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The Brn-3a Transcription Factor Induces Neuronal Process Outgrowth and the Coordinate Expression of Genes Encoding Synaptic Proteins

MARTIN D. SMITH, SALLY J. DAWSON, AND DAVID S. LATCHMAN*

Medical Molecular Biology Unit, Department of Molecular Pathology, University College London Medical School, London W1P 6DB, United Kingdom

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The Brn-3a POU family transcription factor is expressed only in postmitotic neurons in the central nervous system and identifies the first differentiated neurons to appear in the midbrain, hindbrain, and spinal cord during development. This factor is also induced when undifferentiated proliferating ND7 cells cease dividing and differentiate to a mature neuronal-like phenotype bearing numerous neurite processes. We show that overexpression of Brn-3a in undifferentiated ND7 cells induces a mature neuronal phenotype characterized by process outgrowth and the induction of genes encoding synaptic proteins, although the cells continue to proliferate. In contrast, the closely related factors Brn-3b and Brn-3c do not have this effect. Although the N-terminal activation domain of Brn-3a is required for maximum induction of neurite outgrowth and gene expression, these effects are primarily dependent on the DNA binding POU domain, which also acts as an activation domain. Overexpression of the isolated POU domain of Brn-3a is sufficient to induce neurite outgrowth, while the ability of full-length Brn-3a to do so is abolished by mutating a single amino acid in the Brn-3a POU homeodomain to its equivalent in Brn-3b. Thus, Brn-3a appears to play a critical role in the specification of the mature neuronal phenotype, acting by stimulating the expression of genes whose products are required for process outgrowth and synapse formation.

During development and in the adult organism, the outgrowth of neurites is a tightly regulated process playing an essential role in axonal targeting, synapse formation, plasticity, and regeneration. This process involves the expression of a number of different growth-associated proteins (GAPs) in the neuronal cell body which are likely to be essential for neurite outgrowth (21). In agreement with this idea, the overexpression of one of these proteins, GAP-43, in transgenic mice has recently been shown to promote enhanced nerve sprouting (1). Similarly, the presynaptic nerve terminal protein SNAP-25 (20) has also been shown to be essential for neurite outgrowth both in vitro and in vivo (19). This finding suggests that exocytosis of synaptic vesicles which is required for synaptic transmission and the constitutive exocytosis which is required for axon outgrowth may have components such as SNAP-25 in common.

Hence, neurite outgrowth is likely to require the activation of a number of genes encoding GAPs as well as others encoding synaptic proteins. In turn, such gene activation will require the enhanced activity and/or synthesis of one or more cellular transcription factors which regulate the expression of these genes. Clearly, the identification of such key regulatory factors would be of critical importance both for our understanding of neurite outgrowth itself and for successful attempts at enhancing it following nerve injury.

To identify such transcription factors, we have focused initially on the ND7 cell line, which was obtained by fusing primary dorsal root ganglion neurons with C1300 neuroblastoma cells and which retains many of the characteristics of sensory neurons (31). Although these cells proliferate indefinitely in serum-containing medium, following removal of serum or treatment with cyclic AMP, they cease dividing and differentiate to a mature neuronal phenotype bearing long neurite processes (22). This differentiation event is accompanied by the redistribution of numerous proteins to the tips of the process (30) and the induction of specific genes such as that encoding SNAP-25 (14).

In addition, during the course of this differentiation, changes occur in the expression of the members of the Brn-3 subfamily of POU domain transcription factors (for a review of POU factors, see references 28 and 29), which are closely related to one another but are encoded by distinct genes (26). Thus, the levels of the Brn-3a factor (also known as Brn-3 [11] or Brn-3.0 [10]) increase from very low in the proliferating ND7 cells to high in the differentiated cells (2, 14). Similarly, the levels of the closely related POU factor Brn-3b (also known as Brn-3.2 [27]) fall from high in the proliferating cells to low in the undifferentiated cells (2, 14), whereas the levels of the third member of the subfamily, Brn-3c (18) (also known as Brn-3.1 [10]), remain unchanged.

Such findings are of considerable interest, since although Brn-3a and Brn-3b are closely related to one another, they have antagonistic effects on the activities of specific gene promoters. Brn-3a has been shown to activate several promoters, including those of the genes encoding pro-opiomelanocortin (10) and α-internexin (3) as well as a thymidine kinase (tk) promoter containing an added synthetic binding site for the Brn-3s (tk-Oct) (2, 17). In contrast, Brn-3 represses the α-internexin and tk-Oct promoters (2, 17) and also inhibits their activation by Brn-3a in cotransfection experiments. Similarly, the third member of the Brn-3 subfamily, Brn-3c, has only a weak activating effect on these promoters (3, 17).

These findings therefore suggested the possibility that the rise in Brn-3a and fall in Brn-3b expression which occur upon ND7 differentiation plays a causal role in the differentiation process, with specific genes whose protein products are re-

* Corresponding author. Mailing address: Medical Molecular Biology Unit, Department of Molecular Pathology, The Windeyer Building, Cleveland Street, London W1P 6DB, United Kingdom.

quired for differentiation being activated by the enhanced expression of the activating Brn-3a factor and a fall in the level of the Brn-3b repressor. In agreement with this idea, we have shown that Brn-3a can activate the SNAP-25 gene promoter in cotransfection assays (13), acting via a region of the promoter located between −288 and −126 relative to the transcription start site (17a). Moreover, in vivo, Brn-3a is expressed only in postmitotic neurons in the central nervous system and identifies the first differentiated neurons to appear in the midbrain, hindbrain, and spinal cord during development (9–11). Hence, Brn-3a may also play a critical role in neuronal differentiation and process outgrowth in vivo.

To determine whether overexpression of the Brn-3a factor is able to induce differentiation, we have prepared ND7-derived cell lines overexpressing each of the forms of Brn-3 under control of an inducible promoter and have used these cell lines to study the effects of the various forms of Brn-3 on the growth of ND7 cells and their ability to put out processes under conditions which do not normally induce differentiation.

MATERIALS AND METHODS

Isolation of cell lines and analysis of Brn-3 expression. cDNA clones of wild-type and mutant forms of Brn-3a, Brn-3b, and Brn-3c (6, 25) as well as the POU domains of Brn-3a and Brn-3b were inserted in the sense orientation under the control of the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter of the mammalian expression vector pJ5 (16). Following cotransfection into ND7 cells (31) together with a plasmid conferring neomycin resistance (pM5G-NEO), stable transfectants were selected by supplementation of the culture medium with G418 to a final concentration of 800 μg/ml, and individual clones were isolated after 7 to 14 days of selection. Putative clones were treated with dexamethasone at a final concentration of 1 μM for 24 h to induce expression of the MMTV promoter to allow screening for clones capable of expressing the exogenous construct. RNA was isolated from cells by the guanidinium isothiocyanate method (4), treated with DNase to remove any contaminating DNA, and subsequently used as the template for cDNA synthesis. Resultant cDNA was amplified by PCR essentially as described by Kawasaki (12). In initial screening experiments to confirm that exogenous constructs were producing sense mRNAs in the cell lines, PCR was performed by using a forward primer internal to the POU domain (Brn-3a, 5′ GACTCGGACACGGACCCC GCG-3′; Brn-3b, 5′-GACGTGGATGCAGACCCGCGG-3′; or Brn-3c, 5′-GA TGTGGAGTCAGACCCTCGA-3′) and a reverse primer internal to the vector sequence (5′ AGATCTGGTACCATCGAT-3′) so as not to amplify endogenous Brn-3 mRNA, with product detected by Southern hybridization with homologous probes. cDNA from clones capable of expressing the exogenous construct were subsequently subjected to PCR to determine the total level of expression of that member of the Brn-3 family by using forward (see above) and reverse (Brn-3a, 5′-CCCTCCTCAGTAACTGGC-3′; Brn-3b, 5′-CTAAATGCCGGAGAGTAT TTCAC-3′; and Brn-3c, 5′-CAATCGTCCACAGCAGAGTATTT-3′) primers internal to the POU domain, thus amplifying both exogenous and endogenous mRNAs.

Sample pairs were equalized by using primers designed to amplify the mRNA species encoding the invariant L6 ribosomal protein (5′-ATCGCTCCTCAAAC TTGACC-3′ and 5′-AACTACAACCACCTCATGAA-3′).

Measurement of neurite outgrowth and cell proliferation. In all cases, control uninduced cultures were analyzed in parallel with induced experimental cultures, and both were subsequently compared to uninduced and induced lines expressing the pJ5 vector with an empty expression cassette. To determine neurite process length, cells were subject to immunocytochemistry using a primary monoclonal antibody to α-tubulin, with detection by the peroxidase-diaminobenzidene color reaction. The length of the longest process for each of 400 cells was determined for each sample by using video capture NIH Image software (version 1.59), with the operator unaware of the nature of the sample. Statistical analysis was performed with Microstat software. The cell doubling rates of experimental and control cultures were determined, allowing the construction of growth curves; the S-phase proliferative fraction of each culture was determined by bromodeoxyuridine (BrdU) incorporation and subsequent immunocytochemical detection, using a BrdU labeling and detection kit as instructed by the manufacturer (Boehringer Mannheim). The number of positive nuclei in a population of 500 cells per sample was determined for the purpose of statistical analysis.

Measurement of RNA levels. cDNAs were first subjected to PCR designed to amplify the mRNA species encoding the L6 ribosomal protein (see above). Following subsequent Southern hybridization to allow the equalization of cDNA input between both uninduced and induced control and experimental samples, PCR amplification using primers to specifically amplify the mRNA species encoding SNAP-25 (5′-TGACCAGCTGGCTGATGAGTC-3′ and 5′-CCCATGT CTAGGGGGCCATATGA-3′) and GAP-43 (5′-GTGCTCTGGTTTCCTTAG CA-3′ and 5′-GATATAGCCCTCATCCATCA-3′) was performed. Similar amplifications were performed with primers designed to specifically amplify synapsin Ia (5′-ATGGAGACTACCGCAGTTTG-3′ and 5′-CACAACTACAG GGTATGTTG-3′), synaptotagmin I (5′-TCGCCATGGCTGTGGTTGCT-3′ and 5′-CTGGAAATCATAGTCCAGAG-3′), and synaptophysin I (5′-CAGAA ACAAGTACCGAGAGAA-3′ and 5′-CCAAACACCACTGAGGTGTT-3′).

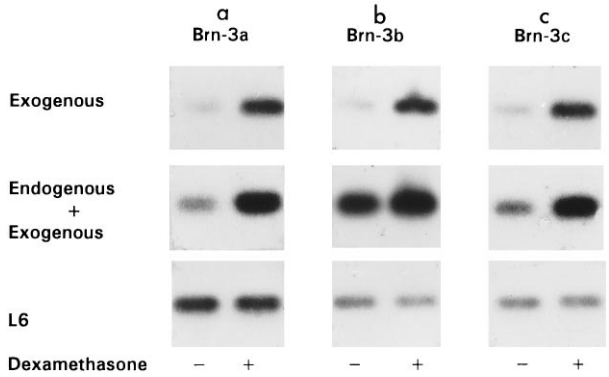

FIG. 1. (A) PCR amplification of the exogenous Brn-3a mRNA derived from the transfected gene (top), the exogenous and the endogenous Brn-3a mRNAs (middle), and the control mRNA encoding the L6 ribosomal protein (bottom). The mRNA was prepared from a representative cell line (3A.2) obtained by transfection with a Brn-3a cDNA clone maintained in either the presence (+) or absence (−) of dexamethasone. (B) Similar amplification of the exogenous Brn-3b mRNA, the exogenous and endogenous Brn-3b mRNAs, and the L6 mRNA in a representative cell line (3B.1) obtained by transfection with a Brn-3b cDNA clone. (C) Similar amplification of the exogenous Brn-3c mRNA, the exogenous and endogenous Brn-3c mRNAs, and the L6 mRNA in a representative cell line (3C.5) obtained by transfection with a Brn-3c cDNA clone.

Western blotting. For Western analysis, 100 μg of total protein was submitted to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electroblotted to a Hybond C nitrocellulose membrane as previously described (7). Antigens of interest were detected by probing filters with appropriate antisera. To confirm equal loading, Coomassie blue staining of parallel gels was performed; each filter was stripped and reprobed with an antibody recognizing neuron-specific PGP9.5.

RESULTS

To investigate the effect of overexpressing Brn-3a, a full-length Brn-3a cDNA clone (25) was inserted into the vector pJ5 (16), placing it under the control of the steroid-inducible MMTV promoter. Similar constructs were prepared for Brn-3b and Brn-3c. All of these constructs were transfected into proliferating ND7 cells, and stably transfected cells were selected on the basis of their neomycin resistance encoded on a cotransfected plasmid. Three cell lines of each type were selected to control for any clonal variation. Each showed basal and steroid-inducible expression of the appropriate transfected form of Brn-3, as assayed by PCR using one primer specific for the RNA transcript derived from the transfected plasmid and one primer specific for the appropriate form of Brn-3 (Fig. 1). These and all other PCR assays were carried out under conditions (cycle numbers and amount of RNA) which had been shown in preliminary experiments to be in the range of the assay where the signal obtained is linearly related to the amount of input RNA (data not shown).

Similarly increased basal levels and steroid inducibility of each form of Brn-3 could be demonstrated by PCR using primers which would specifically amplify both the endogenous and exogenous mRNAs encoding each form of Brn-3 (Fig. 1). Brn-3a-transfected cells displayed an approximately fivefold increased level of total Brn-3a. The elevation of Brn-3b in the Brn-3b-transfected cells was somewhat smaller (approximately twofold) due to the high endogenous level of Brn-3b in undifferentiated ND7 cells. However, when these cells were differ-

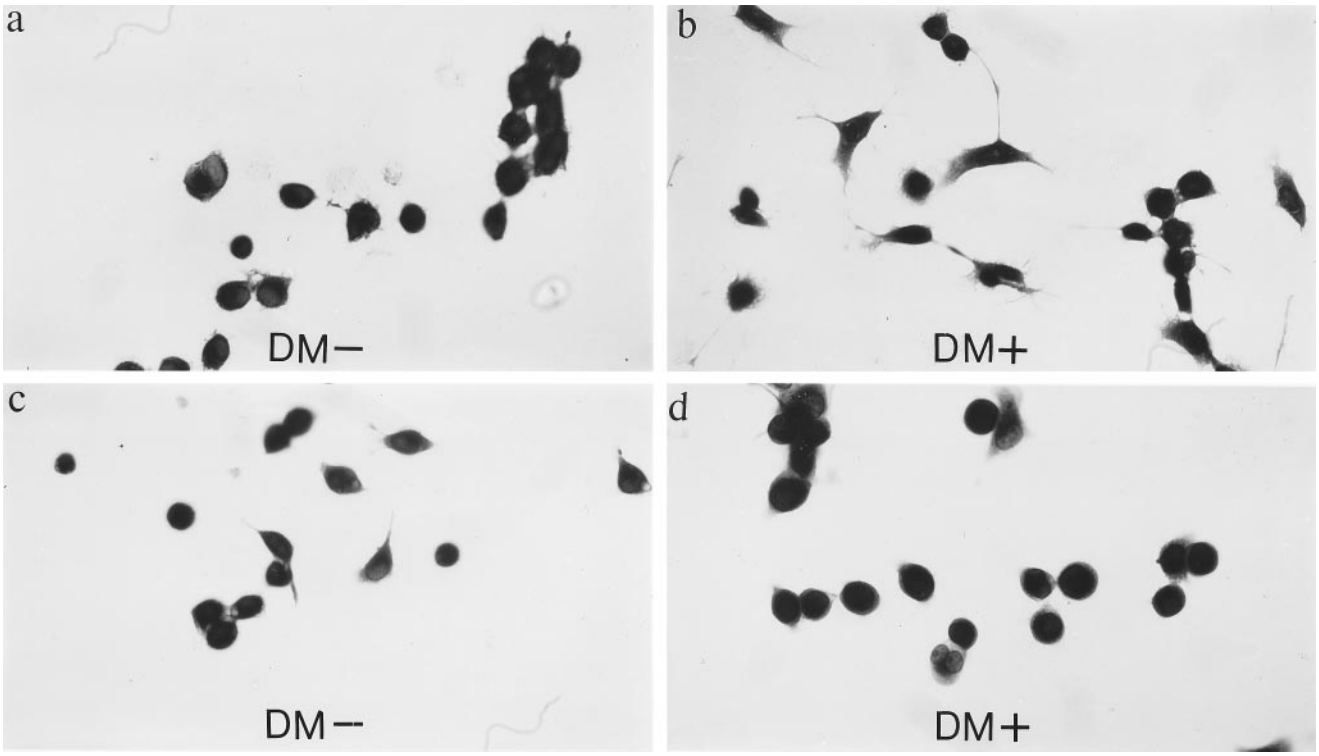

FIG. 2. Brn-3a expression regulates neurite outgrowth but not cellular differentiation and growth arrest in ND7 cells. Shown are representative photomicrographs from fields of cell line 3A.2 overexpressing Brn-3a (a and b) and cell line 3B.1 overexpressing Brn-3b (c and d) when grown in the absence (a and c) or presence (b and d) of dexamethasone (DM), demonstrating an increase in both the proportion of process-bearing cells and the length of those processes.

entiated by transfer to serum-free medium, endogenous Brn-3b levels fell as expected whereas the expression of exogenously delivered Brn-3b remained unchanged, resulting in these cells having much higher levels of Brn-3b than control differentiated cells (data not shown).

Under normal conditions, ND7 cells grown in full serum-containing medium do not form significant numbers of processes (22, 31). Having established that the cell lines showed some basal overexpression of the appropriate form of Brn-3 which could be further induced by glucocorticoid treatment, we investigated whether any of the lines formed neuritic processes when grown in full serum. Most interestingly, all three of the Brn-3a-expressing lines showed a significant enhancement ($P < 0.005$) in the proportion of cells bearing processes when expression of Brn-3a was induced by steroid treatment in serum-containing medium (Fig. 2 and 3a). In contrast, little or no change in the proportion of cells bearing processes was observed in cells overexpressing Brn-3b or Brn-3c compared to those expressing the PJ5 expression vector in the absence of any insert. Similarly, none of these lines showed the dramatic change in process formation upon steroid induction characteristic of Brn-3a-expressing cell lines, in agreement with our previous conclusion that steroid treatment itself has no effect on process formation in ND7 cells (unpublished data). The level of process formation in the steroid treated Brn-3a-expressing cells (on average, 37% of cells bearing processes) was comparable to that observed in control cells differentiated normally by removal of serum (Table 1).

Hence, overexpression of Brn-3a can induce process formation in ND7 cells grown under conditions which do not normally allow significant process formation. In addition, when we measured the lengths of the processes which form in the various cell lines grown in serum-containing medium, a significant increase ($P < 0.01$) in process length was observed in the Brn-3a-expressing cell lines even under uninduced conditions; the length was further increased upon steroid induction of Brn-3a expression ($P < 0.001$) (Fig. 2 and 3b). As before, no significant effect was observed in the Brn-3b- or Brn-3c-overexpressing cells. The average length of processes formed in the Brn-3a-expressing cells was 32 μm, which was approximately 60% of the average length observed in control cells differentiated in the absence of serum (Table 1).

The in vitro differentiation of ND7 cells by cyclic AMP treatment or serum removal involves not only process outgrowth but also growth arrest and cessation of cell division. However, only a small decrease in growth rate or BrdU incorporation was observed in the Brn-3a-overexpressing cells growing in full serum medium compared to the levels observed in cells transfected with pJ5 vector or overexpressing Brn-3b or Brn-3c (data not shown).

These results show that overexpression of Brn-3a can induce outgrowth of greater numbers of longer processes in proliferating ND7 cells but does not induce significant growth arrest. Hence, the rise in Brn-3a levels which occurs in ND7 cells upon serum removal or cyclic AMP treatment is likely to play a critical role in the formation of processes under these circumstances. In view of the known role of Brn-3a as a transcription factor activating the expression of specific genes (3, 10, 17), it is likely that this effect is produced via the activation of genes whose protein products are required for process outgrowth.

We therefore compared the expression of GAP-43 and SNAP-25, two different types of outgrowth factors, in the Brn-3a-overexpressing cells to that observed in the other cell lines or in control cells. In these experiments, no significant overex-

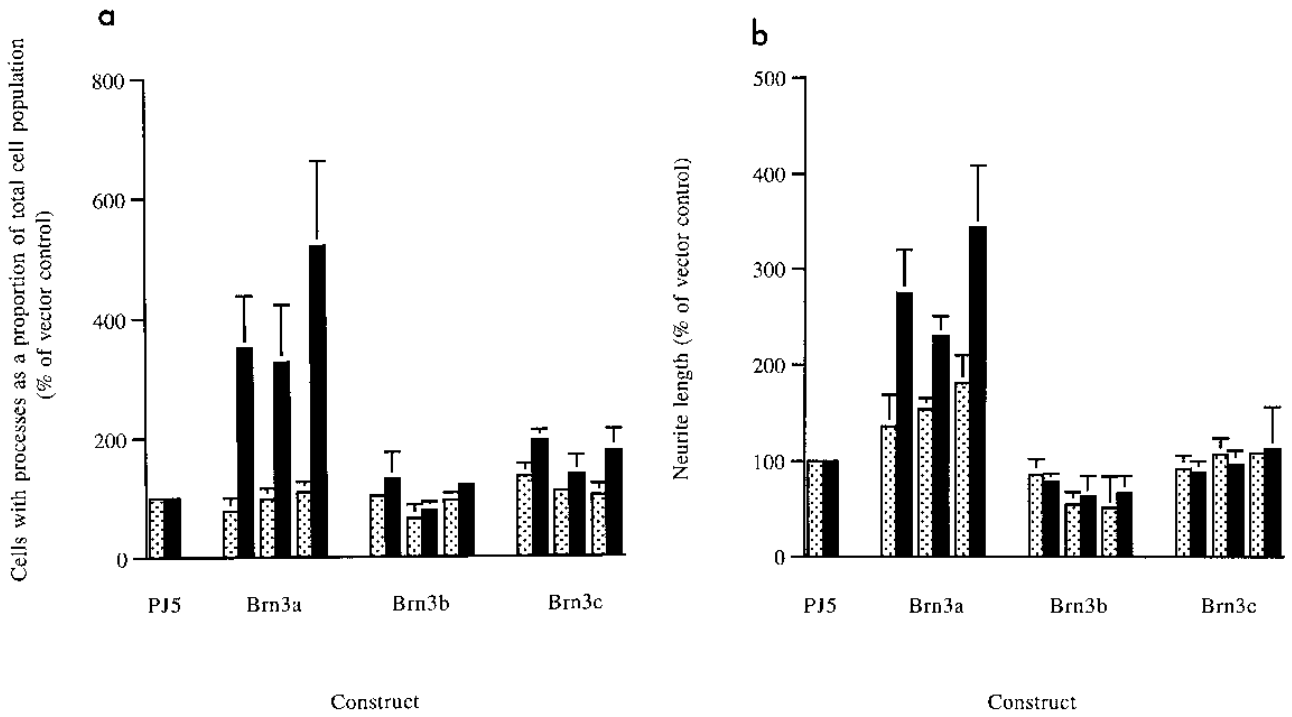

FIG. 3. Effect of overexpressing the indicated form of Brn-3 on the number of cells with processes (a) and length of the processes (b) relative to the vector control. In each case, values are shown for three independent clones overexpressing each construct and are compared to the level observed in ND7 cells transfected with vector alone (PJ5). Stippled boxes, uninduced promoter; solid boxes, dexamethasone-induced promoter. Values are the means of two independent experiments, each analyzed in duplicate; bars indicate standard deviations of the means.

pression of GAP-43 was observed at either the mRNA (Fig. 4a) or protein (Fig. 4b) level in the Brn-3a-overexpressing cells. This is in agreement with our previous observations that the level of GAP-43 does not increase when endogenous Brn-3a levels rise during ND7 cell differentiation, while cotransfection of fibroblasts with a Brn-3a expression vector and a GAP-43 promoter/reporter construct does not result in activation of the GAP-43 promoter (unpublished data).

In contrast, the Brn-3a-expressing cells exhibited a clear upregulation of the SNAP-25 mRNA (Fig. 5a) ($P < 0.05$) and protein (Fig. 5b) ($P < 0.01$) which was particularly marked following induction of Brn-3a with glucocorticoid. This effect was not observed following expression of Brn-3b or Brn-3c. This finding is in agreement with the ability of Brn-3a to induce the SNAP-25 promoter upon cotransfection of fibroblasts (13), suggesting that the rise in Brn-3a which occurs during ND7 cell differentiation directly activates the SNAP-25 promoter and is thus responsible for the rise in SNAP-25 expression which occurs during the differentiation event (13). In view of the role of SNAP-25 in neuronal process outgrowth (19), it is therefore likely that its induction by Brn-3a is, at least in part, responsible for the ability of this transcription factor to induce neurite outgrowth.

TABLE 1. Numbers and lengths of processes formed in various cell lines[a]

Cells transfected with:	% of cells with neurites		Length (μm) of neurites	
	−Dex	+Dex	−Dex	+Dex
pJ5 vector	11 ± 3	13 ± 6	9 ± 4	11 ± 5
Brn-3a	16 ± 3	37 ± 9	14 ± 3	32 ± 9
Brn-3b	9 ± 7	15 ± 4	12 ± 6	8 ± 7
Brn-3c	13 ± 4	21 ± 8	13 ± 8	22 ± 12
pJ5 vector differentiated by serum removal	48 ± 6	52 ± 7	62 ± 8	57 ± 11

[a] Values indicate the percentages of cells with neurites and the average lengths of the neurites in the different cell lines cultured in serum containing medium in the absence or presence of dexamethasone (Dex) and for comparison in control cells differentiated by removal of serum. Values are the means ± standard errors of the means in triplicate determinations in each of three different clonal cell lines of each type.

As the role of SNAP-25 in the synaptic vesicle cycle and in the constitutive exocytosis at the growth cone may involve distinct classes of vesicle (19), we wished to determine whether the levels of other components of the synaptic vesicle cycle (23) were also elevated in Brn-3a-expressing cells. We therefore measured in Brn-3a-overexpressing cells the levels of the synaptic vesicle proteins synapsin I, synaptophysin, and synaptotagmin I, each of which plays a distinct role at different stages of the synaptic vesicle cycle (23, 24). All of these proteins were found to be overexpressed at both the mRNA (Fig. 6a, c, and e) and protein (Fig. 6b, d, and f) levels in the Brn-3a-overexpressing cells compared to control cells ($P < 0.05$ for synapsin mRNA and $P < 0.01$ for protein; $P < 0.01$ for synaptotagmin mRNA and $P < 0.005$ for protein; $P < 0.05$ for synaptophysin mRNA and $P < 0.05$ for protein). Interestingly, overexpression of synapsin I but not of synaptotagmin I or of synaptophysin was observed in the Brn-3b-overexpressing cells. This finding is in accordance with our recent identification of the synapsin I promoter as the first promoter which can be activated by all three forms of Brn-3 in fibroblast cotransfection experiments (17a).

These findings therefore indicate that stable expression of Brn-3a can activate the expression of a number of different genes involved in the synaptic vesicle cycle and also promote

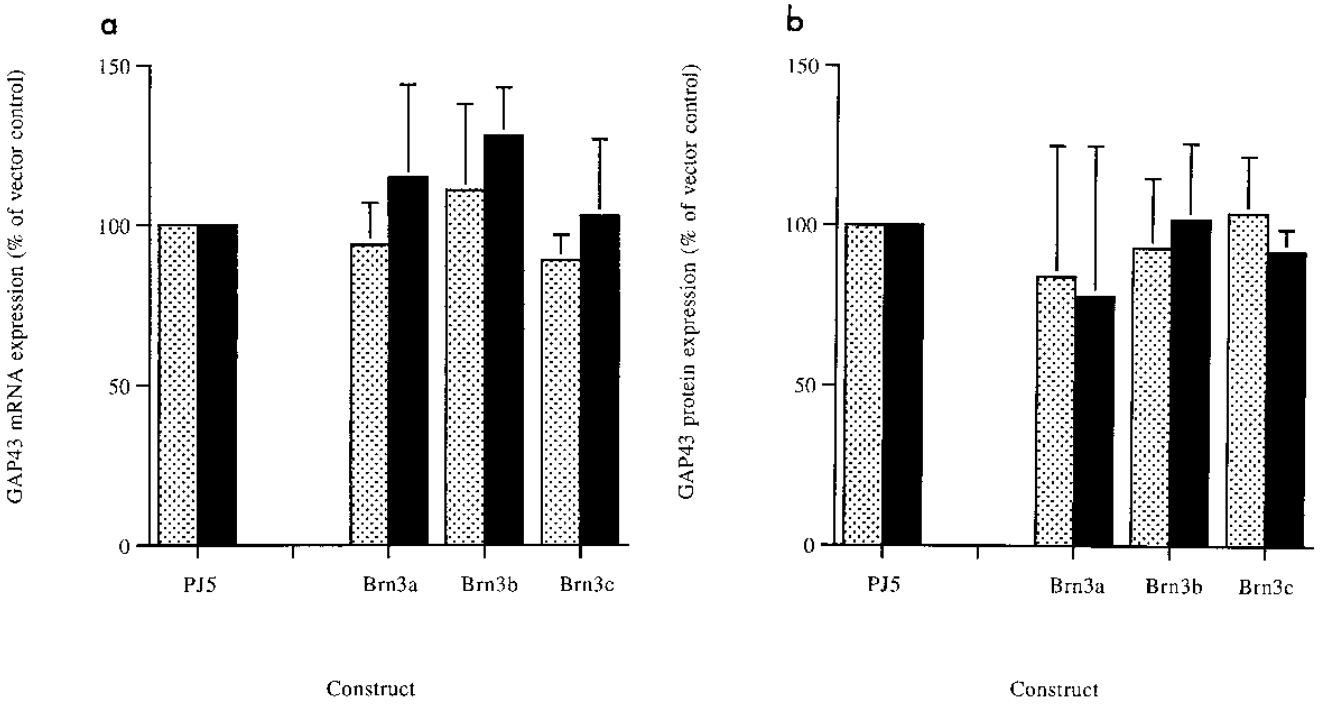

FIG. 4. GAP-43 mRNA (a) and protein (b) expression is unaffected in ND7 cells expressing Brn-3a, Brn-3b, or Brn-3c. Stippled boxes, uninduced; solid boxes, induced. Each value is the average of that obtained from two independent preparations, each analyzed in duplicate. Data for three individual clones expressing each construct have been combined. Bars show the standard deviations of the means.

neurite outgrowth, whereas Brn-3b does not do this except in the case of synapsin I. In our previous cotransfection experiments, the ability of Brn-3a to activate target promoters was dependent on the presence of two activation domains which were absent in Brn-3b. Activation of the tk-Oct promoter requires only the DNA binding POU domain, which is also able to act as an activation domain (3, 17). In contrast, this domain had no effect on the α-internexin promoter, whose activation

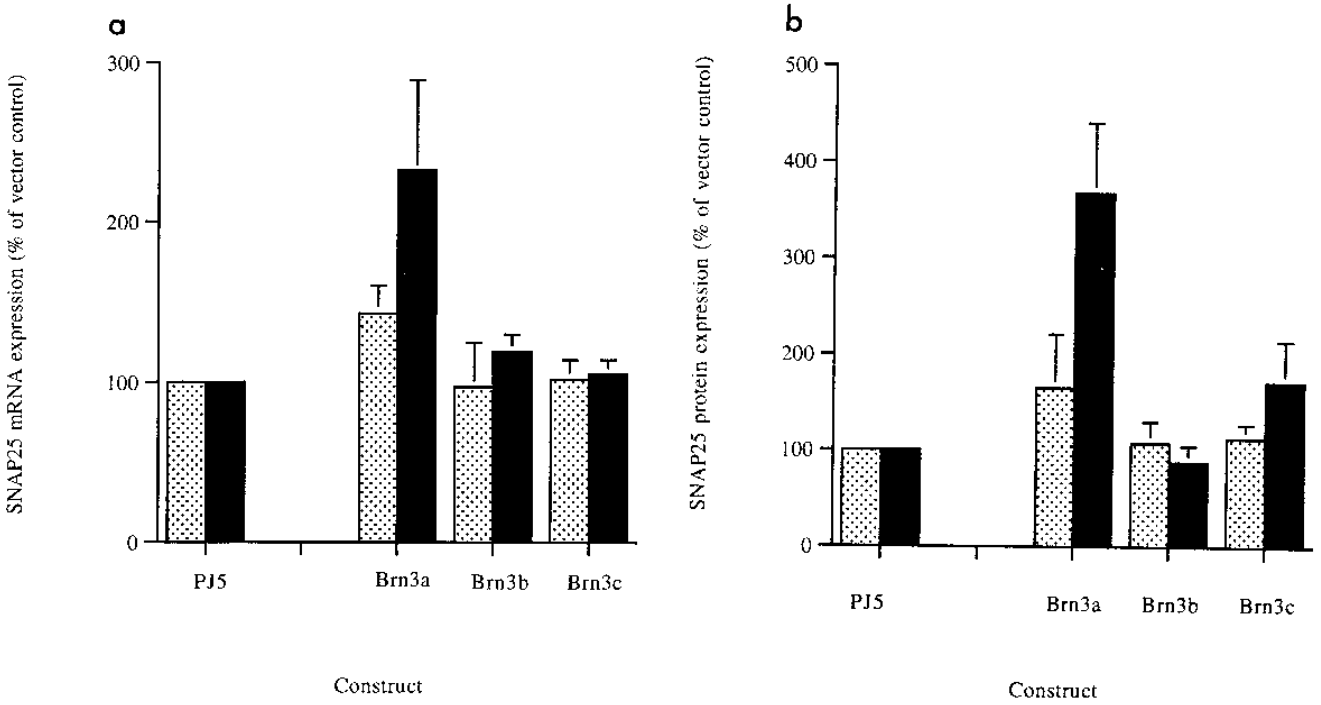

FIG. 5. SNAP-25 mRNA (a) and protein (b) are upregulated in ND7 cells expressing Brn-3a. Stippled boxes, uninduced; solid boxes, induced. Each value is the average of that obtained from two independent preparations, each analyzed in duplicate. Data for three individual clones expressing each construct have been combined. Bars show the standard deviations of the means.

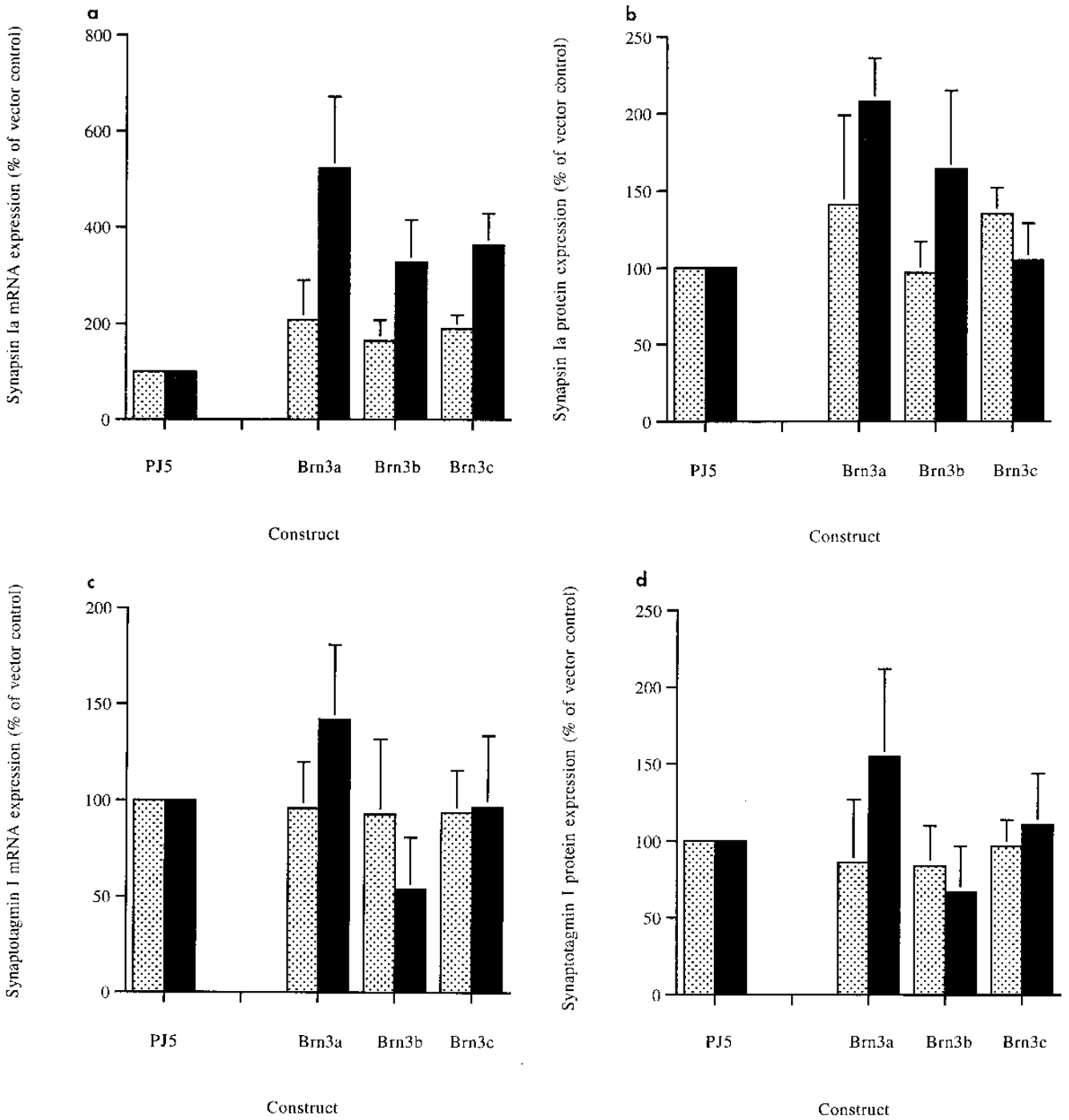

FIG. 6. The synaptic proteins synapsin Ia, synaptotagmin I, and synaptophysin I are coordinately regulated by Brn-3a. Levels of synapsin Ia mRNA (a) and protein (b), synaptotagmin I mRNA (c) and protein (d), and synaptophysin I mRNA (e) and protein (f) in the various cell lines overexpressing different forms of Brn-3 are compared to those in ND7 cells transfected with the pJ5 vector alone. Stippled boxes, uninduced; solid boxes, induced. Each value is the mean of that obtained from two independent preparations, each analyzed in duplicate. Data for three individual clones expressing each construct have been combined. Bars show the standard deviations of the means.

requires a distinct domain at the N terminus of Brn-3a (3). Interestingly, activation of the SNAP-25 promoter in cotransfection experiments can be achieved by either activation domain alone, the maximal effect being observed when both domains are present (17a).

To test the effect of each of these domains, we prepared pJ5-based vectors either containing cDNA encoding the isolated POU domain of Brn-3a or Brn-3b or containing cDNA encoding a naturally occurring short form of Brn-3a (15, 25) which lacks the N-terminal activation domain. These constructs were used to prepare stable cell lines as before. When these cell lines were analyzed for the number and length of neurites, the cells expressing the short form of Brn-3a lacking the N-terminal activation domain showed a similar increase in

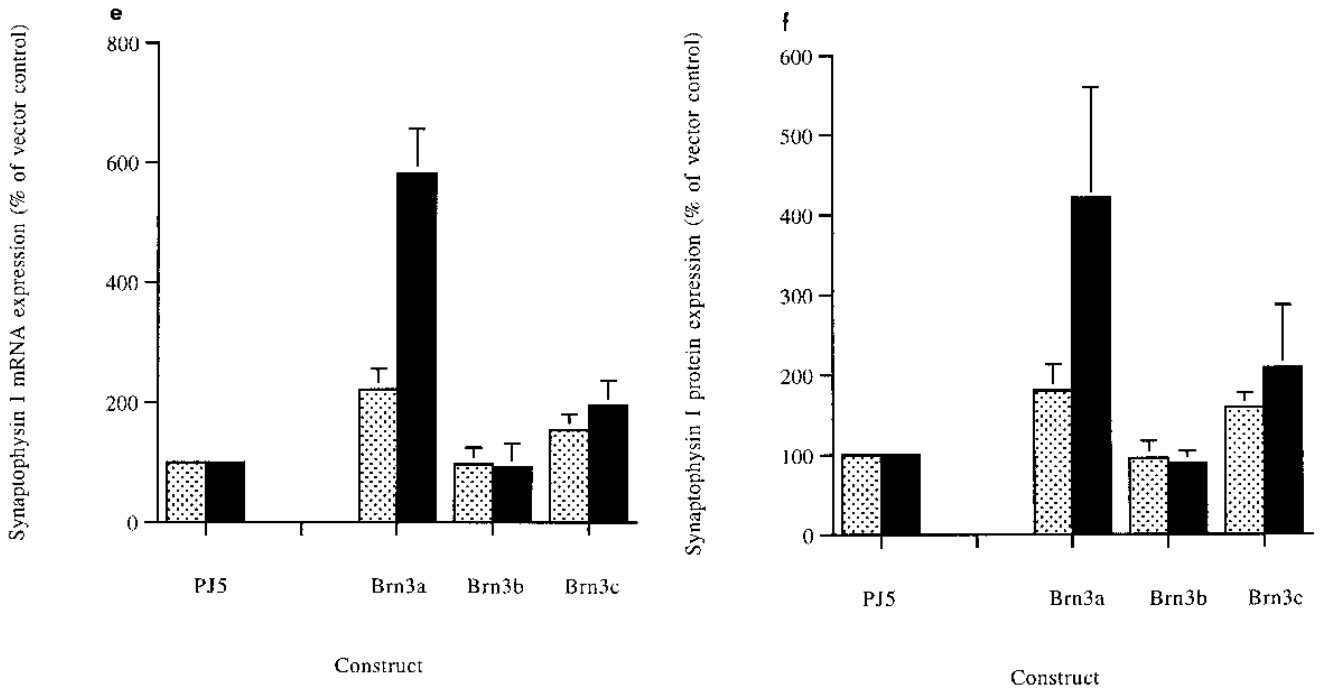

FIG. 6—*Continued.*

the number of processes which formed over control levels ($P < 0.01$) compared to cells expressing the long form of Brn-3a used in all our previous experiments (Fig. 7), suggesting that the N terminus of Brn-3a is not required for process formation. These cells showed some reduction in process length compared to those expressing the long form of Brn-3a (Fig. 7), indicating that the N-terminal activation domain may play some role in this effect. However, a clear enhancement of process length above control levels was still observed, and this effect was enhanced in the steroid-treated cells ($P < 0.05$). Hence, process formation could still be induced in the absence of the N-terminal domain. In agreement with this possibility, a clear enhancement of both process number (Fig. 7a) and length (Fig. 7b) which was steroid inducible was also observed in the cells overexpressing the isolated POU domain of Brn-3a compared to control cells, indicating that this domain alone could induce process formation, although less effectively than intact Brn-3a.

Similar effects were observed when we examined SNAP-25 expression in the cell lines. The cell lines expressing either the short form of Brn-3a or the POU domain both showed enhanced SNAP-25 mRNA expression relative to the control (Fig. 8; $P < 0.05$ in each case), indicating that the POU domain alone can activate SNAP-25 expression.

Interestingly, no effect on length or number of processes (Fig. 7) or SNAP-25 expression (Fig. 8) was achieved by overexpressing the POU domain of Brn-3b. Thus, one or more of the seven amino acid differences in the POU domain sequences of Brn-3a and Brn-3b (14, 17) must play a critical role in this effect. We have previously shown (6) that in terms of the ability to activate a target promoter in cotransfection assays, a critical role is played by the difference at position 22 in the homeodomain, where the valine residue in Brn-3a is replaced by an isoleucine in Brn-3b. Thus, although alterations at this position have no effect on the DNA binding ability of the proteins, the alteration of this residue in Brn-3b to the valine present in Brn-3a confers upon Brn-3b the ability to activate the tk-Oct promoter (6).

We therefore prepared stable ND7-derived cell lines expressing full-length Brn-3a in which this amino acid had been changed to isoleucine (Brn-3aI) or expressing full-length Brn-3b containing valine at this position (Brn-3bV). It should be noted that since the difference at position 22 is the only difference between the Brn-3a and Brn-3b homeodomains, introduction of this mutation effectively exchanges the homeodomains of Brn-3a and Brn-3b, resulting in constructs containing all possible combinations of the Brn-3a or -b linker region with the Brn-3a or -b homeodomain (Fig. 9). Moreover, since the POU-specific domains of Brn-3a and Brn-3b are identical, these constructs represent all various combinations of the different subregions of the POU domain (Fig. 9).

Most interestingly, the mutation of this single residue to isoleucine completely eliminated the ability of Brn-3a to promote neurite outgrowth as assayed by process number (Fig. 7a) or to induce SNAP-25 expression (Fig. 8). Hence, the presence of valine rather than isoleucine at this position in the Brn-3a homeodomain is absolutely critical for the production of increased numbers of processes and SNAP-25 expression. Conversely, Brn-3bV showed enhanced ability to promote increased neurite length (Fig. 7b) ($P < 0.05$) or SNAP-25 expression (Fig. 8) ($P < 0.01$) compared to unmutated Brn-3b. However, Brn-3bV did not promote the formation of increased numbers of processes (Fig. 7a), whereas Brn-3aI still promoted enhanced neurite length (Fig. 7b). These data (summarized in Fig. 9) suggest that for enhanced numbers of processes to form, both the linker region and the POU homeodomain must be derived from Brn-3a, whereas for enhanced length of processes, it is sufficient for either the linker or the homeodomain to be derived from Brn-3a.

DISCUSSION

In previous cotransfection studies, we and others have shown that the Brn-3a transcription factor can activate the promoters of a number of different neuronally expressed genes

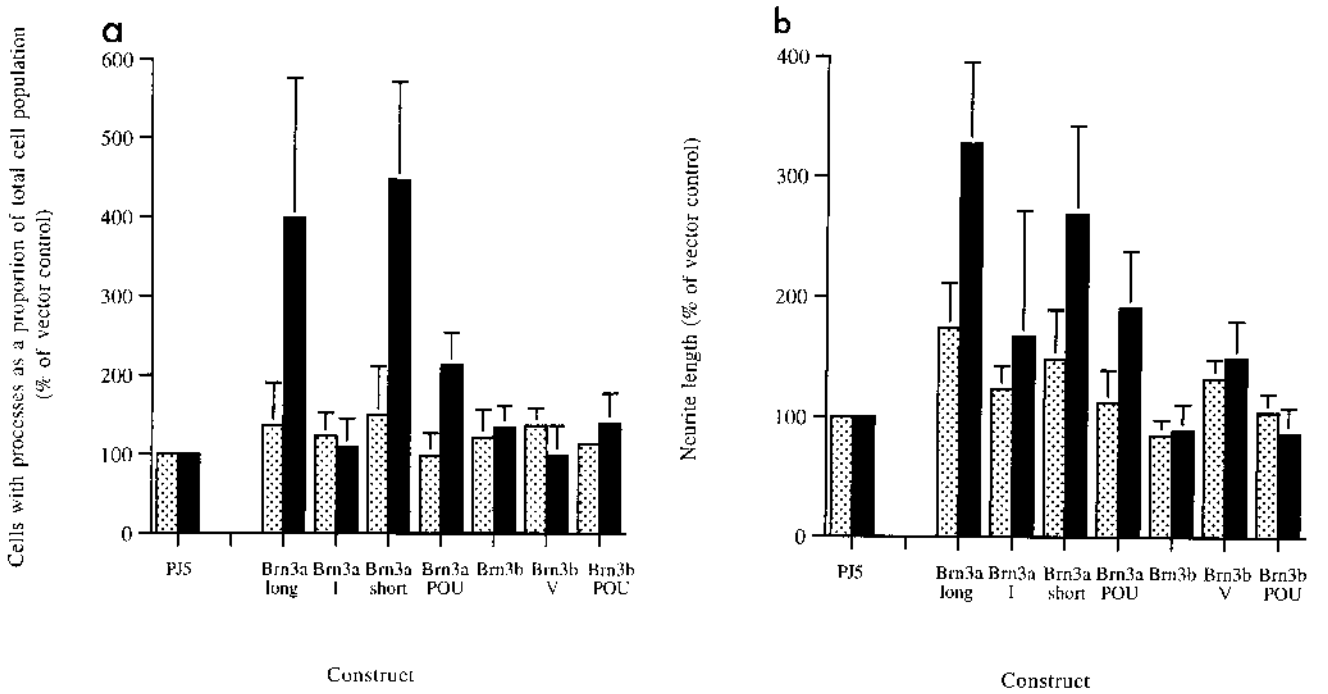

FIG. 7. Effect of overexpressing the indicated forms of Brn-3 on the number of cells with processes (a) and their length (b) relative to the vector control. Stippled boxes, uninduced; solid boxes, induced. Each value is the average of two independent experiments, each analyzed in duplicate and compared to the level in cells transfected with pJ5 vector alone. Data for three independent clones expressing each construct have been combined. Bars show the standard deviations of the means. Brn-3a long and Brn-3a short indicate the results of overexpressing each of the two alternatively spliced forms of Brn-3a. The long form (used in all previous experiments) contains an additional N-terminal exon absent in the short form. Brn-3a I indicates the result with a mutant form of Brn-3a in which the valine at position 22 in the POU homeodomain has been changed to isoleucine; Brn-3b V indicates the result with Brn-3b containing the converse mutation.

(3, 10, 13). Here we have extended these studies to show that this factor can also enhance the expression of endogenous genes when it is overexpressed in a neuronal cell line. Thus, overexpression of Brn-3a can result in the induction of at least four proteins involved at different stages in the synaptic vesicle cycle (23, 24): a presynaptic membrane component (SNAP-25) and synaptic vesicle components involved in docking with the membrane (synaptophysin), fusion and exocytosis (synaptotagmin I), and regulation of trafficking (synapsin I).

Most importantly, overexpression of Brn-3a is sufficient to induce neurite outgrowth, the first time, to our knowledge that this ability has been demonstrated for any neuronally expressed factor. In contrast, Brn-3b had no effect on neurite outgrowth and only induced the expression of the synapsin I gene, paralleling its ability to activate the promoter of this gene in cotransfection experiments. Interestingly, we have recently shown (21a) that the ND7 cells overexpressing Brn-3b show decreased neurite outgrowth following exposure to a differentiation-inducing stimulus. Hence, the normal differentiation process of ND7 cells involves both the observed rise in the differentiation-inducing Brn-3a factor and the fall in the differentiation-inhibiting Brn-3b factor.

Although the N-terminal activation domain of Brn-3a may play some role in its ability to induce SNAP-25 expression and neurite outgrowth, significant effects on both of these processes can be observed in the absence of this domain, and indeed these effects are produced by overexpression of the POU domain alone. In particular, the POU homeodomain of Brn-3a, which differs from that of Brn-3b at a single position, appears to play a critical role in this effect. Thus, altering the valine at position 22 in the POU homeodomain of Brn-3a to its isoleucine equivalent in Brn-3b virtually abolishes the ability of

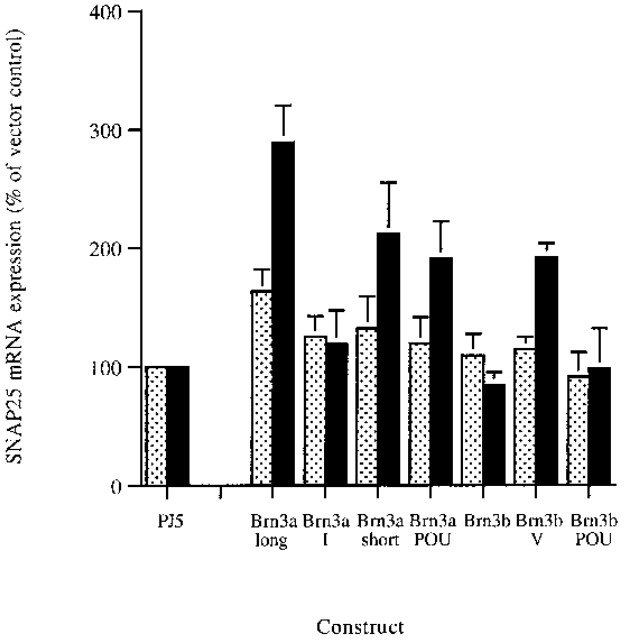

FIG. 8. Effect of overexpressing the indicated form of Brn-3 (as described in the legend to Fig. 8) on SNAP-25 mRNA levels compared to the effect observed in ND7 cells transfected with pJ5 vector alone. Stippled boxes, uninduced; solid boxes, induced. Each value is the mean of that obtained from two independent preparations, each analyzed in duplicate. Data for three individual clones expressing each construct have been combined. Bars show the standard deviations of the means.

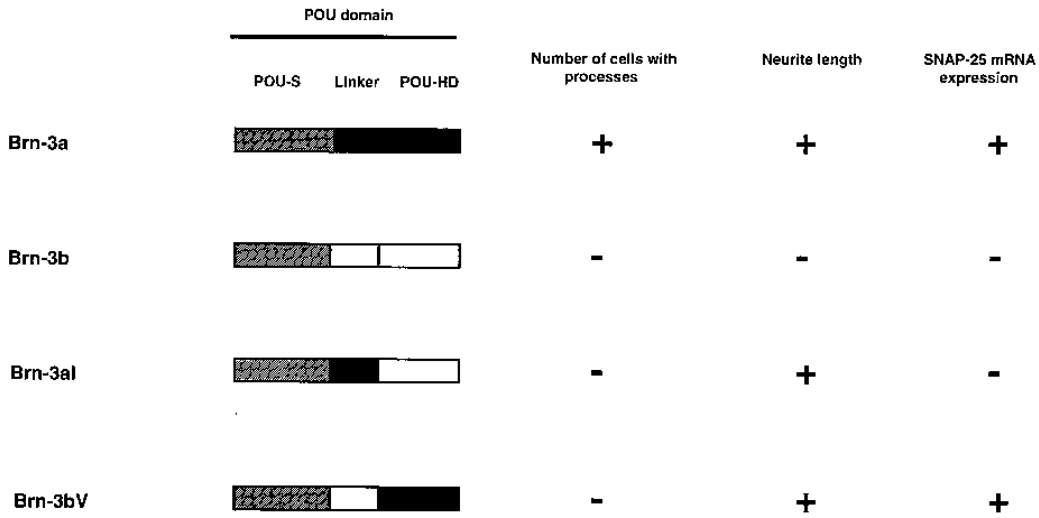

FIG. 9. Schematic diagram of the POU domains in the various constructs and their effects on the number of cells with processes, neurite length, and SNAP-25 expression. The common POU-specific domain of Brn-3a and Brn-3b is shown as a shaded box; the Brn-3a linker and POU homeodomain are shown as solid boxes, and those of Brn-3b are shown as open boxes. Note that the isoleucine-to-valine change converts the POU homeodomain of Brn-3a to that of Brn-3b and vice versa. +, increased relative to control cells; −, no increase.

full-length Brn-3a to induce increased numbers of neurites and SNAP-25 expression, although it does not alter the DNA binding ability of the protein. Conversely, alteration of this amino acid in Brn-3b to valine produces enhancement of neurite length and SNAP-25 expression, paralleling the ability of this mutant Brn-3b to activate a test promoter in cotransfections (6). Thus, a single amino acid identified as critical in cotransfection experiments also plays a critical role in the ability to induce neurite outgrowth and endogenous gene expression.

Thus, close parallels exist between the ability of Brn-3a to activate gene expression and its ability to induce neurite outgrowth. This finding indicates that the induction of neurite outgrowth by overexpression of Brn-3a or following the rise in its expression during ND7 cell differentiation is likely to be dependent on the ability of Brn-3a to activate specific target genes whose protein products are required for neurite outgrowth. Given the ability of Brn-3a to induce several different components of the synaptic vesicle cycle as well as the role of SNAP-25 in this cycle and its role in the constitutive exocytosis required for neurite outgrowth (19, 20), it is likely that the ability of Brn-3a to induce SNAP-25 and potentially other components held in common between these two processes underlies its role in promoting neurite outgrowth. Indeed, we previously showed that antisense-mediated inhibition of Brn-3a expression prevented the neurite outgrowth and SNAP-25 induction which normally occur when ND7 cells are transferred to serum-free medium, indicating that Brn-3a is necessary for these effects (13).

The findings presented here extend these findings to show that overexpression of Brn-3a alone is sufficient to induce neurite outgrowth and the coordinate induction of synaptic vesicle proteins in ND7 cells. Such findings closely parallel the role of the MyoD transcription factor in muscle differentiation. This factor was originally identified on the basis of its ability to induce the 10T1/2 immortalized cell line to differentiate to a skeletal muscle-like phenotype (5) and was subsequently shown to play a key role in skeletal muscle differentiation in vivo (8). Although further experiments will be required to show that Brn-3a can also regulate neurite outgrowth in primary neurons in vitro and in vivo, it is of interest that Brn-3a is expressed in the earliest postmitotic neurons which form in the central nervous system, supporting a critical role in the specification of the mature neuronal phenotype (9). Obviously, in view of our observations that increased (this report) or decreased (13) expression of Brn-3a has little or no effect on neuronal cell division, other factors must be involved in the growth arrest characteristic of terminal neuronal differentiation. Indeed, the finding that in the peripheral nervous system Brn-3a can be expressed in dividing neuronal progenitors (9) suggests that in different systems, growth arrest and the induction of neuron-specific gene expression by Brn-3a may occur at different times relative to one another.

Whatever the case, the observation that overexpression of a single transcription factor can induce both neurite outgrowth and the expression of synaptic proteins suggests that Brn-3a is likely to play a critical role in these processes during development and that its artificial overexpression may be of benefit in cases of spinal injury, when nerve regeneration is poor.

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