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Cellular localization of proenkephalin mRNA and enkephalin peptide products in cultured astrocytes

Kurt F. Hauser¹, John G. Osborne¹, Anne Stiene-Martin¹, and Michael H. Melner²

¹Department of Anatomy and Neurobiology, University of Kentucky School of Medicine, Lexington, KY 40536 (U.S.A)

²Division of Reproductive Biology and Behavior, Oregon Regional Primate Research Center, Beaverton, Oregon 97006 (U.S.A.)

Abstract

To identify the possible cellular sites of opioid gene expression during ontogeny, proenkephalin mRNA and enkephalin peptide expression were examined, respectively, by in situ hybridization and immunocytochemistry in organotypic explants of rat cerebellum and in astrocyte-enriched cultures of murine cerebral hemispheres. High levels of proenkephalin mRNA and enkephalin immunoreactivity were detected in immature cells identified as astrocytes. Double-labeling studies combining in situ hybridization and immunocytochemical localization of the astrocytic marker, glial fibrillary acidic protein, provided direct evidence that proenkephalin mRNA is expressed by astrocytes in culture. Based on previous studies that Met-enkephalin can inhibit astrocyte growth in vitro, the present results suggest that proenkephalin gene expression by astrocytes is important during central nervous system maturation.

Keywords

Neural development; In situ hybridization; Endogenous opioids; Proenkephalin A; Glial fibrillary acidic protein; Astrocyte; In vitro

The growth of neuronal and non-neuronal cells of the postnatal rat cerebral cortex, hippocampus, and cerebellum is modified by experimental manipulation of endogenous opioid systems (i.e., endogenous opioids and opioid receptors). Cellular differentiation^{17,18}, cell number and packing density^{54,55}, and cortical thickness^{54,55} are modifiable by treatment with opioid antagonist drugs in vivo. In the cerebellum, [³H]-thymidine incorporation by neuroblasts of the external granular layer (EGL) in 6-day-old rats is inhibited by systemic administration of Met-enkephalin, while treatment with the opioid antagonist naltrexone, at dosages sufficient to completely block opioid receptors, is reported to increase [³H]-thymidine incorporation compared to untreated controls⁵⁶. These and other experiments suggest that endogenous opioids are normally available to cells in the developing CNS in sufficient quantities to tonically inhibit growth^{17,18,41,54–56}. In support of this hypothesis, opioid peptide levels and opioid binding are greatly increased in the rat cerebellum^{48,49} and

cerebrum^{4,11,27} during development, and enkephalin immunoreactivity is reported to be transiently present within germinative neuroblasts and glia in the developing rat cerebellar cortex⁵⁷. Recently, endogenous opioids have been reported to directly modify the growth of primary neural cells^{25,46,47} in culture.

Although the above findings suggest that endogenous opioids are present during development and tonically inhibit growth^{17,18,54–57}, few studies have attempted to identify the potential cellular sites of opioid synthesis in the developing central nervous system (CNS). Recent reports have demonstrated the presence of proenkephalin mRNA by Northern analysis of confluent glial cultures that are enriched in astrocytes (i.e., 95–97% astrocytes)^{28,32,43,52}. In the present study, proenkephalin (proenkephalin A) mRNA and enkephalin peptide expression were examined by in situ hybridization and immunocytochemistry, respectively, in primary cultures of the developing CNS (i) to identify the specific cellular sites of proenkephalin gene and peptide expression, and (ii) to determine whether opioids are expressed by replicating astrocytes prior to their reaching confluence. We were particularly interested in determining whether rapidly dividing cells in astrocyte-enriched cultures express the proenkephalin gene prior to reaching confluence, because we have recently found that the proenkephalin peptide product, Met-enkephalin, selectively inhibits type 1 astrocyte proliferation at this time (i.e., at 4 or 6 days in vitro)^{46,47}. Initial studies localized high levels of proenkephalin mRNA in cells with astrocytic morphology irrespective of culture conditions, brain region, or rodent species studied. To unambiguously determine whether astrocytes expressed this opioid gene, in situ hybridization for proenkephalin mRNA was combined with immunocytochemical localization of an astrocytic marker, glial fibrillary acidic protein (GFAP)^{7,37}; and both labels were identified in the same cell. The results provide direct evidence that the proenkephalin gene is expressed by immature astrocytes in cultures isolated from diverse regions of the CNS, but additionally indicate that cells lacking GFAP immunoreactivity and neuronal morphology also express proenkephalin mRNA in vitro.

For explant cultures, a total of 40 cerebella from newborn, male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were dissected in the parasagittal plane into 4–6 symmetrical pairs (8–12 explants/cerebella) and maintained for 7 days in vitro prior to harvesting. The relatively thin parasagittal explants were oriented flat against the substrate surface so that the pial surface, which covers the EGL, was at the peripheral border of the explant. Each explant was maintained in organotypic culture using Maximow double-coverslip assemblies in a rigorously controlled, sterile-environment, as previously described¹⁶; some explants were maintained in 12-well (22 mm diameter) plastic tissue culture chambers.

For astrocyte-enriched dissociated glial cultures, Swiss-Webster mice (ICR Strain, Harlan Sprague Dawley, IN) were used within 24 h of birth to obtain primary cultures of glial cells using a modification^{46,47} of the methods described^{31,33}. Briefly, the cerebral hemispheres were minced and transferred to a tube containing Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) and 2.5% trypsin at 37 °C for 30 min, agitating vigorously every 5 min to disperse the cells. The cell suspension was centrifuged at 40 × *g* for 5 min to separate cells from debris. Pellets were resuspended in 3–4 ml of growth media

(DMEM with 0.5% glucose and 0.06% Na_2CO_3) with 10% fetal calf serum (FCS) (Hazelton, Lenexa, KS) and triturated several times to break up cell aggregates. The cell suspension was then filtered through Nitex 130 (Tetko, Co., Elmsford, NY), centrifuged ($40 \times g$ for 3 min), and the cell pellet resuspended in growth media with 10% FCS. The cells were diluted to a density of approximately 5×10^5 cells/ml with growth media containing 10% FCS. Glass coverslips (15 mm diameter) were coated with poly-L-lysine (62.5 kDa; Sigma, St. Louis, MO), placed into 22 mm wells, and seeded with 1.0 ml of cell suspension. Cultures were incubated at 34–34.5 °C in 5% CO_2 /95% air and high humidity for 5 days. The serum-containing nutrient media used for astrocyte-enriched dissociated cultures, as well as explant cultures, selects for type 1 astrocytes^{31,33}. Therefore, the description and discussion that follows refers to opioid expression by cultures enriched with type 1 astrocytes.

All cultures were fixed for 30–60 min in ice-cold 4% paraformaldehyde (freshly made) in pH 7.4 PBS. Because the cellular outgrowth from cerebellar explants forms a zone 1–2 cell layers thick at the periphery of the explant, approximately half of the cerebellar explants and all of the astrocyte-enriched cultures were processed as whole-mounts so that labeled cells and their processes could be viewed in their entirety. Remaining cerebellar explants were cryoprotected (10% sucrose overnight), embedded (OTC compound), and frozen-sectioned (10 μm thick). The distribution of astrocytes was studied in 10 whole-mount explants using rabbit anti-GFAP antisera (BioGenex, Dublin, CA) at a dilution of 1:400 or 1:600 and immunocytochemical methods described for enkephalin (vide infra).

Whole-mount and frozen-sectioned explants were reacted with primary rabbit antisera against Met-enkephalin (1:4000 or 1:3000 dilution) in 0.4% Triton X-100 in PBS for 48–72 h at 4 °C. An avidin-biotin-peroxidase method (Vectastain; Vector Laboratories, Burlingame, CA) with 0.003% diaminobenzidine (DAB; Sigma) was used to visualize immunoreactivity. Endogenous peroxidase activity was quenched by treatment with 0.03% H_2O_2 in 1:3 methanol: H_2O or 0.01% sodium *m*-periodate in 0.1 M Tris buffered saline, pH 7.4. The antiserum against [Met⁵]-enkephalin used in this study (a gift from Dr. B. Maley) has been previously characterized^{14,30} and exhibits limited cross-reactivity to [Leu⁵]-enkephalin (<5%). Thus, the general term enkephalin was used to describe the immunoreactivity. Staining was not observed when the anti-Met-enkephalin antisera were preabsorbed with both 10 μM Met-enkephalin (Peninsula) and 10 μM Leu-enkephalin (Sigma).

Whole-mount and frozen-sectioned explants were hybridized for 16 h at 37 °C with an [³⁵S]-labeled cRNA probe (5×10^4 cpm/slide) specific for rat proenkephalin mRNA^{28,39} according to previously described methods^{3,44}. Tissues were washed (0.2% SSC final wash at room temperature), coated with NTB-2 emulsion (Kodak) (diluted 1:1 with 0.2 M acetate buffer, pH 8.0) and exposed for 4 weeks at 4 °C. The probe used was transcribed from a 1070 bp cDNA in pSP64 plasmid. The plasmid was linearized with *Eco*RI and transcribed with SP6 RNA polymerase using [³⁵S]-UTP and the remaining unlabeled nucleotides. The probe was subjected to mild alkaline hydrolysis to fragment the RNA (0.1 M Na_2CO_3 /NaHCO₃, pH 10 for 40 min at 60 °C) and precipitated to remove small fragments. This produced a range of fragments from 50–200 bp, which has been previously demonstrated to be specific for rat proenkephalin mRNA²⁸. Briefly, this probe recognizes proenkephalin mRNA (approximately 1.4 kb) on Northern blots, can be selectively displaced by an excess

of the unlabeled probe and is sensitive to RNase I tissue pretreatment (Osborne and Hauser, unpublished). Moreover, we find that the distribution of hybridization signal in the adult rat cerebellum using our cRNA probe¹⁹ is consistent with the distribution of proenkephalin peptide immunoreactivity^{36,42} and of proenkephalin mRNA using a different cDNA probe⁴⁵ reported by other laboratories. Lastly, an additional control was performed by hybridizing with an [³⁵S]-labeled cRNA probe (5×10^4 cpm/slide) to prodynorphin mRNA¹⁰ which has some sequence homology to proenkephalin mRNA^{10,22,39,50}. Vilijn et al.⁵² and Low et al.²⁸ have previously demonstrated the absence of prodynorphin mRNA in astrocyte-enriched cultures by Northern analysis.

The method for GFAP immunocytochemistry and proenkephalin in situ hybridization double labeling was modified for the use of cRNA probes with tissue culture cells (Stiene-Martin and Hauser, in preparation) from a previously described method⁴⁴. Briefly, GFAP immunocytochemistry was performed as described above in 0.1% diethylpyrocarbonate (DEP; Sigma) pretreated solutions. After visualization of the DAB immunoprodukt, whole-mount explants or sections were washed 5x (50 min total) with DEP-treated water, air dried and immediately placed into prehybridization solution for 2–3 h. Subsequent in situ hybridization steps were performed as described above.

Cerebellar astrocytes with GFAP immunoreactivity were either differentiated (possessing flat, polygon-shaped cytoplasmic processes) or undifferentiated (lacking cytoplasmic processes) after 7 days in vitro (Fig. 1A). Large numbers of both differentiated and undifferentiated astrocytes in cerebellar explant cultures contained enkephalin immunoreactivity (Fig. 1B) or proenkephalin mRNA hybridization signal (Fig. 1C). Differentiated astrocytes that expressed either opioid peptide or mRNA products had flattened polygonal-shaped cytoplasmic extensions and were readily identified as type 1 astrocytes. Undifferentiated astrocytes could be distinguished from other types of undifferentiated cells in cerebellar explants. Undifferentiated astrocytes were of distinct size (11.8 ± 1.4 μ m perikaryal diameter); often had multiple small nucleoli within a nucleus that was eccentrically positioned within the perikarya; and were distributed at the periphery of the outgrowth at a position normally occupied almost exclusively by astrocytes in cerebellar explant cultures after 7 days in vitro^{1,23} (Hauser, unpublished). In addition, double-labeling studies have demonstrated that these cells both incorporate [³H]-thymidine and are enkephalin immunoreactive (Osborne and Hauser, in preparation). Except for EGL cells which are neuronal progenitors, all other intrinsic cerebellar neurons are postmitotic in the postnatal rat² and therefore do not incorporate [³H]-thymidine. EGL cells have been previously characterized in vitro^{1,23,34} and in vivo³⁵, and are of distinct size (7.2 ± 0.7 μ m), morphology, and distribution (within the explant) from immature astrocytes. Astrocytes were not labeled for proenkephalin mRNA when pretreated with RNase or an excess of unlabeled probe, or when using an [³⁵S]-labeled RNA probe complementary to prodynorphin mRNA with the same hybridization conditions, exposure time, and specific activity as used for the proenkephalin cRNA probe (Fig. 1D).

A majority of the cells in astrocyte-enriched cultures from mouse cerebrum was GFAP immunoreactive after 5 days in vitro, and all of the GFAP immunoreactive cells containing opioid gene and peptide products were identified as type 1 astrocytes (Fig. 1E). Double-

labeling studies confirmed the coexistence of proenkephalin mRNA hybridization signal in GFAP-immunoreactive astrocytes (Figs. 1E & F). However, the quantitative determinations of the number of GFAP immunoreactive cells indicate that there was variable expression of proenkephalin mRNA between individual astrocytes (Table I). It was noteworthy that similar proportions of astrocytes expressed proenkephalin mRNA, despite differences in brain regions, rodent species, astrocyte density, type of tissue culture (explant vs. dissociated and astrocyte-enriched) and culture conditions (Table I). In general, fewer cells were enkephalin immunoreactive compared to those exhibiting proenkephalin mRNA (discussed below).

Proenkephalin mRNA has been previously detected in confluent astrocyte-enriched (95–98% type 1 astrocytes) cultures from the rat cerebral cortex^{28,32,43} and cerebellum⁴³, or rat striatum⁵² by Northern analysis, but not at the cellular level. The present results confirm and extend previous reports by localizing proenkephalin mRNA in astrocytes at the cellular level by in situ hybridization, and thereby provide direct evidence that the enkephalin mRNA previously identified in Northern blots is expressed by astrocytes and not by a small number of contaminating non-astrocytic cells which are present in the astrocyte-enriched cultures reported in previous studies^{37,43,52}. In particular, cells double-labeled for both proenkephalin mRNA and GFAP intermediate filaments provide first direct evidence that astrocytes express the proenkephalin gene during development. The findings also indicate that astrocytes express proenkephalin mRNA during the sustained period of active replication prior to reaching confluence when their growth in vitro is modifiable by Met-enkephalin (see below). Moreover, the heterogeneous nature by which astrocytes express proenkephalin can only be appreciated by examining this expression at the cellular level. For example, the results indicate that: (i) proenkephalin mRNA and enkephalin peptide expression levels vary greatly between individual astrocytes; (ii) an astrocyte subpopulation (morphologically similar to type 1 astrocytes), in particular, is capable of opioid gene expression; and (iii) some non-GFAP immunoreactive cells also expressed proenkephalin mRNA. Non-GFAP-immunoreactive cells that might express the proenkephalin gene include immature (e.g., astrocyte progenitor cells, i.e., O-2A cells) or mature glia (e.g., oligodendrocytes, ependymal cells, or microglia) (however, see ref. 28), or non-glial cells (e.g., fibroblasts, endothelial cells, or neurons), any of which are potentially present in low numbers (0.01–5%) in our cultures.

Levels of enkephalin mRNA or peptide expression varied greatly between individual cells with astrocytic morphology. The reasons for this are uncertain, but several possibilities exist: (i) cells may express enkephalin only at certain critical periods during their development or at selective times during the cell cycle. Age-dependent differences in proenkephalin expression are evident in astrocyte-enriched cultures derived from gestational day 17 or newborn rat striatum⁵²; (ii) only a subpopulation of cells may express proenkephalin, (iii) local factors, such as relative proximity of other neurons, glia (including other astrocytes), or non-neural cells (e.g., a small number of fibroblasts, endothelial cells, and macrophages are typically present in primary neural cultures) may differentially affect enkephalin levels. However, similarities in the percentage of astrocytes expressing proenkephalin mRNA were observed (Table I) despite differences in brain regions, rodent species, culture conditions, or cell density suggesting that possibility (iii) is not correct. It is also noteworthy that fewer astrocytes displayed immunoreactivity for the enkephalin peptide compared to those that had

detectable levels of proenkephalin mRNA. Recent RIA/HPLC studies indicate that in cultures of type 1 astrocytes significant amounts of immunoreactive proenkephalin is secreted as intact, unprocessed proenkephalin precursor³². This suggests that only small amounts of Met-enkephalin may be present in the cytoplasm of astrocytes expressing the proenkephalin gene, and may, in part, explain why fewer astrocytes have detectable enkephalin immunoreactivity than detectable proenkephalin mRNA. Therefore, estimates of the relative numbers of astrocytes that express proenkephalin mRNA were determined from the in situ hybridization results alone (Table I).

In parallel studies conducted in vivo, we have not observed high levels of proenkephalin expression by cerebellar astrocytes during the first 3 weeks of postnatal development in the rat compared to proenkephalin expression in vitro¹⁹. Other investigators have noted similar increases in the expression of other opioid genes in vitro compared to in vivo. For example, increased expression of the mRNA from another opioid gene, proopiomelanocortin (POMC), has been observed in primary cultures of the rat hypothalamus²⁶ and spinal cord⁵ when compared to in vivo. The reasons for increased gene expression in vitro compared to in vivo are uncertain. There are isolated reports that mRNA has increased stability in vitro¹² and the processing of the peptide precursors might also be altered. However, there is additional evidence that opioid genes are specifically repressed by external factors. For example, POMC mRNA is expressed by motoneurons during development^{5,20}, but only in adult motoneurons following axotomy^{5,21}. This and other evidence have prompted speculation that POMC expression is inhibited by factors that increase with progressive maturation, such as those provided by through afferent and/or efferent synaptic connections. Similarly, in a tumor cell model, the expression (derepression) of proenkephalin mRNA by rat C6 glioma cells in vitro⁵³ is reported to not be due to amplification of the proenkephalin gene during malignant transformation⁴⁰, but is postulated to be due to the loss of feed-back inhibition by an external signal⁴⁰.

Although endogenous opioid peptides are involved in numerous functions, these neuropeptides have recently been proposed as negative trophic regulators of neuronal proliferation⁵⁶ and differentiation^{17,18}. Therefore, opioid production by astrocyte cells might be one mechanism by which glia might influence the growth of opioid-receptor containing neurons during development. Interactions between neurons and glia have been shown to modify neuronal migration^{13,15,38} and differentiation including dendrite elaboration^{9,24}. In contrast, astrocytes themselves may be targets for opioid action. Astrocytes isolated from chick cerebral cortex²⁹, as well as GFAP-positive mammalian pituitary cells (pituicytes)^{6,8}, contain opioid receptors. Glial cell metabolism⁵¹ is altered by the presence of opioid peptides and/or opiate drugs in culture. Recently, we have found that the proliferation of GFAP-immunoreactive cells cultured from the murine cerebral cortex is inhibited by Met-enkephalin in a naloxone reversible manner^{46,47}, suggesting that astrocytes are a target for opioid action during development. Therefore, the present findings imply that the proenkephalin gene might be involved in the paracrine or autocrine regulation of astrocyte growth, and suggest that astrocytes and perhaps other non-neuronal cells are critical intermediaries in opioid-dependent growth of the CNS.

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Fig. 1.

(A-D) Rat cerebellar explant cultures after 7 days in vitro. (A) Astrocytes immunostained with antisera against glial fibrillary acidic protein (GFAP) are identical in morphology and distribution to enkephalin-immunoreactive cells in (B) or cells containing proenkephalin mRNA as identified by in situ hybridization (C). (C) An intense proenkephalin hybridization signal was observed in type 1 astrocytes in cerebellar explants. However, note the relative differences in signal intensity between astrocytes. Darkfield micrograph. (D) No cells were labeled by in situ hybridization using an [35S]-labeled cRNA probe against prodynorphin with the same exposure time, specific activity, and cpm/hybridization as the proenkephalin cRNA probe. Darkfield micrograph. (E-F) Combined proenkephalin in situ hybridization and GFAP immunocytochemistry in astrocyte-enriched cultures dissociated from the mouse cerebrum after 5 days in vitro. (E) Brightfield micrograph of astrocytes (whole-mount preparations) with GFAP-immunoreactivity in cytoplasmic processes astrocytes. (F) Darkfield micrograph of the same field as Fig. 1E (an arrowhead marks the same cell in Figs. 1E & F), demonstrating the presence of proenkephalin mRNA hybridization signal in the same astrocytes as labeled with GFAP in Fig. 1E. (A-D) Identical magnification, scale bar = 10 μ m; (E-F) Identical magnification, scale bar = 10 μ m.

TABLE I
Levels of proenkephalin mRNA expression vary greatly between individual type 1 astrocytes in culture.

	Labeled ^a	Unlabeled ^b	% Labeled	n	Days in vitro
Cerebellum	990 ± 180	630 ± 120	61 ± 5	6	7
Cerebrum	2.7 ± 0.24	1.5 ± 0.24	64 ± 6	9	5

The number of type 1 astrocytes that were unlabeled or labeled with an [³⁵S]-cRNA probe to proenkephalin mRNA was determined in primary cultures of rat cerebellar explants (cerebellum) and in dissociated astrocyte-enriched cultures of mouse cerebral hemispheres (cerebrum) after 7 or 5 days in vitro, respectively. Similarities in the percentage of astrocytes labeled with proenkephalin mRNA were noted despite differences between cerebellar and cerebral cultures (see text and below).

^aThe number of type 1 astrocytes per mm² expressing proenkephalin mRNA hybridization signal. Astrocytes were considered to be expressing proenkephalin mRNA (labeled) if they had >25 autoradiographic grains/cell; background counts were 3–7 grains/cell. Determinations were made from 400–700 μm² areas in histologic sections (10 μm thick) of the region of glial outgrowth from rat cerebellar explants, or from 204 mm² astrocyte-enriched monolayer cultures of mouse cerebrum.

^bThe number of unlabeled type 1 astrocytes per mm².