Developmental Studies Hybridoma Bank and anti-β-tubulin at 1:500 in TNB buffer) were added on serial sections on separate slides. Anti-nestin was incubated 2 h at 37°C and anti-β-tubulin at 4°C overnight followed by incubation at 37°C with rhodamine-conjugated goat-anti-mouse antibodies at 1:200 and Hoechst at 1:100 (diluted in TNB buffer for anti-nestin and PBS for anti-β-tubulin). The slides were washed three times in PBS and coverslips mounted with FluorSave Reagent (Calbiochem).

Western blotting

Cells were washed with ice-cold PBS, pelleted and extracted in sodium dodecyl sulfate (SDS)-lysis buffer (60 mM Tris–HCl pH 6.8, 100 mM 2-mercaptoethanol and 2% SDS). After protein quantification, equal amounts were run on a 10% SDS–PAGE gel and electroblotted onto nitrocellulose membranes (Bio-Rad). The blots were blocked in blocking buffer (25 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.3% Tween 20 and 5% non-fat skim milk) and incubated with primary antibodies: anti-Brn-4 rabbit polyclonal antibodies (1:5000), anti-HA mouse monoclonal antibodies (1:500; Boehringer Mannheim) or anti-actin goat polyclonal antibodies (1:5000; Santa Cruz) in the blocking buffer overnight at 4°C. Blots were washed and then incubated with goat-anti-mouse IgG and/or rabbit IgG conjugated to horseradish peroxidase (Chemicon). After final washing, the blots were developed using Enhanced Chemiluminescence (Amersham).

RT–PCR Southern blot

Total RNA was isolated from EGF-generated stem cell progenies, E14 cultured striatal cells or E14 brain by using Trizol reagent (Gibco-BRL). First-strand cDNA was synthesized utilizing Superscript (Gibco-BRL), then amplified using Taq-DNA polymerase (Gibco-BRL) with 20 cycles of denaturation (94°C, 45 s), primer annealing (60°C for Brn-2, 4 and SCIP; 55°C for Brn-1, 45 s) and extension (72°C, 45 s) in the presence of 5% dimethylsulfoxide (DMSO). Primers were: Brn-1, upstream 5′-CCGCAGAGTCTGCTGTACTCGCAG-3′ and downstream 5′-GGTG-ATGATGCTCCGCCAACTCG-3′ (Hara *et al.*, 1992; DDBJ/EMBL/GenBank accession No. M88299); Brn-2, upstream 5′-GACATCAA-GCCCTCGGTGGTGGTAC-3′ and downstream 5′-TCTGCATGGTG-TGGCTCATCGTGG-3′ (Hara *et al.*, 1992; DDBJ/EMBL/GenBank accession No. M88300); Brn-4, upstream 5′-CTGCAACTGGGCG-CAATCATCC-3′ and downstream 5′-CTTCATCAGAGTGGTCCTGG-CAGTG-3′ (Hara *et al.*, 1992; DDBJ/EMBL/GenBank accession No. M88301); SCIP, upstream 5′-GCGAGCACTCGGACGAGGATGC-3′ and downstream 5′-GGGGTCATGCGCTTCTCCTTCTGC-3′ (Hara *et al*., 1992; DDBJ/EMBL/GenBank accession No. M88302); β-actin, upstream position 182–202 and downstream corresponds to 424–404 (Tokunaga *et al*., 1986; DDBJ/EMBL/GenBank accession No. X03672). PCR products (Brn-1, 171 bp; Brn-2, 382 bp; Brn-4, 300 bp; SCIP, 474 bp and β-actin, 243 bp) were run on 1.5–2% agarose gels, blotted onto positively charged nylon membranes (Amersham), and hybridized with fluorescein-labeled cDNA probes corresponding to each gene prepared using Gene Image Labeling Kit (Amersham). The cDNAs, sizes as above, were prepared from PCR-based direct cloning. Thirty cycles of each PCR reaction described above were carried out and the products were purified using Geneclean II kit (Bio 101) and ligated into pGEM-T vector plasmids (Promega). Correct plasmid clones were identified by sequencing. The signals were detected using Gene Image Detection Kit (Amersham). The linearity of all the PCR amplifications including the β-actin signal for each condition was ascertained prior to the actual experiment, so that those experiments are quantitative (Chelly *et al*., 1990). The relative difference of the signal intensity was analyzed using an NIH image after scanning the autoradiographs. PCR primers for IGF-I cDNA were upstream 5′-CACTCTGACCTGCTGTGTAA-3′, downstream 5′-GGAGACTGGAGATGTACTGT-3′ (Bell *et al*., 1986; DDBJ/EMBL/GenBank accession No. X04482). The 318 bp DNA fragment was amplified by 35 cycles of denaturation (94°C, 45 s), annealing (63°C, 45 s) and extension (72°C, 60 s). The RT–PCR of the IGF-I receptor was performed as described previously (Arsenijevic and Weiss, 1998). We checked for the contamination of DNA by RT–PCR without reverse transcription in these experiments. The results presented here were replicated in at least three separate experiments.

Antisense oligonucleotides

Phosphorothionate-modified oligonucleotides purified by gel filtration were obtained from the University Core DNA Services (University of Calgary, Canada). The sequence of antisense oligonucleotide to Brn-4 mRNA corresponds to the position from –9 to +10 which includes the proximal ATG initiation codon (5′-CTGTGGCCATGGTCGATGAG-3′). The control oligonucleotides chosen were the complementary sense strands and a missense (5′-CTGTGGCGTTGGTCGATGAG-3′) of the antisense one.

Transient transfection of expression vectors

The expression plasmid vectors, pCMV and pCMVBrn-4 (Mathis *et al*., 1992) were transfected into NIH 3T3 cells grown in DMEM medium supplemented with 10% FBS using LipofectAMINE^{TM} reagent (Gibco-BRL) according to the manufacturer's protocol. Briefly, 1 μg of plasmids was incubated for 45 min with 6 μl of the reagent in 200 μl of serum-free medium to form liposome–DNA complex, diluted with 800 μl of the medium and then the cells (2×10^5) were incubated with the complexes in a 35 mm culture dish. After 5 h incubation, the medium was replaced with 2 ml of medium containing 10% serum. The cells were harvested 48 h after starting the transfection and extracted for Western blot analysis.

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