Expression of preproenkephalin mRNA by cultured astrocytes and neurons

(glial heterogeneity/opioid peptide/prodynorphin)

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ABSTRACT  Expression of preproenkephalin mRNA by developing glia and neurons was examined in cultures of embryonic and neonatal rat brain. Cultured glia from specific regions of embryonic day 17 and neonatal day 1 rat brain were identified as astrocytes on the basis of both morphology and expression of immunoreactivity for glial fibrillary acidic protein. The level of preproenkephalin mRNA in cultured neonatal hypothalamic astrocytes was comparable to levels present in cultured embryonic striatal and hypothalamic neurons. Levels of the mRNA were significantly higher in astrocytes derived from neonatal hypothalamus compared to astrocytes derived from other areas of the brain. Thus, there is heterogeneity among astrocytes with respect to preproenkephalin expression. Levels of preproenkephalin mRNA in cultured neonatal striatal astrocytes were only one-third as high as levels in embryonic striatal astrocytes; this observation suggests that glial expression of the gene may be down-regulated during development. Although cultured hypothalamic neurons contained substantial levels of prodynorphin mRNA, levels of this mRNA were not detectable in cultured astrocytes from any brain region or in cultured striatal neurons. Thus, glia do not express all opioid peptide genes during development. These observations suggest that expression of the preproenkephalin gene by astrocytes may play a role in development of the brain.

Astrocytes are the most numerous cellular type in the brain and appear to subserve multiple functions. Astrocytes may help to regulate the ionic environment of the extracellular space (1–3), to inactive neurotransmitters by accumulating them (3–7), and to transfer nutrients and neurotransmitter precursors to neurons (8, 9). Proliferation of astrocytes plays an integral part in the healing of brain injury, and interactions between neurons and astrocytes regulate neuronal differentiation and process elongation (9–13). Studies of cell surface molecules expressed by astrocytes have shown at least two identifiable subtypes (14), and there is some evidence that there are differences among astrocytes from different regions of the brain (10).

Although astrocytes express receptors for neurotransmitters (6, 15–19) and contain systems for neurotransmitter uptake (3–7), they do not appear to synthesize neurotransmitters themselves. With the exception of the amino acid neurotransmitters, it is generally accepted that neurotransmitters in brain are localized within neurons. However, neurotransmitter genes may be expressed by nonneuronal cells, particularly in the endocrine and immune systems (for a review, see ref. 20), and there is some evidence that glial precursors may express opioid peptides. Enkephalin-like immunoreactivity was detected in glia-like and neuron-like cells and in the germ layer of developing rat cerebellum but was not detected in fully differentiated cerebellar glia or neurons (21). Cells derived from the C6 glioma line contain abundant amounts of preproenkephalin mRNA and preproenkephalin, although they contain little Met-enkephalin (22). These observations raise the possibility that the preproenkephalin gene might be expressed by glia. The present study reports that cultured embryonic and neonatal astrocytes, identified by immunoreactivity for glial fibrillary acidic protein (GFAP), contain significant levels of preproenkephalin mRNA. Moreover, astrocytes from different areas of the brain differ in their levels of preproenkephalin mRNA, suggesting regional differences among astrocytes that are otherwise indistinguishable by morphological or standard immunocytochemical criteria.

METHODS

Tissue Culture Techniques. Neuronal cultures and glial cultures were prepared from 17-day-old rat embryos (E17 cells) by using striatum and hypothalamus. Glial cultures were also prepared from 1-day-old rat neonates by using striatum, hypothalamus, frontal cortex, hippocampus, or cerebellum. Brain regions were rapidly dissected in ice-cold calcium/magnesium-free phosphate-buffered saline (PBS), pH 7.4. Tissue was then minced and incubated in PBS containing trypsin (2 mg/ml) and DNase I (0.01 mg/ml) for 20 min at 37°C, washed twice with culture medium, and disrupted by trituration through a Pasteur pipet. Cells were plated on 60-mm poly(D-lysine) (Sigma)-coated dishes (Falcon) and grown in 45% (vol/vol) minimal essential medium (GIBCO)/45% (vol/vol) Ham’s F-12 medium (GIBCO)/5% (vol/vol) fetal calf serum (KC Biological, Lenexa, KS)/5 μg of insulin per ml. Cultures were maintained at 37°C in 95% air/5% CO2 at nearly 100% relative humidity.

For preparation of neuronal cultures, striatal and hypothalamic cells were plated at densities of 5 × 10⁶ and 3 × 10⁶ cells per 60-mm dish, respectively. To minimize glial proliferation, the cells were treated with cytosine arabinoside (0.1 mM) on day 7 for striatal cultures or on day 3 for hypothalamic cultures. For glial culture preparation, cells were plated at a density of 1 × 10⁶ per dish. Three days after plating and then once a week, the culture medium was replaced by fresh ice-cold medium. To prevent glial overproliferation, the neonatal and E17 confluent cells were treated on day 9 with 2′-deoxy-5-fluorouridine (0.1 mM) and cytosine arabinoside (0.1 mM), respectively.

Extraction of Total Cellular RNA. Ice-cold PBS was added to each plate, and cells were scraped with a rubber policeman. The cells were centrifuged at 3000 × g for 5 min, and the pellets were homogenized in glass/glass homogenizers in 0.4 ml of lysis solution: 10 mM Tris-HCl/1 mM EDTA/350 mM NaCl/2% (vol/vol) NaDodSO₄/7 M urea, pH 8.0.

Abbreviations: GFAP, glial fibrillary acidic protein; E17, embryonic day 17.

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Homogenized samples were then extracted twice with an equal volume of 50% (vol/vol) phenol/48% (vol/vol) chloroform/2% (vol/vol) isoamyl alcohol and once with one volume of 96% (vol/vol) chloroform/4% (vol/vol) isoamyl alcohol. Total nucleic acids were precipitated overnight with 0.1 volume of 3 M NaOAc and 2.5 volumes of ethanol and were quantitated by ultraviolet absorption at 260 nm. An average yield of 10–15 µg of total nucleic acids per dish was obtained for neuronal cultures, and 20 µg of total nucleic acids per dish was obtained for glial cultures.

**RNA Gel and Blot Techniques.** Denatured RNA samples (10 µg of total nucleic acids) were run on a 1.5% agarose gel containing 16.6% formaldehyde. RNAs were transferred to GeneScreenPlus (New England Nuclear) by overnight capillary blotting in 1.5 M sodium chloride/0.15 M sodium citrate. The blot was then baked in vacuo at 80°C for 2 hr. A 32P-labeled RNA probe (22) synthesized by using phage SP6 RNA polymerase was used to detect preproenkephalin mRNA (1.5 kilobases (kb)). Prodynorphin mRNA (2.4 kb) was detected by a Riboprobe containing the entire 3' main exon of the rat prodynorphin gene (23). The 1B15 mRNA (1.0 kb) was detected with a probe supplied by James Douglass (Oregon Health Sciences University, Portland, OR) (24).

RNA blot were prehybridized for 6 hr at 65°C in 50% formamide/1 M NaCl/1% NaDodSO4/0.1 mg of denatured herring sperm DNA per ml. Blots were hybridized at 65°C overnight in prehybridization solution to which 1–4 × 106 dpm one of the probes had been added per ml. Blots were then extensively washed at 70°C and exposed to Kodak XAR-5 film at −70°C. Spots were quantitated by using a Quantimet (Cambridge Institute, Cambridge, U.K.) 920 image analysis system. Blots were then prepared the same way for the second and third hybridizations. Each experiment was repeated at least three times.

**Immunofluorescence Staining.** Indirect immunofluorescent labeling of cells was performed as described by Fields et al. (25). Fixed cells (methanol at −20°C for 3 min) were labeled with rabbit anti-GFAP (diluted 1:100) (14, 26) followed by rhodamine-conjugated goat anti-rabbit immunoglobulin (diluted 1:300). Anti-fibronectin (27), anti-galactocerebroside, and A2B5 monoclonal antibody (28, 29) were used with the appropriate second layer reagent under the same conditions on live cells.

**RESULTS**

**Characterization of Cultures.** Primary cultures of glial cells or neurons were established from E17 cells and neonatal (1-day-old) rat hypothalamus, striatum, frontal cortex, hippocampus, and cerebellum. Since interpretation of measurements of preproenkephalin mRNA depended upon proper identification of the cells present in the cultures, detailed morphological and immunocytochemical examinations of the cultures were performed. For immunofluorescence staining, the following cell-type specific markers were used: GFAP, an intracellular marker for astrocytes (26, 30); galactocerebroside, a cell surface marker for oligodendrocytes (30); fibronectin, a surface marker for fibroblasts (27); and A2B5 monoclonal antibody, a cell surface marker for neurons and astrocytic precursors (14, 26).

In the high-density glial cultures, all of the cells assumed a flat and elongated or polygonal morphology (Fig. 1 A). The glial cultures were almost entirely astrocytic, as judged by immunofluorescence staining with antisera against GFAP (Fig. 1 B). There were no A2B5-positive or galactocerebroside-positive cells in the glial cultures; thus, these cultures were almost entirely devoid of neurons and oligodendroglial cells. Virtually 100% of the cells in the striatal and frontal cortex glial cultures contained GFAP immunoreactivity, and there was no detectable fibronectin immunoreactivity. These findings indicate that the striatal and frontal cortex glial cultures consisted almost entirely (>99%) of astrocytes (30). Most cells in the hypothalamic and hippocampal glial cultures were positive for GFAP, but there were a few cells, presumably fibroblasts, that contained fibronectin immunoreactivity. Finally, cerebellar glial cultures were predominantly GFAP positive, but a substantial number of cells contained fibronectin immunoreactivity.

In the neuronal cultures, the large majority of cells assumed a process-bearing morphology typical of neurons (Fig. 1 C). In the hypothalamic neuronal cultures, 95% of the cells were labeled with A2B5 monoclonal antibody (Table 1). A2B5-positive cells were not labeled by antibodies against GFAP, galactocerebroside, or fibronectin. However, approximately 5% of the cells were GFAP-positive, A2B5-negative astrocytes (Fig. 1 D). By comparison, in the striatal neuronal cultures, a larger percentage of GFAP-positive astrocytes was found (as many as 25% of the cells) compared to the cultures of hypothalamic neurons.

**Preproenkephalin mRNA in Cultured Glia and Neurons.** To determine whether preproenkephalin mRNA was present in cultured neurons or glia, each of the primary cultures was examined by RNA blot analysis (Fig. 2 A). Densitometry of autoradiograms of representative blots from several separate experiments was utilized to compare cell populations quantitatively (Fig. 2 B). All of the glial and neuronal cultures contained significant amounts of preproenkephalin mRNA. By contrast, liver contained no detectable preproenkephalin mRNA. Cultured neonatal glia contained levels of preproenkephalin mRNA comparable to levels detected in
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Table 1. Immunocytochemical examination of glial and neuronal cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>GFAP</th>
<th>A2B5</th>
<th>Fibronectin</th>
<th>GalCer</th>
</tr>
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<tbody>
<tr>
<td>Glia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>++</td>
<td>-</td>
<td>+ (&lt;10%)</td>
<td>-</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>++</td>
<td>-</td>
<td>+ (&lt;10%)</td>
<td>-</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>++</td>
<td>-</td>
<td>+ (&lt;20%)</td>
<td>-</td>
</tr>
<tr>
<td>Neurons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>+ (5%)</td>
<td>+</td>
<td>-</td>
<td>+ (&lt;1%)</td>
</tr>
<tr>
<td>Striatum</td>
<td>+ (25%)</td>
<td>+</td>
<td>-</td>
<td>+ (&lt;1%)</td>
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</tbody>
</table>

Glia cultures were established from neonatal rat brain and were examined immunocytochemically after 3 weeks in culture for the presence of GFAP, A2B5, fibronectin, and galactocerebrosidase (GalCer) immunoreactivity. Neuronal cultures were established from E17 brains and were similarly examined after 2 weeks in culture. Values in brackets represent the percent of total cells positive for immunoreactivity. +, Positive immunoreactivity; −, negative immunoreactivity.

cultured embryonic hypothalamic and striatal neurons. There was heterogeneity among glial populations with hypothalamic and hippocampal glia expressing significantly more preproenkephalin mRNA than striatal, cortical, or cerebellar glia. Hypothalamic glia contained 3 times as much preproenkephalin mRNA as striatal, cortical, or cerebellar glia, and hippocampal glia contained about 1.5 times the level detected in striatal, cortical, or cerebellar glia. Cultured striatal neurons contained slightly higher levels of preproenkephalin mRNA than did hypothalamic neurons.

Equal amounts of RNA (as determined by measurement of optical density at 260 nm) were loaded onto each lane of the gels. However, to better quantitate the amount of RNA in each lane, the blot described in Fig. 2 was rehybridized to the neutral probe, 1B15 (refs. 28, 31, and 32; Fig. 3); differences in the autoradiographic density among the groups were small with only ±19% variation from the mean density. Thus, the differences among populations of glia in levels of preproenkephalin mRNA are not likely to be due to differences in the amount of RNA loaded onto the gels. Normalization of preproenkephalin mRNA levels for the amount of RNA loaded was accomplished by taking the ratio of preproenkephalin mRNA to 1B15 mRNA for each sample (Table 2). These values ranged from a high of 1.56 for hypothalamic glia to a low of 0.39 for striatal glia. Thus, neonatal hypothalamic glia expressed 4 times as much preproenkephalin mRNA as did striatal glia and slightly less preproenkephalin mRNA than did embryonic hypothalamic neurons.

Prodynorphin mRNA in Cultured Glia and Neurons. Do neonatal glia express the mRNAs encoding opioid peptides, or is expression specific for preproenkephalin mRNA? To approach this question, the RNA blot shown in Fig. 2 was rehybridized to a Riboprobe for prodynorphin (Fig. 4). Cultured embryonic hypothalamic neurons contained significant levels of prodynorphin mRNA. However,

FIG. 2. Blot-hybridization analysis of preproenkephalin mRNA in cultured glia and neurons. (A) Total RNA was prepared from neuronal or glial cells after 2 or 3 weeks in culture, respectively. Ten micrograms of total RNA was analyzed in each lane. Lanes: 1, hypothalamic glia; 2, hippocampal glia; 3, striatal glia; 4, cerebellar glia; 5, frontal cortex glia; 6, hypothalamic neurons; 7, striatal neurons; 8, liver. A representative autoradiogram from one of several experiments of an 8- hr exposure at −70°C is shown. The arrowhead indicates the position of preproenkephalin mRNA. (B) Densitometry of the representative blot shown in A. Total density measurement (OD × area) was performed to obtain a quantitative measure of relative preproenkephalin mRNA levels. Hypo, hypothalamus; Hipp, hippocampus; FC, frontal cortex; CBM, cerebellum; Str, striatum; LV, liver.

FIG. 3. Blot-hybridization analysis (A) and densitometry (B) for levels of 1B15 mRNA. The RNA blot analyzed in Fig. 2 for levels of preproenkephalin mRNA was rehybridized with a Riboprobe for 1B15, and levels of 1B15 were quantitated by densitometry of an autoradiogram after a 3-hr exposure at −70°C. Abbreviations are as in Fig. 2.
Table 2. Ratio of preproenkephalin mRNA to 1B15 mRNA in cultured glia and neurons

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Ratio preproenkephalin mRNA/1B15 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured glia</td>
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</tr>
<tr>
<td>Hypothalamus</td>
<td>1.56</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.03</td>
</tr>
<tr>
<td>Striatum</td>
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</tr>
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<td>Frontal cortex</td>
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</tr>
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<td>Cerebellum</td>
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</tr>
<tr>
<td>Cultured neurons</td>
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</tr>
<tr>
<td>Hypothalamus</td>
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</tr>
<tr>
<td>Striatum</td>
<td>2.29</td>
</tr>
<tr>
<td>Liver</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Densitometric analysis of levels of preproenkephalin mRNA and 1B15 mRNA (see Figs. 2 and 3) was performed, and the value obtained for preproenkephalin mRNA was divided by the value obtained for 1B15 mRNA to normalize for possible differences in the amount of mRNA loaded onto each lane of the gel.

There was no detectable prodynorphin mRNA in glial cultures from any brain region, nor was there detectable prodynorphin mRNA in cultured embryonic striatal neurons or liver. Thus, neonatal glia express preproenkephalin mRNA but not prodynorphin mRNA.

Developmental Regulation of Preproenkephalin mRNA Expression. To determine whether glial expression of preproenkephalin mRNA is regulated during development, embryonic (E17) and neonatal striatal glia were compared for levels of the mRNA. Cultures of striatal glia were utilized for this aspect of the study because these cultures contained exclusively astrocytes (Table 1). Levels of preproenkephalin mRNA in neonatal glia were only one-third of the levels in embryonic glia (Fig. 5). By contrast, levels of the neutral probe 1B15 were virtually identical in embryonic and neonatal striatal glia. Thus, levels of preproenkephalin mRNA appear to be down-regulated during development.

**DISCUSSION**

Although cultured astrocytes have a characteristic morphology, they cannot be distinguished from other cell types, such as fibroblasts, by morphologic criteria alone (30). For this reason cell type-specific markers were utilized to characterize the cells in our cultures. GFAP is widely used as a relatively specific marker for vertebrate astrocytes both in culture (30) and in tissue sections (26). Virtually all cells in the striatal and cortical glial cultures and 90–95% of cells in hypothalamic and hippocampal glial cultures were GFAP positive. This observation confirms them to be astrocytes.

Although no cells in these cultures had the morphologic appearance of neurons, neuron-like cells that bind GFAP have been described (33). However, all of the cells in the glial cultures were A2B5 negative, an observation that substantiates the morphologic observation that neurons were absent from these cultures. Since fibroblasts cannot be distinguished morphologically from astrocytes in culture, fibronectin antibody was used to define the numbers of fibroblasts in the astrocyte cultures. Only rare fibronectin-positive cells (<1% of total cells) were observed in striatal and cortical astrocyte cultures, although cultures derived from other brain regions contained greater numbers of fibroblasts. The glial cultures were all negative for galactocerebroside. Thus, the striatal and cortical cultures appeared to consist almost completely (>99%) of astrocytes. The absence of binding of A2B5 suggests that the astrocytes in these cultures correspond to the type I astrocytes described by Raff et al. (14) in cultures of optic nerve glia.

Results with the neuronal cultures differed strikingly. As expected, the majority of cells had morphologic characteristics of neurons, and they were labeled with A2B5. Moreover, they were negative for GFAP, galactocerebroside, and fibronectin. However, there were some large flat GFAP-positive astrocytes in both the striatal and hypothalamic neuronal cultures. The percentage of astrocytes in the cultures of hypothalamic neurons was small (about 5%) but was nearly 25% in the cultures of striatal neurons.

We initially began these studies to examine regulation of preproenkephalin mRNA development in cultured neurons; glial cells were examined as controls. Unexpectedly, we found that cultured astrocytes contained preproenkephalin mRNA and that levels in cultured astrocytes were comparable to levels detected in cultures of striatal and hypothalamic neurons. The size of the mRNA (1.5 kb) in glia and neurons was identical (see Fig. 2). Moreover, the absence of preproenkephalin mRNA in liver indicates some tissue specificity for expression of the mRNA in glia and neurons. Finally, hypothalamic neuronal cultures contained prodynorphin mRNA, but glial and striatal neuron cultures contained no detectable prodynorphin mRNA. This observation further substantiates the specificity of glial expression of preproenkephalin mRNA. Since the cultures of striatal and cortical glia contained only the large flat GFAP-positive cells, we conclude that differentiated astrocytes can express preproenkephalin mRNA.

It is intriguing that astrocytes from hypothalamus and hippocampus contained substantially higher levels of preproenkephalin mRNA than did astrocytes from other areas of brain. The 3-fold difference in levels of the mRNA was not large, but it was consistently observed in multiple sets of cultures established from different litters. Moreover, levels of 1B15 mRNA were reproducibly the same in astrocytes cultured from different brain regions, indicating the specificity of the differences in levels of preproenkephalin mRNA. These observations provide biochemical evidence for the suggestion that there are region-specific differences among
Astrocytes from different areas of the brain (10). There are two possible interpretations of these observed differences. First, astrocytes may simply develop more rapidly in some areas of the brain, and the differences in levels of preproenkephalin mRNA may represent differences in stages of development. Alternatively, the final phenotypes of astrocytes from different areas may differ with respect to levels of expression of this and other genes. More detailed study of astrocytes from different ages including adults will help to resolve this issue.

Levels of preproenkephalin mRNA in striatal glia cultured from neonates (day 1) were only one-third as high as levels in astrocytes cultured from embryonic (E17) striatum, suggesting that preproenkephalin mRNA expression is down-regulated during development. This conclusion is consistent with the observation that glial-like cells in neonatal cerebellum but not adult cerebellum contained enkephalin-like immunoreactivity (21). Previous studies have shown that some opioid receptors may also be down-regulated during development (34). This observation, together with our findings, suggests that preproenkephalin gene expression may play a role in normal brain development. However, additional studies will be necessary to determine whether astrocytes in vivo express preproenkephalin mRNA during development, or whether expression of the gene occurs only in cultured glia. It will also be necessary to determine what peptides are processed from preproenkephalin mRNA in glia.

Since astrocytes may contain substantial levels of preproenkephalin mRNA, measurements of levels in brain cultures must be interpreted carefully since most cultures of brain neurons contain at least some astrocytes. Further, these observations raise the possibility that astrocytes in vivo express preproenkephalin mRNA during embryogenesis and in the neonatal period. Consequently, studies of the regulation of the preproenkephalin gene in the developing brain must control for the possibility that significant portions of the mRNA might be localized within glia. Finally, these findings raise the possibility that other neuropeptide genes may be expressed by developing glia and increase the importance of correlating biochemical measurements of mRNA levels with in situ localization.

We thank Dr. Kay Fields for her help with the immunocytochemical characterization of cultures and for providing the GFAP, fibronectin, galactocerebroside, and A2BS antibodies. We thank Dr. James Douglass for providing the preproenkephalin cDNA, the main exon of the prodynorphin gene, and the 1B15 cDNA; and we thank Dr. Lloyd Fricker for help in preparing Riboprobes. This work was supported by National Institutes of Health Grants NS20013, NS20778, NS14580, DA 04439, and DA 00069.