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Regional, Developmental, and Cell Cycle-Dependent Differences in μ , and δ , and κ -Opioid Receptor Expression among Cultured Mouse Astrocytes

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Abstract

The diversity of opioid receptor expression was examined in astrocytes in low-density and non-dividing (confluent) cultures from the cerebral cortex, hippocampus, cerebellum, and striatum of 1-day-old mice. μ , δ , and κ Opioid receptor expression was assessed in individual cells immunocytochemically, by using flow cytometry, and functionally by examining agonist-induced changes in intracellular calcium ($[Ca^{2+}]_i$). Significant spatial and temporal differences were evident in the pattern of expression of μ , δ , and κ receptors among astrocytes. In low-density cultures, greater proportions of astrocytes expressed μ -opioid receptor immunoreactivity in the cerebral cortex and hippocampus (26-34%) than in the cerebellum or striatum (7-12%). At confluence, a greater percentage of astrocytes in cerebellar (26%) and striatal (30%) cultures expressed μ -immunoreactivity. Fewer astrocytes possessed δ -immunoreactivity in low-density striatal cultures (8%) compared to other regions (16-22%). The proportion of δ receptor-expressing astrocytes declined in the cerebellum but increased in the hippocampus. κ -Opioid receptors were uniformly expressed by 27-34% of astrocytes from all regions, except in cortical cultures where the proportion of κ expressing cells was 38% at low-density and decreased to 22% at confluence. Selective μ (PLO 17; H-Tyr-Pro-Phe (N-Me) -D-Pro-NH₂, δ ([D-Pen², D-Pen⁵] enkephalin) or κ (U50,488H; *trans*-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide methanesulfonate) opioid receptor agonists increased $[Ca^{2+}]_i$ in subpopulations of astrocytes indicating the presence of functional receptors. Lastly, opioid receptor immunofluorescence varied during the cell division cycle. A greater proportion of astrocytes in the G₂/M phase of the cell cycle were μ or δ receptor immunofluorescence than at G₀/G₁. When astrocytes were reversibly arrested in G₁, significantly fewer cells expressed δ receptor immunofluorescence; however, upon reentry into the cell cycle immunofluorescent cells reappeared. In conclusion, opioid phenotype varies considerably among individual cultured astrocytes, and this diversity was determined by regional and developmental (age and cell cycle

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dependent) differences in the brain. These in vitro findings suggest astroglia contribute to regional and developmental idiosyncrasies in opioid function within the brain.

Keywords

Mu opioid receptors; Delta opioid receptors; Kappa opioid receptors; Intracellular calcium; cell proliferation; Drug Abuse

INTRODUCTION

The endogenous opioid system consists of multiple receptors (μ , δ , and κ) and peptide genes (proenkephalin, proopiomelanocortin, and prodynorphin) (Simon and Hiller, 1994; Knapp et al. 1995). This system is expressed in the developing brain and typically acts by inhibiting neural cell proliferation (Hauser and Stiene-Martin, 1993; Hammer, Jr. 1993; Zagon and McLaughlin, 1983, 1991).

Astrocytes are targets of opioid action in the developing CNS (Stiene-Martin et al. 1991; Stiene-Martin and Hauser, 1990; Hauser et al. 1996). Not only do opioids inhibit the genesis of new astrocytes, astrocytes themselves synthesize and release opioid peptides (Melnor et al. 1990; Spruce et al. 1990; Batter et al. 1991; Stiene-Martin et al. 1991; Stiene-Martin and Hauser, 1990; Hauser et al. 1996). Although the developmental consequences of opioids on astrocytes themselves are intrinsically important, astrocytes may also affect neuronal development (Sivron et al. 1993; Rakic, 1995; Muller, 1995; Lin et al. 1993; Gasser and Hatten, 1990; Fishman and Hatten, 1993). In the adult CNS, astroglia affect neuronal metabolism (Dietzel et al. 1989; Anderson et al. 1992), and there is some evidence that astrocytes modulate neuronal function through direct signaling (Nedergaard, 1994; Parpura et al. 1994).

There is considerable diversity in the form and function of individual astrocytes in the CNS. This diversity is exemplified by the multiplicity of neurotransmitter receptor types that can be expressed within different astrocyte subpopulations (Wilkin et al. 1995; Pearce, and Wilkin, 1995; Shao and McCarthy, 1994; McCarthy et al. 1995). An unexpected finding has been that the opioid system is widely expressed by astroglia. Astrocytes express μ , δ , and κ opioid receptors and/or functional responsiveness to opioids, and there is substantial phenotypic heterogeneity among individual cells (Gurwell et al. 1996; Hauser et al. 1996; Ruzicka et al. 1995; Eriksson et al. 1990, 1991, 1992, 1993; Batter and Kessler, 1991; Van Bockstaele et al. 1996; Cheng et al. 1996; Svingos et al. 1994). Through their interactions with astroglia, it has been inferred that opioids, in part, affect CNS development and injury, and may modulate neuroimmune function. However, despite the widespread expression of astrocytes in opioid function, few studies have attempted to identify the origin and function of μ , δ , and κ receptor diversity.

Regional differences in the brain are thought to contribute to the phenotypic heterogeneity in opioid receptor expression among astrocytes (Ruzicka et al. 1995). In addition, age-related factors also affect opioid expression by individual astrocytes (Gurwell et al. 1996; Shinoda et al. 1992). There is an increase and subsequent fall in the proportion of astrocytes

expressing κ receptors with maturation in vitro (Gurwell et al. 1996). The ability of opioids to inhibit astrocyte growth is age-related and suggests that developmental factors also affect receptor expression (Hauser and Stiene-Martin, 1991; Gurwell et al. 1996). We assessed regional and temporal shifts in opioid receptor phenotype in astrocytes. Our results demonstrate that opioid receptors are extensively expressed among astroglia. The opioid phenotype of individual astrocytes appears to be highly plastic, and shaped by regional and developmental (age-related or cell-cycle-dependent) differences in the CNS.

MATERIALS AND METHODS

Cell Culture

One-day old ICR mice (Harlan Sprague Dawley, IN) were anesthetized with ether and sacrificed by decapitation according to NIH and IACUC guidelines. The cerebellum, cerebral cortex, hippocampus, and striatum were isolated using techniques described before (Messer, 1989; Robertson et al. 1989). Tissue from each region was mechanically and enzymatically dissociated in 0.25% trypsin (Difco, Detroit, MI) containing DNase (1 μ g/ml) (Hauser et al. 1996). The cell suspensions were centrifuged at $40 \times g$ and the pellets resuspended in basal medium consisting of DMEM with glucose (0.027M), Na_2HCO_3 (0.006 M) and 10% fetal calf serum (FCS) (KC Biological, Lenexa, KS). The cell suspensions were triturated, filtered through Nitex 130, and centrifuged again at $40 \times g$ for 3 min. Resuspended cells were diluted to 1.5×10^5 cells/ml. For studies of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), 1 ml of cell suspension was added to 35 mm diameter glass-bottomed plastic dishes (MatTek Co., Natick, MA) or 25 mm diameter coverslips previously coated with poly-L-lysine. Cultures were incubated at 35°C in 5% CO_2 /95% air at high humidity.

Astrocytes were assessed in low density or confluent cultures. In low-density cultures (4-7 days in vitro), 95-98% of the cells were glial fibrillary acidic protein (GFAP) immunoreactive. Individual cells were physically isolated from one another with no or infrequent cell-cell contacts. In confluent cultures (typically 10-12 days in vitro), 98-99% of the cells were astrocytes and there was minimal unoccupied space between cells as well as a cessation of cell division. Non-astroglial cells consist of macroglial precursors, oligodendroglia, and some microglia. Few neurons were present after 3-4 days.

Immunocytochemical Detection of μ , δ and κ Opioid Receptors

Cultures were fixed for 30 min in 4% paraformaldehyde in Sorenson's phosphate buffer (pH 7.2 at 4°C) and rinsed 3×20 min in PBS, pH 7.2. Cultures were incubated in phosphate buffer containing crystalline bovine serum albumin (BSA) (1%), goat serum (1%), and Triton $\times$ 100 (0.1%). Purified rabbit-antiserum directed against μ (MOR1, Arvidsson et al. 1995b), δ (DOR1, Arvidsson et al. 1995a), or κ (KOR, Arvidsson et al. 1995c) opioid receptors was diluted, 1:5000, 1:5000, or 1:2500, respectively, in PBS containing Triton-X 100 (0.1%) and BSA (0.1%) (Calbiochem, San Diego, CA). These antibodies were generated against unique epitopes of their respective cloned opioid receptors and affinity purified. These antibodies were originally characterized by Western blot analysis, by examining immunoreactivity in known (positive and negative) opioid-receptor expressing regions throughout the brain, and by co-localization with other markers in chimeric

receptors transfected into test cells (Arvidsson et al. 1995a; 1995b; 1995c). Cultures were incubated in diluted primary antiserum at 4°C on an orbital shaker (40-60 rpm) for 24 h. Secondary, biotinylated goat-anti-rabbit antibodies conjugated to avidin-peroxidase were used as directed (Vectastain-ABC kit, Vector Laboratories, Burlingame, CA) to detect MOR1, DOR1 or KOR primary antibodies. Nickel-intensified DAB consisting of 2.5% nickel ammonium sulfate, 0.35% diaminobenzidine (DAB), and 0.012% H₂O₂ in 0.1 M sodium acetate (pH 6.0) was used as a substrate for peroxidase. In some cases, secondary, biotinylated goat-anti-rabbit antibodies conjugated to fluorescein (FITC) were used to detect MOR1, DOR1 or KOR1 primary antibodies by immunofluorescence microscopy. After 3 × 5 min rinses in PBS, cultures were incubated in the secondary FITC-tagged antibodies for 1 h at room temperature. Cultures were then rinsed (3 × 5 min) in PBS, and mounted in ProLong Antifade mountant (Molecular Probes). Preabsorbed controls and controls where the primary antibody was omitted were included to assure specificity of the primary antibodies. To assess regional and age-related differences in μ and δ opioid receptor expression, about 500 flat, polyhedral cells were sampled in each culture. Individual cultures consisted of independent cell samples derived from the brain regions of separate mice; data are the mean of at least 3 cultures. Cells that had (i) an immunoreactive intensity unambiguously above background and (ii) a characteristic subcellular distribution for particular receptor type were considered immunopositive. A digital color camera was used to acquire fluorescent images (Optronics DEI-470, Goleta, CA).

Opioid-Induced Alterations in $[Ca^{2+}]_i$

H-Tyr-Pro-Phe (N-Me)-D-Pro-NH₂ (PLO-17) (Chiron, San Diego, CA), [D-Pen², D-Pen⁵]enkephalin (Sigma, St. Louis), and *trans*-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide methanesulfonate (U50,488H) (NIDA Research Technology Branch), respectively, were used to activate μ , δ , or κ opioid receptors.

$[Ca^{2+}]_i$ was measured in flat, polyhedral cells in astrocyte-enriched cultures as previously described (Hauser et al. 1996). Ninety-five to ninety-eight percent of the *flat, polyhedral* cells in these cultures are GFAP immunoreactive after day 5 in vitro (unpublished). Cells were loaded with 10 μ M fura-2/AM (Molecular Probes, Eugene, OR) for 45 min at 35 °C in growth media that included 10 mM Hepes buffer (pH 7.2) and 2% DMSO. Following incubation, cells were rinsed 3-times and incubated for 30 min in growth media containing 10 mM Hepes buffer to permit complete hydrolysis of the fura-2/AM. Cells were grown in MatTek dishes or on 25 mm diameter glass coverslips and imaged in a perfusion chamber (RC-21; Warner Instrument Corp., Hamden, CT) which allowed continuous monitoring of cells while exchanging drug solutions. Cells were perfused at a rate of about 1 ml/min (100 μ L bath volume) using a syringe pump (MD-1001; Bioanalytical Systems, W. Lafayette, IN). Ratiometric $[Ca^{2+}]_i$ measurements were acquired at about 28 °C using an inverted Nikon microscope with an oil immersion, fluoro 40× (1.3 N.A. objective), a Dage 72 CCD camera (Michigan City, IN), and a Hamamatsu C2400 intensifier (Hamamatsu Photonics K.K., Hamamatsu-City, Japan). MCID MI or M4 imaging systems (Imaging Research Inc., St. Catharines, Ontario) with fura-2 ratiometric software were used to acquire and process the images. $[Ca^{2+}]_i$ was determined from the ratio of fluorescence at 340 and 380 nm excitation wavelengths (Grynkiewicz et al. 1985) as previously described (Hauser et al.

1996). A computer-controlled, optical filter wheel (Lambda-10, Axon Instruments, Novato, CA) was used to change excitation filters. Repeated measures were made at 15 s intervals on the same cell before and after treatment with 100 nM concentrations of opioid agonists. We have previously found that PL017, DPDPE, and U50,488H, at 100 nM concentrations, cause highly selective functional changes in astrocytes by activating μ , δ , or κ -receptors, respectively (Stiene-Martin and Hauser, 1991; Gurwell et al. 1996; Hauser et al. 1996). Opioid-induced increases in $[Ca^{2+}]_i$ are concentration dependent, can be prevented by selective antagonists, and are highly selective, especially at 1 to 100 nM concentrations. $[Ca^{2+}]_i$ increases are maximal at 100 nM agonist concentrations. $[Ca^{2+}]_i$ measurements were taken from the glial cytoplasm, away from peripheral processes but not over the nucleus. The criteria for a positive response was at least a 50% increase in $[Ca^{2+}]_i$ within 20 s of agonist administration. About 50 flat, polyhedral cells were sampled in each culture. Individual cultures consisted of independent cell samples derived from the brain regions of separate mice; data are the mean $\pm$ SEM of 6 cultures.

Flow Cytometry

Astrocytes were isolated from the cerebral forebrain as previously described (Hauser et al. 1996), rather than individual brain regions, to obtain sufficient numbers of cells for flow cytometry. Opioid receptor expression and cell cycle parameters were assessed by combining opioid receptor immunofluorescence and propidium iodide staining in the same cells (Brons et al. 1994; Braylan, 1982). Briefly, 50,000 cells/ml (7 ml total) were added to 25 cm² flasks, cultured for 6 days, and harvested for 30 min at 34°C in flow cytometry assay buffer consisting of sodium citrate (38 mM), Nonidet P-40 (0.1% v/v), spermine 4 HCl (1.5 mM), and Tris-[hydroxymethyl]aminomethane (0.5 mM) adjusted to pH 7.4 with HCl. To detach cells the assay buffer additionally contained EDTA (0.1%) with or without trypsin (0.003%). The cell suspension was centrifuged (500 $\times$ g) for 5 min at 4°C and the cell pellet rinsed 3-times with PBS. Cells from each flask were resuspended in 200 μ L of PBS and fixed with 1 ml of paraformaldehyde (1%) in PBS, which was added gradually with repeated vortexing to prevent clumping of the cell suspension. Cells were fixed in paraformaldehyde (1%) at 4°C for 5 h, centrifuged (1500 $\times$ g) at 4°C for 5 min, and rinsed 3-times with PBS. Opioid receptor antibodies were detected using FITC secondary antibodies (1:250 dilution; Jackson ImmunoResearch, West Grove, PA) (Babcock and Dawes, 1994) as described earlier. Immunofluorescent cells were treated with ribonuclease A solution (0.01%) in Tris-HCl (10 mM pH 7.5) at 37°C for 30 min followed by treatment with propidium iodide (10 μ g/ml) for 1 h at 4°C in flow cytometry assay buffer.

Flow cytometry was performed using a dual-emission fluorescence-activated cell sorter (FACScan, Becton-Dickinson, San Jose, CA). Immunofluorescence was determined using log amplification. Background fluorescence intensity was determined in control populations of cells in which the primary antibodies were preabsorbed or omitted from the reaction. Cells were considered immunopositive if they had fluorescence intensity greater than the control (background) population. Cell cycle analysis was performed using linear amplification of the FL2 area signal. Data analysis was performed using Modfit LT software (Verity Software House, Topsham, ME). In samples with anti- μ and δ immunofluorescent

cells, cell cycle analysis was performed on the ungated, and fluorescein-positive and negative populations.

Astrocytes isolated from 1-day-old mouse cerebra were arrested in the G₁ phase of the cell cycle by exposure to growth medium containing hydroxyurea (0.5 mM), with or without reduced FCS (5%), for 24 h before harvesting (Langan et al. 1994; Langan and Slater, 1991). It was determined using flow cytometry that astrocytes reentered the cell cycle following the reintroduction of basal growth medium without hydroxyurea.

Statistics

Regional and age-related differences in opioid receptor immunoreactivity were assessed using ANOVA and Newman-Keuls test; while differences in $[Ca^{2+}]_i$ responsiveness were examined using Kruskal-Wallis ANOVA (General ANOVA programs, Statistica, StatSoft, Tulsa, OK). Data were reported as the mean $\pm$ SEM.

RESULTS

μ , δ , and κ Opioid Receptor Immunoreactivity

μ -Opioid receptor immunoreactivity in cultured astrocytes was typically reticular, and discretely located within a polarized position within the juxtannuclear cytoplasm (Figure 1A). This focal pattern of immunoreactivity has been described for the μ receptor in other cell types, including neurons (Arvidsson et al. 1995b), astrocytes (Hauser et al. 1996), and oligodendrocytes (Knapp and Hauser, 1996). Based on the subcellular localization in adult rat neurons, it has been suggested that μ -opioid-receptor immunofluorescence is present in the endoplasmic reticulum and Golgi apparatus (Arvidsson et al. 1995b). In contrast, the pattern of δ -opioid-receptor immunoreactivity was more diffuse and evenly distributed within the boundaries of the cell membrane (Figure 1B), while κ receptor immunoreactivity had both patchy and diffuse attributes. Preabsorbed controls lacked specific immunofluorescence (Figure 1D-F).

Opioid-Induced Alterations in $[Ca^{2+}]_i$

The activation of μ , δ , and κ opioid receptors increased free intracellular Ca^{2+} (Ca^{2+})_i in individual flat, polyhedral astrocytes (Figures 1G-H & 2A-F). Individual astrocytes did not respond uniformly to μ , δ , and/or κ opioid agonists, and opioids failed to increase $[Ca^{2+}]_i$ in many cells. Often only a single astrocyte within a cluster was affected (Figure 2B-D) by a particular agonist, and there was little cross reactivity among agonists for different receptor types (Figure 2A-D). The nature of the response was similar for μ , δ , or κ receptor activation. Typically, this response consisted of an initial $[Ca^{2+}]_i$ spike lasting 60-90 sec, followed by a moderate elevation in $[Ca^{2+}]_i$ above resting levels lasting 60-120 sec (Figure 2) (see also Jin et al. 1994).

Regional differences in μ , δ and κ Opioid Receptor Immunoreactivity

In low-density cultures, there were regional differences in the percentage of astrocytes that expressed μ or δ opioid receptor immunoreactivity (Figure 3). Regional dissimilarities in the proportion of astrocytes expressing μ -receptors were as follows: hippocampus (34%) >

cerebral cortex >> cerebellum > striatum (7%). At confluence, the percentage of μ -immunoreactive astrocytes increased significantly in cerebellar and striatal cultures ($P < 0.05$), but remained unchanged in cultures of the cerebral cortex and hippocampus (Figure 3).

In contrast, a significantly greater percentage of astrocytes expressed δ -immunoreactivity in cerebral cortical or cerebellar cultures than in other regions ($P < 0.05$) (Figure 3). The rank order of δ abundance by region was cerebral cortex (22%) > cerebellum > hippocampus > striatum (8%). Compared to low density cultures, at confluence the proportion of δ receptor-expressing astrocytes decreased in the cerebellum, increased in the hippocampus, and remained unchanged in the cerebral cortex and striatum (Figure 3).

Some aspects of the developmental patterns of κ -opioid receptor expression by astrocytes isolated from the cerebral cortex have been previously described (Gurwell et al. 1996) and agree with the present findings. In low-density cultures, about 38% of the astrocytes were κ opioid receptor immunoreactive, while in confluent cultures significantly fewer (22%) astrocytes were immunoreactive (Gurwell et al. 1996). Otherwise, a similar proportion of astroglia in cerebellar, hippocampal and striatal cultures expressed κ immunoreactivity (Figure 3).

At 5-8 days in vitro, the response of individual astrocytes to opioid-induced changes in $[Ca^{2+}]_i$ was both agonist and region specific (Figure 4). Moreover, the response to μ , δ , and κ opioid agonists varied greatly among individual astrocytes within a culture resulting in a high degree of statistical variability. Despite the variability, significant regional differences in the proportion of astrocytes responding to μ receptor agonists were noted, and significantly greater numbers of hippocampal astrocytes responded to μ agonists in confluent compared to low density cultures (Figure 4). Several trends were also evident: (i) The activation of μ , δ , or κ opioid receptors increased Ca^{2+} within distinct astrocyte populations. Even within the same brain region, the response of individual astrocytes to particular opioid agonists was non-uniform. In most cases, isolated cells showed discrete responses to agonist treatments (Figure 2). Less frequently, responsive and non-responsive astrocytes were segregated into clusters—with multiple astrocytes within a cluster often responding similarly to an agonist challenge. (ii) Fewer astrocytes in striatal cultures exhibited increases in $[Ca^{2+}]_i$ in response to μ , δ , or κ agonists compared to cultures from other brain regions. (iii) The proportion of astrocytes responding to δ agonists tended to be greater than those responding to κ or μ agonists—irrespective of region. (iv) A greater proportion of astrocytes displayed μ , δ , or κ immunoreactivity than displayed μ , δ , or κ agonist-induced increases in $[Ca^{2+}]_i$. The rank order of $[Ca^{2+}]_i$ responsiveness by receptor type was $\delta > \kappa \cong \mu$ (Figure 4).

In confluent cultures, μ , δ , and/or κ receptor activation increased $[Ca^{2+}]_i$ in subpopulations of astrocytes from most brain regions indicating the presence of functional receptors (Figure 4). Although confluent astrocytes were sometimes segregated into discrete clusters of responsive and non-responsive cells, most cells displayed isolated increases in $[Ca^{2+}]_i$. Some astrocytes responded to more than one agonist. When the effects of opioids on astrocytes from all brain regions are considered together, a majority of cells only responded to a single agonist type (μ , δ , or κ). About 10% of the cells responded to two separate

agonist types, and all three agonist types (μ , δ , or κ) affected about 10% of astrocytes, suggesting that these cells co-expressed all three receptor types (Figure 1 F&G).

Flow Cytometry

Of the total population of cerebral forebrain astrocytes, greater than 26% of the cells in astrocyte-enriched cultures were in the S or M/G₂ stages of the cell cycle. Correspondingly, about 73% of the remaining cells were in G₀/G₁ at 6 days in culture (Table 1). Interestingly, there was a significantly greater percentage of μ or δ receptor-immunofluorescent astrocytes in the M or G₂ phases of the cell cycle compared to non-receptor immunofluorescent astrocytes, and correspondingly fewer receptor positive cells in G₀/G₁ (Table 1). It is noteworthy that the cell cycle distribution of the population of astrocytes lacking μ or δ receptor immunofluorescence did not differ significantly from the total population, whereas μ or δ immunopositive astrocytes differed markedly from the total population. There are two reasons for this. First, only a small percentage of astrocytes possessed μ or δ immunoreactivity intensity above background in the cerebral cortex (See Figure 3). Second, to avoid measuring false-positive cells, of the small percentage of astrocytes above background the cell cycle distribution was only determined in 15-20% of the most intensely immunofluorescent cells.

Arresting cells in G₁ by treating with hydroxyurea (0.5 mM) and reduced serum (5%) for 24 h significantly reduced both the proportion of astrocytes in G₂/M ($P < 0.05$) and the percentage cells expressing δ receptor immunofluorescence ($P < 0.05$) (Table 2). When cells were permitted to reenter the cell cycle and progress to G₂/M (by removing hydroxyurea for 24 h), the proportion of δ opioid receptor-immunoreactive astrocytes increased significantly. There was a similar reduction in the proportion of astrocytes expressing μ opioid receptor immunofluorescence, but this trend was not significant. Despite a significant reduction in astrocyte division following hydroxyurea (0.5 mM) treatment, attempts to completely block astrocyte replication (e.g., increased hydroxyurea and/or reduced serum levels) caused severe cytotoxicity—especially in the low density cultures. Thus, the proportion δ receptor-expressing astrocytes might be reduced further by completely blocking cell division.

DISCUSSION

Astrocytes provide intimate trophic, metabolic, and physical support for neurons (Sivron et al. 1993; Rakic, 1995; Muller, 1995; Lin et al. 1993; Dietzel et al. 1989; Anderson et al. 1992; Nedergaard, 1994; Parpura et al. 1994). Considering the variety of neuronal forms and functions, and the interrelatedness of neurons and astroglia, it is not surprising that astroglia themselves exhibit substantial phenotypic diversity (McCarthy et al. 1995; Wilkin et al. 1995; Pearce, and Wilkin, 1995; Rakic, 1995). Our results show that there is considerable heterogeneity in μ , δ , and κ -opioid receptor expression among individual astrocytes, and this heterogeneity is dependent on regional and developmental factors (days in vitro and position within the cell cycle). In addition to opioid receptors, astroglia also express endogenous opioid peptides in vivo and in vitro (Spruce et al. 1990; Shinoda et al. 1989; Hauser et al. 1990). Thus, astroglia contribute to the diversity of the endogenous opioid system

throughout the brain, and opioid expression within this glial type is highly plastic and modifiable during ontogeny.

Our results confirm and extend the findings of Ruzicka et al. (1995) and Eriksson et al. (1991), who described regional differences in μ , δ , and/or κ opioid receptor mRNA levels and/or function in rat astrocyte cultures. Collectively, the above studies provide novel biochemical and functional evidence that μ , δ , and κ receptors are expressed by astrocytes throughout the brain. The present study unambiguously demonstrates a high degree of diversity in opioid phenotype at the cellular level—supporting the belief that opioid diversity among astrocytes is rivaled only by neurons (Ruzicka et al. 1995). Our findings infer that astroglia participate in the complex tapestry of intercellular opioid signaling.

Despite essential similarities, however, it was not surprising that there were differences among studies regarding the relative abundance of opioid receptors. Ruzicka et al., (1995) assessed mRNA levels biochemically in rat astroglia maintained using different culture conditions, whereas our study assayed immunocytochemical and functional studies in individual mouse cells. One inconsistency between our findings and past studies relates to the extent to which μ receptors are expressed by astrocytes. We found evidence that large numbers of astrocytes expressed μ opioid receptors, especially in cerebellar and hippocampal cultures. In contrast, Ruzicka et al. (1995) found small relative amounts of μ receptor mRNA in confluent rat astrocyte cultures—irrespective of brain region. Eriksson et al. (1990, 1991, 1992) found δ and κ , but not μ , agonist-induced changes in cAMP in confluent rat astrocyte cultures. Methodological differences may contribute to inconsistencies assessing μ -receptors.

Alternatively, a lack of opioid-induced reductions in cAMP levels (Eriksson et al. 1990; 1992), a common assay for opioid function, may reflect an absence of μ receptor coupling to adenylate cyclase, rather than the absence of μ receptors themselves. Opioid receptors can be promiscuous in their interaction with particular intracellular effectors (Prather et al. 1995; Jin et al. 1993; 1994; Fields et al. 1995). In mature neurons, opioids typically restrict cAMP and $[Ca^{2+}]_i$ which contrasts their effects in developing astroglia (Eriksson et al. 1993; Thorlin et al. 1994; Gurwell et al. 1996; Hauser et al. 1996). Moreover, opioid receptors can differentially couple into alternative G-protein-signaling pathways within a single cell type (Prather et al. 1995), and may only couple to $[Ca^{2+}]_i$ in a subset of astroglia (Hauser and Mangoura, 1997; Hauser et al. 1996). This could explain why opioids only increased $[Ca^{2+}]_i$ in a small proportion of astrocytes expressing μ , δ , or receptor immunoreactivity, and was particularly apparent in confluent striatal cultures where few, if any, astrocytes responded to opioids. Therefore, in the present study, a strict one-to-one correspondence was not expected when comparing the proportion of immunoreactive cells to the proportion of cells displaying functional changes in $[Ca^{2+}]_i$.

Besides restricted coupling of opioid receptors to $[Ca^{2+}]_i$, our procedure may have underestimated the number of cells expressing opioid receptors. Because we were concerned about erroneously recording false-positive responses, the criteria for a positive response were intentionally difficult but unambiguous. Saturating concentrations of μ , δ , and κ receptor agonists were used to induce maximal and sustained rises in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$

responses less than 50% above resting levels within 20 s of drug addition were not counted. Alternatively, gap junctions could permit Ca^{2+} transients to spread into astrocytes lacking opioid receptors resulting in an overestimation of opioid-expressing cells. Cells in low-density cultures rarely contacted each other. However, in confluent cultures, cells did contact one another. Although most responses were by isolated cells—occasionally $[\text{Ca}^{2+}]_i$ increases were segregated into discreet clusters of responsive and non-opioid-responsive cells. For this reason, the Ca^{2+} data from confluent cultures must be interpreted with some caution. On the other hand, the proportion of responsive astrocytes did not increase at confluence—except in hippocampal astrocytes in response to μ receptor activation—suggesting intercellular signaling is not an issue. Interestingly, adjacent clusters of astrocytes often possessed μ , δ or κ receptor immunoreactivity and are perhaps clonally related (e.g., Figure 1A-C shows clusters of phenotypically similar cells). Irrespective of the above concerns, these studies provide functional evidence that μ , δ , and κ receptors are potentially expressed by astrocytes throughout the brain and these receptors can be coupled to increases in $[\text{Ca}^{2+}]_i$. The findings are important because Ca^{2+} *per se* mediates opioid-induced changes in astrocyte proliferation and differentiation (Hauser et al. 1996).

Regional variations in the expression of the opioid system have been described extensively (Mansour et al. 1994; 1988; Arvidsson et al. 1995a; 1995b; 1995c). These differences have almost exclusively been attributed to neuronal variability. The potential glial contribution to opioid diversity was often not considered (Mansour et al. 1994; Arvidsson et al. 1995a; Arvidsson et al. 1995b; Arvidsson et al. 1995c; Mansour et al. 1988). Recently, Pickel and coworkers localized μ -opioid receptor immunoreactivity on the plasmamembrane of astroglia in the locus coeruleus and in the nucleus of the solitary tract (Van Bockstaele et al. 1996; Cheng et al. 1996). This supports the notion that μ opioid receptors are expressed by astroglia *in vivo*. In the brain regions examined in the present study, μ , δ , and κ opioid receptors are generally abundant within the striatum and cerebral cortex, although κ receptors are less abundant than μ and δ receptors in the cerebral cortex (Mansour et al. 1988; 1994). Moreover, all three receptor types are expressed regionally within the hippocampus, but are absent (or expressed at low levels) in the cerebellum of adult rats (Mansour et al. 1988; 1994). In general, the regional variability seen *in vitro* was consistent with the distribution of opioid receptors described *in vivo*—with some exceptions. Most notably, we found that cerebellar astrocytes expressed opioid receptors, which is inconsistent with the traditional view that cerebellar cells lack opioid receptors. As mentioned previously, the failure of opioids to increase $[\text{Ca}^{2+}]_i$ in striatal astrocytes may result from opioid receptors coupling into alternative signaling pathways in this cell population.

Another potential inconsistency is few studies have systematically examined opioid receptor expression during development, which can differ from adult patterns of expression. In the adult cerebellum, the medial and interposed nuclei express μ and κ receptors at low levels, and a small percentage of internal granular layer cells express δ receptor mRNA (Mansour et al. 1994; 1988). In contrast, opioid receptors are expressed by germinative cells (Kinney and White, 1991; Zagon et al. 1991). Immature cerebellar astrocytes may be more likely to

express opioid receptors. Intact and partially processed-proenkephalin peptides have been identified in cerebellar astroglia in immature rats (Spruce et al. 1990).

Progression through the cell cycle requires the coordination of intracellular and extracellular events (Pardee, 1989). The periodicity in opioid receptor immunofluorescence during the cell cycle suggests that there are numerical and/or functional differences in opioid receptors as cells divide. Two earlier studies demonstrated that δ opioid receptor levels increase significantly in NG108-15 cells during the S, G₂ and/or M phases of the cell cycle (Scheideler et al. 1983; Ornatowska and Glasel, 1991). This generally agrees with our present findings that δ opioid receptor immunofluorescence increased during the G₂/M phases of the cell cycle in primary astrocytes, although we did not see significant increases in immunofluorescence during the S phase. The possibility that opioid receptor expression and/or function change during the cell cycle is provocative and may be relevant during cell division. We found that the activation of μ , κ , and perhaps δ , opioid receptors inhibits DNA synthesis in astrocytes (Stiene-Martin and Hauser, 1991; Gurwell et al. 1996; Hauser et al. 1996). Based on these findings, it is interesting to speculate that the response of astrocytes changes during the cell cycle. Alternatively, increasing the number of receptors does not necessarily heighten a cell's response to opioids. The receptors must be appropriately coupled. For example, despite finding increases in δ receptor binding during the S + G₂, portion of the cell cycle, these receptors couple far less efficiently to adenylate cyclase at this time (Scheideler et al. 1983).

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Figure 1.

(A-F) Opioid receptor immunofluorescence (green FITC product) in cerebral cortical astrocytes counterstained with propidium iodide (red nuclear product) at 6 days *in vitro*—similar to those used for flow cytometry. Astrocytes can express μ , δ , and/or κ opioid receptor immunoreactivity (A-F). μ -Opioid receptor immunoreactivity is reticular and highly polarized within individual astroglia (A), δ receptor immunoprotein is more diffuse and uniformly distributed throughout the cytoplasm (B), while κ -Opioid receptor immunoreactivity has both punctate and diffuse attributes (C); scale bar in A = 25 μ m (A-C). Figure 1A-C is intended to illustrate the cellular pattern of opioid receptor immunoreactivity, rather than the actual proportion of receptor positive cells in these cultures. Astrocytes possessing opioid receptor immunoreactivity were not distributed uniformly in cell clusters. Specific immunoreactivity was not seen in preabsorbed controls (D-F). Scale bar in C = 20 μ m (D-F). (G-H) μ , δ , and/or κ opioid receptor activation increased $[Ca^{2+}]_i$ in some astrocytes. Functional changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$), in response to opioid agonist treatment, varied greatly among individual astrocytes (repeated measures in cerebral forebrain astrocytes at 6 days in culture). (G) Exposure to the δ opioid receptor agonist, DPDPE, caused transient increases in $[Ca^{2+}]_i$ within a subset of astrocytes (arrows). Alternatively, treatment with a κ agonist (U50,488H) did not increase $[Ca^{2+}]_i$; while PL017, a μ receptor agonist, elevated $[Ca^{2+}]_i$ in cells (arrow) that had previously responded to DPDPE. (H) DPDPE exposure elevated $[Ca^{2+}]_i$ in a subpopulation of astrocytes (hatched arrow), while U50,488H treatment recruited sustained $[Ca^{2+}]_i$ increases in several additional cells (arrows) that also responded to PL017 treatment. Drug concentrations were 100 nM (G-H).

Figure 2.

Effect of opioid receptor agonists on free intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in individual flat, polyhedral astrocytes (A-F). Astrocytes do not respond uniformly to μ , δ , or κ opioid agonists—individual cells display unique responses to μ , δ , and/or κ receptor activation. The effects of opioid receptor stimulation on $[\text{Ca}^{2+}]_i$ are selective, often only a single astrocyte within a field was affected by a particular agonist type (B,C, D), and there was little cross reactivity among agonist types (A-D). The topography of the response was generally similar irrespective of whether μ , δ , or κ opioid receptors were activated. Agonist concentrations were 100 nM and agonist effects could be prevented by opioid antagonists (E-F). For example, naloxone pretreatment attenuated the response to U50,488 (F). Responsive cells (—) or (---); Non-responsive cells (.....).

Figure 3.

Effect of regional or age-related (low-density vs. confluent) differences on the percentage of astrocytes expressing μ , δ or κ opioid receptor immunoreactivity. The mean $\pm$ SEM was determined from at least 3 cultures. About 500 astrocytes were assessed per culture; each culture consisted of an independent sample of cells from separate mice. The proportion of μ or δ receptor-expressing astrocytes differed significantly across regions ($P < 0.05$), and differed among low density and confluent cultures (* $P < 0.05$ vs. confluent astrocytes).

Figure 4.

Effect of regional or age-related (low-density vs. confluent) differences on the percentage of astrocytes exhibiting changes in $[Ca^{2+}]_i$ in response to μ , δ , or κ opioid agonists. The mean $\pm$ SEM was determined from at least five cultures. Forty-sixty astrocytes were assessed in each culture; each culture consisted of an independent sample of cells from separate mice. The proportion of astrocytes displaying increases in $[Ca^{2+}]_i$ in response to μ receptor activation differed significantly across regions ($P < 0.05$), and low density vs. confluent cultures of the hippocampus ($*P < 0.05$ vs. confluent astrocytes). Because, opioid receptors may or may not couple to increases in $[Ca^{2+}]_i$, a strict one-to-one correspondence was not expected when comparing the proportion of immunoreactive cells (Figure 3) to the proportion of cells displaying functional changes in $[Ca^{2+}]_i$.

Table 1

Cell Cycle-Distribution (Percentage of Cells in G₀/G₁, S, or G₂/M) of Opioid and Non-Opioid Receptor-Immunofluorescent Astrocyte Subpopulations.

	<i>No. of exp-eriments</i>	<i>G₀/G₁ (%)</i>	<i>S (%)</i>	<i>G₂/M (%)</i>
Astrocytes				
<i>Total population</i>	5	72.2 ± 1.1	6.0 ± 1.8	21.8 ± 2.0
<i>μ receptor negative</i>		72.5 ± 1.9	9.7 ± 1.5	18.5 ± 1.7
<i>μ receptor positive</i>		54.1 ± 2.8*	5.4 ± 1.5	40.5 ± 2.0*
<i>Total population</i>	6	73.5 ± 1.4	6.0 ± 2.0	20.5 ± 1.9
<i>δ receptor negative</i>		75.2 ± 1.3	5.6 ± 1.2	19.2 ± 0.9
<i>δ receptor positive</i>		51.7 ± 5.9*	7.7 ± 0.8	40.6 ± 6.1*

A greater proportion of μ or δ immunofluorescent astrocytes were in the G₂ or M phase of the cell cycle than non-μ or δ immunofluorescent astrocytes. Conversely, a smaller percentage of μ or δ immunofluorescent astrocytes were in G₀ or the G₁ phase of the cell cycle compared to non-μ or δ immunofluorescent astrocytes.

* P < 0.05 Vs cells lacking μ or δ receptor immunofluorescence.

Table 2

Changes in Percentage of μ and δ Opioid Receptor Immunoreactive Astrocytes Following Arrest in G₁ and Reentry into the Cell Cycle (%) as Determined by Flow Cytometry

Treatment	<u>Opioid receptor immunoreactive astrocytes (%)</u>	
	μ	δ
Untreated	53.4 $\pm$ 5.3	34.2 $\pm$ 6.5
Hydroxyurea (24 h)	26.0 $\pm$ 6.9	19.8 $\pm$ 1.4 *
Hydroxyurea (24 h) (16 h washout)	41.9 $\pm$ 1.7	42.4 $\pm$ 2.3

The cultures sampled demonstrated significant (about 56%) reductions in the proportion of cells in G₂/M following hydroxyurea treatment.

* $P < 0.05$ vs. untreated cells or following hydroxyurea washout for 16 h; n = 3 experiments.