Molecular Cloning and Characterization of a Novel Form of Neuropeptide Gene as a Developmentally Regulated Molecule*

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To examine the molecular basis controlling neuronal differentiation, subtraction library construction and differential screening were used to identify cDNAs whose mRNA levels are regulated in mouse NS20Y cells by dibutyryl cyclic AMP treatment. One of them, N27K, whose mRNA increases transiently during both neuronal differentiation in NS20Y cells and development in mouse brain. The deduced amino acid sequence of N27K comprises 212 amino acid residues and is a novel form of a precursor protein for a new neuropeptide nociceptin/orphanin FQ, which we independently cloned as N23K. That is, the putative protein encoded by N27K is 25 amino acids longer than that encoded by N23K. Using an antibody against a C-terminal peptide of the N27K protein that recognizes a 27-kDa protein in Western blot analysis, a punctate structure in the perinuclear region and areas near the tip of neurites is visualized in neurally differentiating NS20Y cells. The time of maximal expression correlates with periods of neurite extension, and expression decreases as the neuritic network develops. Immunohistochemistry of tissue sections of the mouse central nervous system revealed that reactivity for the anti-N27K protein antibody can detected in early generated neurons at embryonic day 14, in virtually all immature neurons at postnatal day 1, and in subsets of neurons of discrete brain regions such as the hypothalamus and spinal cord in adults. This remarkable redistribution suggests that N27K may be involved in a process in neurite outgrowth and nervous system development.

Differentiation and development of the vertebrate nervous system require temporally and spatially dynamic interactions between gene expression and external signals. The identification of regulatory molecules that program specific developmental transitions has attracted much attention. There are two common approaches to analyzing the dynamic molecular mechanisms underlying differentiation and development of the nervous system. One is the isolation and analysis of mammalian homologues to developmentally important molecules first identified in lower eukaryotes (1, 2). Another is the direct identification of novel genes that are expressed differentially during neurogenesis (3, 4). However, attempts to use vertebrate in vivo systems are complicated by the large number of cell types in the nervous system and the complexity of their interactions. NS20Y cells are derived from mouse neuroblastoma C1300 cells (5). In the presence of agents that increase intracellular cyclic AMP, NS20Y cells differentiate from replicating cells to nonreplicating cells with a neuronal phenotype by the extension of long, branching neurites (5). The biochemical and electrical characteristics of differentiated NS20Y cells have been described by a number of investigators, and NS20Y cells have been used as an in vitro model for differentiation and development of neuronal cells (6–8). Here we report the isolation of a nervous system-specific gene, N27K, from neurally differentiating NS20Y cells using a subtractive hybridization approach. The N27K mRNA and protein show transient expression during differentiation in NS20Y cells and development in mouse brain. The encoded protein is a novel form of precursor protein for a newly discovered neuropeptide, nociceptin (9, 10), also called orphanin FQ (11). We present unique features of N27K that suggest an important role in neurite outgrowth and development of the nervous system. We also show that mouse NS20Y cells may provide a useful cellular model in which to study how nociceptin is up-regulated and silenced in the cyclic AMP-responsive signal transduction pathway.

MATERIALS AND METHODS

Cell Cultures—Mouse NS20Y cells were grown in high glucose Dulbecco's modified Eagle’s essential medium plus 5% heat-treated horse serum and 5% fetal bovine serum. Neural differentiation was induced by the addition of 1 mM dibutyryl cyclic AMP (dbcAMP)1 (Yamasa, Chiba, Japan) (5).

RNA Extraction—Total cellular RNA was extracted from cultured cells and various organs of male ddY mouse by the differential ethanol precipitation method with modifications (12). The amounts of RNA were quantified by measuring UV absorption at 260 nm.

Subtraction Cloning and Differential Hybridization—Poly(A)1 RNA was purified on oligo(dT) latex (Nihon Roche) from the total RNA of dbcAMP1-treated and dbcAMP NS20Y cells. A cDNA library from the oligo(dT)-primed reverse transcription of dbcAMP+ mRNA pool was constructed in the ZAP system (Stratagene), and single-stranded (antisense) cDNA in the pBluescript vector was rescued by helper phage infection. Common sequences between the dbcAMP+ and dbcAMP− pools were removed from the dbcAMP+ cDNA pool by hybridization with biotinylated dbcAMP mRNA followed by proteolytic degradation.

1 The abbreviations used are: dbcAMP, dibutyryl cyclic AMP; PBS, phosphate-buffered saline; CNS, central nervous system; kb, kilobase(s).
Molecular Cloning of a Developmentally Regulated Gene

DNA Extraction—DNA samples were blotted on membranes (Hybond-N, substracted library. About 300 colonies were randomly selected from the library. Plasmid DNA samples were blotted on membranes (Hybond-N, Amersham Corp.) and screened by differential hybridization with 32P-labeled cDNA probes synthesized from the dabcAMP or dabcAMP mRNA pools. A plasmid, which contained a 0.9-kilobase (kb) insert, was selected from those that showed a higher signal intensity upon hybridization with the dabcAMP cDNA probe than that with the dabcAMP-DNA probe. A larger cDNA insert (N27K) was isolated by screening two independent cDNA libraries constructed from the dabcAMP mRNA pools.

Northern Blot Analysis—Total RNA was electrophoresed on 6% formaldehyde/1% agarose gels containing 0.5 μl ethidium bromide and blotted onto nylon membranes (Hybond-N, Amersham Corp.). RNA blots were fixed on the membranes by UV irradiation and hybridized with 32P-labeled cDNA that hybridized specifically to the 1.7 kb band. Hybridization and washing were performed under the conditions reported previously (12).

DNA Sequencing—DNA sequences were determined as double-stranded plasmids in both directions by the dideoxy chain termination method (14).

Expression of N27K-encoded Protein in COS Cells—A 1.0-kb EcoRl-Xhol fragment of N27K was subcloned into the unique EcoRl-Xhol site of the expression vector pS1023 (15) to construct pN27K. COS-1 cells were transfected with pN27K by the DEAE-dextran method followed by chloroquine shock treatment (16). After 72 h of incubation, pN27-transfected COS cells were sonicated in Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 150 mM NaCl, 15 mM 2-mercaptoethanol, 0.25 mg of bromphenol blue/ml) and analyzed as described for immunoblotting.

Generation of Polyclonal Antiserum—Rabbits were immunized with a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to residues 203–217 (C-terminal) of the deduced amino acid sequence of N27K. The rabbits were injected intramuscularly with 50 μg of the synthetic peptide conjugated with 100 μg of keyhole limpet hemocyanin (KLIH) emulsified with 0.5% defatted milk overnight at 4°C, and 3) horseradish peroxidase-conjugated secondary antibody described previously (17) or a 1:100 dilution for 1 h at 37°C. Finally, the immunoreaction was visualized with 0.01% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 50 mM Tris-HCl buffer, pH 7.6, for 5–15 min at 37°C, dehydrated, and mounted under coverslips. To confirm the specificity of the immunoreaction, the immunostaining procedure was carried out with the antibody solution preabsorbed by excess antigen peptide (100 μg/ml).

RESULTS

Cloning of a Differentially Expressed Gene—A cDNA of 0.9 kb, denoted N27K, was identified by differential screening of a subtracted CDNA library prepared from 48-h dabcAMP-treated NS20Y cells. This fragment detects a major transcript of approximately 1.7 kb on Northern blots of NS20Y cells treated with dabcAMP for 48 h (Fig. 1A). We examined the expression of N27K mRNA in NS20Y cells during neuronal differentiation induced by dabcAMP treatment. The level of mRNA was markedly increased after dabcAMP treatment, reaching a maximum at 48 h and slightly decreasing thereafter. Fig. 1 (B and C) shows the distribution of N27K mRNA isolated from embryonic brain and adult mouse tissues; N27K mRNA (1.7 kb) is enriched in brain and spinal cord but not in any non-neuronal tissues examined. N27K mRNA was detected in embryonic day 14 (E14) brain, and larger amounts were detected in 1-day postnatal (P1) brain. As compared with P1 brain, the amount of N27K mRNA in P8 brain was lower. A prominent decrease was detected in adult brain. These results indicate that the levels of N27K mRNA decrease gradually as mice grow.
Molecular Cloning of a Developmentally Regulated Gene

Fig. 2. Primary structures of N27K and the N27K protein. A, nucleotide and predicted amino acid sequences of N27K. Numbers to the right of the nucleotide sequence refer to the last nucleotide in each line beginning from the first residue of the ATG triplet encoding the initiating Met. The predicted amino acid sequence is displayed under the corresponding nucleotide sequences, and the residue numbers are indicated from the initiating Met. Asterisks indicate a stop codon. The N-terminal hydrophobic segment is underlined. The bold wavy line indicates the amino acid sequence of nociceptin/orphanin FQ. The wavy line indicates the amino acid sequence of the peptide used to raise the peptide antibody used as an immunogen. B, alignment of the amino acid sequences of the N27K protein and the N23K protein. C, inserted nucleotide sequence of N23K. The long open reading frame predicts a 212-amino acid protein (calculated molecular weight, 23320; Fig. 2). A putative polyadenylation signal AATAAA before the poly(A) stretch. The longest open reading frame was confirmed by the detection of a protein as described below. We found that N27K is a novel form of N23K (9), a newly discovered neuropeptide nociceptin or orphanin FQ (10, 11) (Fig. 2A). The difference between N23K and N27K lies in a 57-base pair nucleotide sequence in N23K, the sequence is inserted between Gly595 and Gly596 of N23K protein. Consequently, 2 amino acids are added to the common 185 amino acids in the N23K protein, whereas 27 amino acids are added to the common sequence in the N27K protein (Fig. 2B). A short stretch of hydrophobic residues near the N terminus (20), three repeatsof the novel repetitive motif (DAEFG) in the central region, and a nociceptin heptadecapeptide \((\text{GFFGTGARKSARKLANQ})\) that resembles opioid dynorphin A are included in the N27K protein. There is one potential site of phosphorylation by protein kinase A (177RRRT180) (21), and there are two potential MAP kinase phosphorylation sites (73SP74 and 207PPSP210) (22) in the N27K protein. Consequently, 2 amino acids are added to the common 185 amino acids in the N23K protein, whereas 27 amino acids are added to the common sequence in the N27K protein (Fig. 2B). A short stretch of hydrophobic residues near the N terminus (20), three repeats of the novel repetitive motif (DAEFG) in the central region, and a nociceptin heptadecapeptide \((\text{GFFGTGARKSARKLANQ})\) that resembles opioid dynorphin A are included in the N27K protein. There is one potential site of phosphorylation by protein kinase A (177RRRT180) (21), and there are two potential MAP kinase phosphorylation sites (73SP74 and 207PPSP210) (22) in the N27K protein. Consequently, 2 amino acids are added to the common 185 amino acids in the N23K protein, whereas 27 amino acids are added to the common sequence in the N27K protein (Fig. 2B). A short stretch of hydrophobic residues near the N terminus (20), three repeats of the novel repetitive motif (DAEFG) in the central region, and a nociceptin heptadecapeptide \((\text{GFFGTGARKSARKLANQ})\) that resembles opioid dynorphin A are included in the N27K protein. There is one potential site of phosphorylation by protein kinase A (177RRRT180) (21), and there are two potential MAP kinase phosphorylation sites (73SP74 and 207PPSP210) (22) in the predicted N27K sequence. The potential MAP kinase phosphorylation site at 207PPSP210 is specific to the N27K protein. A data base search using the FASTA program (23) resulted in the identification of a nucleotide fragment sharing extensive sequence homology (nucleotides 163–344, 82.4%) with a putatively transcribed partial gene derived from human fetal brain...
This strongly suggests the existence of an N27K homologue in human brain.

Production of the N27K Protein-specific Antibody—In order to confirm the assignment of the open reading frame, we synthesized the N27K protein by transfecting the cDNA into COS-1 cells (Fig. 3, left, lane 8). An antibody against synthetic peptides corresponding to the C-terminal region of the deduced protein sequence reacted with a 27-kDa band. This band was not present in mock-transfected COS-1 cells (Fig. 3, left, lane 9). This result indicates that the coding region of the N27K cDNA is mostly translated into protein. Western analysis of protein extracts prepared from NS20Y cells during neuronal differentiation showed that the N27K protein is induced by dbcAMP-treatment for 24–120 h, like the similarly sized 27-kDa protein in transfected COS-1 cells and that the level decreases slightly after 7 days and significantly after 10 days (Fig. 3, left, lanes 1–7). Binding of the antibody to the 27-kDa band was inhibited in the presence of the corresponding peptide using immunogen (Fig. 3, right), confirming the specificity of the reaction.

Localization of the N27K Protein in NS20Y Cells—To provide more detailed information on the distribution of the N27K protein, we performed immunocytological studies utilizing an antibody against the N27K protein in 48-h dbcAMP-treated NS20Y cells (Fig. 4). Paraformaldehyde fixation and Triton X-100 permeabilization revealed that the N27K protein is visible in the juxta-nuclear region and neurites with a punctated appearance (Fig. 4, A and B). The specificity of antibody staining was confirmed by the lack of this staining pattern when the antibody was absorbed by the corresponding peptide immunogen (Fig. 4, C and D). The N27K protein was not distributed uniformly in neurites, and the areas at the tips of neurites were more intensely labeled (Fig. 4E). These punctates were colocalized with secretogranin I (24), the most prominent protein in large dense core vesicles (LDVs) (Fig. 4F). These data raise the possibility that the N27K protein is a secretory protein.

We next examined whether the staining pattern for the N27K protein changes during differentiation in NS20Y cells (Fig. 5). Little reactivity toward the antibody was seen in undifferentiated NS20Y cells (Fig. 5, A and B). After 18 h of treatment with 1 mM dbcAMP, the cells showed intense staining of a compact juxta-nuclear structure resembling the Golgi apparatus (Fig. 5C). After dbcAMP treatment for 48 h, the cells extended processes, and the N27K protein was found in the

![Fig. 4. Immunofluorescence detection of the N27K protein in dbcAMP-treated NS20Y cells.](image-url)
juxta-nuclear structure and neurites (Fig. 4A and Fig. 5, E and F). After longer treatment (96 h), neurite length increased, and the N27K protein was clearly found to accumulate on the tips of most neurites (Fig. 5, G and H). After 10 days, while the complexity of the neuritic network increased, N27K protein expression became restricted to a very small proportion of varicosities distributed along thin neurites, and little immunosignal remained in the juxta-nuclear structure (Fig. 5, I and J). The same results were obtained in primary cultured cells taken from E18 rat cerebral cortex. The N27K protein immunosignal with a punctated appearance was detected in neuronal cells at the stage examined, 4 days in vitro (4DIV). At later stages (7DIV), N27K protein expression was less (data not shown). Thus, the N27K protein is also transiently expressed during neuronal differentiation in primary cultured cells. In addition, we previously generated an anti-N23K protein antibody that did not cross-react with the N27K protein (9), and the distribution pattern of the N23K protein in NS20Y cells during differentiation is the same as that of the N27K protein (data not shown).

Localization of the N27K Protein in Mouse Nervous System—In order to examine the developmental distribution of the N27K protein in the mouse central nervous system (CNS), immunohistochemical staining of tissue sections was performed using the affinity-purified N27K antibody. We confirmed that the N27K antiserum reacted with a 27-kDa protein in E14 head, P1, and adult mouse brain that has the same molecular mass as the protein in developing NS20Y cells, and preabsorption with the peptide used as antigen abolished the specific protein band (Fig. 6A). A larger expression of the N27K protein was observed in P1 brain, and this transient nature during development is consistent with the results of Northern blot analysis (Fig. 1C). Upon immunohistochemistry of sections of E14 mouse CNS, N27K protein immunoreactivity was found in the neurons of the peripheral zone throughout the CNS. In the telencephalon, for example, N27K-immunoreactive cells were detected in the marginal zone and subplate of the cerebral cortex in which the early generated neurons of the cortex are situated at this stage (Fig. 6B). At E16, the distribution of immunoreactive cells had spread into a deeper zone. In the cerebral cortex, immunoreactive cells were detected in the late generated cortical plates as well as the marginal zone and subplate derived from the preplate (Fig. 6C). At P1, intense N27K immunoreactivity was ubiquitously localized in most neurons throughout the CNS. In the cerebral cortex, immunoreactive neurons occupied the whole cortical plate (Fig. 6D). During the embryonic and neonatal periods, immunoreactions were localized in the cell bodies and processes of immature neurons.

In adult mice, N27K immunoreactivity was observed in subsets of neurons in discrete regions throughout the CNS, including the telencephalon, diencephalon, midbrain, cerebellum, pons, medulla oblongata, and spinal cord. In the hypothalamus, N27K immunoreactivity was located in the cell bodies of the paraventricular nucleus (Fig. 6E) and arcuate nucleus, indicating the localization of this protein in the hypothalamic neuroendocrine system. In the spinal cord, N27K immunoreactive neurons were detected in both the dorsal and ventral horns (Fig. 6F). These immunoreactions were completely abolished when preabsorbed antibody was substituted for the primary antibody (Fig. 6G). In the peripheral nervous system, N27K immunoreactivity was found in the dorsal root ganglion. In the ganglion, small sized neurons (15–30 μm in diameter) were immunopositive, whereas large sized neurons were immunonegative (Fig. 6H).

DISCUSSION

We have isolated and characterized a gene, N27K, whose expression is regulated both in mouse NS20Y cells by dbcAMP and in mouse brain during development. N27K encodes a novel form of the N23K protein (9), a precursor protein for the newly discovered neuropeptide nociceptin, which resembles opioid dynorphin A (10, 11). The expression of the N27K protein in both NS20Y cells and mouse brain shows remarkable redistribution during development, suggesting that the protein has a dual role as both a source for nociceptin in the mature brain and a regulatory molecule during neuronal differentiation.

Nociceptin was identified as a ligand for an orphan hetero-
Molecular Cloning of a Developmentally Regulated Gene

immunodetection of a 27-kDa protein in whole brain extract (lane 1–3, 20 μg) or differentiating NS20Y cells (lane 4, 5 μg). Samples were electrophoresed on 15% polyacrylamide SDS gels, followed by Western blot analysis with anti-N27K protein antibody (1 μg/ml) (left). Binding of the antibody to the 27-kDa band was competitively inhibited by the antigen peptide (right). Lane 1, embryonic 14 day (E14); lane 2, postnatal 1 day (P1); lane 3, adult; lane 4, NS20Y cells treated with db-cAMP for 48 h. B–D, immunohistochemistry of the N27K protein in the developing mouse cerebral cortex. At E14 (B), N27K protein immunostaining is detected in cells of the marginal zone (M) and subplate (SP). At E16 (C), neurons of the cortical plate (CP) as well as the marginal zone (M) and subplate (SP) exhibit the positive reaction. At P1 (D), many neurons in the cortical plate (CP) are immunoreactive for the N27K protein. Immunoreactions are found in the cell body and the apical dendrite of cortical neurons (arrows). E–H, nervous system of the adult mouse immunostained with anti-N27K protein. E, frontal section of the hypothalamus. Neurons of the paraventricular nucleus (PVN) are immunoreactive for the N27K protein. F, and G, the ventral horn of the spinal cord. All motoneurons (arrows) are immunopositive (F) and these reactions are completely abolished when immunostained with antibody preabsorbed with antigen peptide (G, arrows). H, cervical dorsal ganglion. Small sized (arrows) but not large sized neurons (arrowheads) are trimeric GTP-binding protein (G protein)-coupled receptor (ORL1, for opioid receptor-like 1) (25–31) and may act as a transmitter in the brain by modulating nociceptive and locomotor behavior (10, 11). Nociceptin is pharmacologically unique in the opioid family, because nociceptin has no significant agonist activity at μ-, δ-, or κ-opioid receptors, and classical opioid peptides and broad-spectrum opioid antagonists such as naloxone have no affinity for the ORL1 receptors (10, 11). Although nociceptin is a very recently identified neuropeptide and no information is available about processing intermediates and additional neuropeptide transmitters, we have isolated both larger polyprotein precursors N23K and N27K as developmentally regulated genes.

Fig. 6: Immunodetection of the N27K protein in mouse brain.

A, immunodetection of a 27-kDa protein in whole brain extract (lane 1–3, 20 μg) or differentiating NS20Y cells (lane 4, 5 μg). Samples were electrophoresed on 15% polyacrylamide SDS gels, followed by Western blot analysis with anti-N27K protein antibody (1 μg/ml) (left). Binding of the antibody to the 27-kDa band was competitively inhibited by the antigen peptide (right). Lane 1, embryonic 14 day (E14); lane 2, postnatal 1 day (P1); lane 3, adult; lane 4, NS20Y cells treated with db-cAMP for 48 h. B–D, immunohistochemistry of the N27K protein in the developing mouse cerebral cortex. At E14 (B), N27K protein immunostaining is detected in cells of the marginal zone (M) and subplate (SP). At E16 (C), neurons of the cortical plate (CP) as well as the marginal zone (M) and subplate (SP) exhibit the positive reaction. At P1 (D), many neurons in the cortical plate (CP) are immunoreactive for the N27K protein. Immunoreactions are found in the cell body and the apical dendrite of cortical neurons (arrows). E–H, nervous system of the adult mouse immunostained with anti-N27K protein. E, frontal section of the hypothalamus. Neurons of the paraventricular nucleus (PVN) are immunoreactive for the N27K protein. F, and G, the ventral horn of the spinal cord. All motoneurons (arrows) are immunopositive (F) and these reactions are completely abolished when immunostained with antibody preabsorbed with antigen peptide (G, arrows). H, cervical dorsal ganglion. Small sized (arrows) but not large sized neurons (arrowheads) are trimeric GTP-binding protein (G protein)-coupled receptor (ORL1, for opioid receptor-like 1) (25–31) and may act as a transmitter in the brain by modulating nociceptive and locomotor behavior (10, 11). Nociceptin is pharmacologically unique in the opioid family, because nociceptin has no significant agonist activity at μ-, δ-, or κ-opioid receptors, and classical opioid peptides and broad-spectrum opioid antagonists such as naloxone have no affinity for the ORL1 receptors (10, 11). Although nociceptin is a very recently identified neuropeptide and no information is available about processing intermediates and additional neuropeptide transmitters, we have isolated both larger polyprotein precursors N23K and N27K as developmentally regulated genes.

Many genes encoding neuropeptides display a positive response to the activation of cAMP-dependent second messenger systems in neuroendocrine cells and neuroblastoma cells. Indeed, genes encoding other classes of opioid peptides, proenkephalin (32, 33), prodynorphin (34), and pro-opiomelanocortin (35), exhibit positive regulation in response to cAMP stimulation. However, in all cases, the induction of mRNA expression is not so strong or transient as that of N27K. It is interesting to examine the molecular mechanisms for the regulation of N23K/N27K gene expression during differentiation and the gene silence in NS20Y cells. Not only the expression of the mRNA for N27K but also protein expression is increased in NS20Y cells that are extending neurites in response to db-cAMP. In the use of antibody against the C-terminal peptide of the N27K protein, our immunocytochemical analysis clearly showed that the neuronal differentiation process is characterized not only by the expression of the N27K protein but also by its subcellular compartmentalization. That is, the N27K protein initially appears in the perinuclear area and then moves to the distal region of neurites. Suburo et al. reported that in a sensory neuron-derived cell line, ND7, neuropeptide Y and its C-flanking peptide (CPON) are transported from the cell body to the tips of the processes during differentiation (36). The most remarkable difference between neuropeptide Y, CPON, and the N27K protein is the maximal expression of the N27K protein near the neurite tip in correlation with periods of neurite outgrowth and the decrease in immunoreactivity as neuronal network is formed, phenomena not previously described for other neuropeptides. Thus, the amount and intracellular localization of the N27K protein in NS20Y cells are regulated in parallel with the differentiation stage. In its pattern of transient expression in cultured neuronal cells, the N27K protein shows similarities to the neuronal growth-associated proteins SCG10 (37) and GAP43 (38) rather than to other neuropeptide proteins. However, the sequences of the three proteins are unrelated (37–40), and the localization and timing of GAP43 protein expression are different from those of the N27K protein during differentiation in NS20Y cells (data not shown). These data suggest the possibility of different roles for the N27K protein and SCG10 and GAP43 in neuronal differentiation.

Endogenous opioid systems, like other neuropeptide systems (41, 42), have been implicated in the regulation of nervous system development (43, 44). Indeed, the exogenous addition of opioids or opioid antagonist to neuroblastoma or neuronal cells modulates their proliferation (45–47). Furthermore, there is accumulating evidence for the involvement of neuropeptide precursor forms and their derivatives in cellular growth and differentiation (48, 49). These reports and our observation of the colocalization of secretogranin I with the N27K protein lend
support to the possibility that the N27K protein might be a secretory protein and have a trophic function. However, using an antibody against the C-terminal peptide of the N27K protein, we could not detect a 27-kDa protein or other bands in the medium of NS20Y cells stimulated by dBCAMP (data not shown). This could be due to a technical limitation in the detection of the protein by Western blot analysis. Another important notion may involve the processing of the precursor peptide. Because proenkephalin, prodynorphin, and pro-opiomelanocortin are precursors of several bioactive peptides, it is possible that a uniquely processed peptide without a C-terminal peptide results from processing and that an extracellular pathway exists for processing the peptide. In this regard, one study suggests that astrocytes secrete a processing enzyme carboxypeptidase E (50). It is important to determine whether any unique processing of the N27K protein occurs and if the N27K protein or its processing intermediates or derivative peptides including nociceptin have any as yet uncharacterized functions during differentiation. Future studies of ORL1 and other corresponding receptors for additional neuropeptides in NS20Y cells will allow a more reliable interpretation of the role of the N27K protein during neuronal differentiation.

The tissue distribution of N27K protein immunoreactivity in the developing mouse indicates that this protein is produced by postmitotic neurons throughout entire CNS. At E14, neurons situated in the peripheral region of the CNS (such as preplate neurons in the cerebral cortex) show a positive reaction (Fig. 6B). It is likely that early generated postmigratory and differentiated neurons gain the ability to produce the peptide. At P1, when the amounts of N27K mRNA are at their maximum level during the life of mice, virtually all of the central neurons are immunoreactive, suggesting that the N27K protein may have a function common to all the central neurons during development. On the other hand, the distribution of the N27K protein in the adult CNS differs from that in the developing CNS. The N27K protein-immunoreactive cells are distributed widely but in particular regions in the adult. N27K protein immunostaining is localized exclusively in the neuronal cell body, which is reminiscent of the localization of neuropeptide precursor proteins like pro-TRH (51). Although the N27K protein contains a sequence identical to the bioactive peptide nociceptin/orphanin FQ (10, 11), the immunoreaction of C-terminal region of the N27K protein may represent the distribution of the precursor form, not the processed peptides in adult CNS. The distribution of immunoreactivity in adult CNS overlaps with that of ORL1, mRNA, which has been reported to be prominently expressed in many regions of the adult CNS, such as the hypothalamic paraventricular nucleus and both the dorsal and ventral horns of the spinal cord (26, 27, 29, 31, 32). Furthermore, in the present study, N27K protein immunoreactivity was exclusively found in the small sized primary afferent neurons of the dorsal root ganglion (Fig. 6H), which is known to project to the dorsal horn of the spinal cord and contain substance P, a putative nociceptive transmitter (52). Therefore, it is highly likely that nociceptin processed from the N27K protein functions as a neurotransmitter involved in nociceptive transduction in the adult mouse.

Because little is known about the processes involved in the selective localization of neuropeptide precursor proteins or larger polypeptides during brain development, it is difficult to make comparisons. However, based on in situ hybridization methodology, it is at least clear that preprodynorphin mRNA-positive cells first appear postnatally in the cerebral cortex (53). Preproenkephalin mRNA-positive cells are first detected in the telencephalon of E18 rat brain, and the mRNA is associated with all embryonic cells, both proliferative and postmitotic. After birth, the mRNA is more discretely localized, predominantly in the cerebellum, hippocampus, and caudate putamen (54). On the basis of these reports, the immunostaining of the N27K protein shows its early appearance and novel pattern, suggesting that the N27K protein may represent a new class in the opioid family that functions as a potentially important neuromodulator during differentiation in vivo as well as in vitro in the NS20Y cell system.

In the present study, we could not detect a marked difference in the distributions of the N27K protein and the N23K protein in NS20Y cells during differentiation or in mouse brain during development. Then, at this stage, it is difficult to discuss the possibilities of a functional difference between N23K and N27K, although the N27K protein is 25 amino acids longer than the N23K protein. To determine the biological functions of N27K and N23K, it is now necessary to overexpress the N27K or/and N23K genes in undifferentiated NS20Y cells and various potent cell lines that undergo neuronal differentiation and study their morphological and biochemical properties. Finally, further studies coupled with gene disruption of N27K/ N23K in mice will hopefully yield important insights into brain function.

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Addendum—After submission of the initial version of this article, two independent groups reported their discovery of a novel neuropeptide, nociceptin/orphanin FQ, isolated from rat brain and porcine hypothalamus as an endogenous ligand with orphanin heterotrimeric GTP-binding protein-coupled receptor (10, 11). We therefore incorporated the new information in revising the manuscript while describing N23K, an N27K homologue, elsewhere (9). It is of particular interest that the protein was discovered independently using totally different approaches.

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Molecular Cloning of a Developmentally Regulated Gene

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Molecular Cloning and Characterization of a Novel Form of Neuropeptide Gene as a Developmentally Regulated Molecule

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