Morphine regulates DNA synthesis in rat cerebellar neuroblasts in vitro

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The effects of morphine on DNA synthesis by external granular layer (EGL) neuroblasts was examined in whole-mount organotypic cultures isolated from 10-day-old rat cerebella using bromodeoxyuridine (BrdU). After 24 h in vitro, explants were treated for 24 h with 10 nM, 1 or 100 \( \mu \)M morphine, morphine plus 30 nM, 3 or 300 \( \mu \)M of the opiate antagonist naloxone, respectively, or those concentrations of naloxone alone. BrdU was added during the last 4 h of drug treatment. EGL neuroblasts were unambiguously identified by size and morphology, location and by protein kinase C II immunocytochemistry. The proportion of EGL neuroblasts incorporating BrdU was significantly reduced in the presence of 1 \( \mu \)M morphine, while 100 \( \mu \)M morphine had little additional effect. The concentration of morphine predicted to cause a half-maximal reduction in BrdU labeling index was 22.5 nM. Morphine’s ability to reduce BrdU incorporation by EGL neuroblasts was concentration dependent and was prevented by concomitant treatment with naloxone, implicating the involvement of opioid receptors. The results suggest that morphine can directly regulate the growth of the developing cerebellum by inhibiting neuroblast proliferation within the EGL.

Endogenous opioid systems are present during development4,17,21,22,27,32,47,50,52 (for review, see ref. 29) and can modify nervous system matura-
tion7,15,16,26,36,42,44,49-51 (for review, see ref. 12). During development, endogenous opioid neuropeptides typically act by inhibiting the growth of the nervous system in a regionally dependent manner7,15,16,26,36,44,49-51. Opiate drugs also affect neural development10,13,18,23,30,41,45 presumably by disrupting normal opioid-opioid receptor interactions.

Opioids and opiate drugs affect neural cell numbers in the developing nervous system. Most often this is reported to result from altered cell divi-
sion7,23,26,36,42,44,45. However, there is conflicting evidence whether cell proliferation is directly or indirectly affected18,23,36. Treatment with the opioid antagonist naltrexone causes long-term alterations in cell proliferation that may be mediated in part through non-opioid mechanisms36. Glial numbers in the cerebellum can be altered by opioids in vivo50 and astrocyte numbers can be modified by opiates in cerebellar explant cultures18.

Yet, there are temporal and regional differences in the ability of [Met5]-enkephalin to inhibit the growth of type 1 astrocytes18. These differences may be dependent on local growth factors and/or cell-cell interactions18. Moreover, all opioid-responsive cells do not respond similarly to opioids during development. Opi-
ates affect neuronal numbers in the avian ciliary gan-
glion by altering survival, not proliferation30. Also, whether growth is accelerated or retarded is dependent on the duration of opioid exposure7,16,50,51. For example, in mesencephalic raphe cultures from fetal rats, the number of serotonergic neurons is decreased after chronic exposure, but increased following acute ex-
posure to opioids7. Thus, there are considerable differ-
ences in the cellular response to opioids during ont-
geny. It is as yet unclear whether opioids affect development through a single underlying mechanism.

The rodent cerebellum is likely to provide important clues about the developmental role of opioids. First, subpopulations of developing cerebellar neurons and glia express proenkephalin mRNA and/or peptide...
products\textsuperscript{17,32,40,52}. Second, opioid receptors are present in the developing cerebellum\textsuperscript{21,43,47,48}. Lastly, the growth of cerebellar neurons and/or glia can be modified by manipulating endogenous opioid systems\textsuperscript{15,16,18,20,45,50,51}. The rate of thymidine incorporation by neuroblasts of cerebellar neurons and/or glia can be modified by manipulating endogenous opioid systems\textsuperscript{15,16,18,26,45,50,51}. Second, opioid receptors are present in the external granular layer (EGL) can be affected by opioid agonists and antagonists in vivo\textsuperscript{51}. In culture, opiates can intrinsically affect the outgrowth of cells from cerebellar explant cultures\textsuperscript{18,45} which consist of neurons and glia\textsuperscript{18}. The growth and survival of Purkinje cells is also affected by opiates in explant cultures (Hauser, in preparation). Yet, despite findings that the developing cerebellum is sensitive to opioids, the direct effects of opiates on DNA synthesis in cerebellar neuroblasts in culture has not been previously studied. The preliminary results herein support the hypothesis that opiates per se can inhibit the rate of proliferation of neuronal progenitors within the EGL. These findings have implications regarding chronic licit or illicit opiate drug use during pregnancy and early childhood.

Cerebella obtained from 24, 10-day-old male, Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were dissected into small \([0.3-0.5] \times 1\ \text{mm}^2\) explants and maintained for 48 h in vitro before harvesting. There are considerable differences in the growth of different regions within the rat cerebellum and in the expression of endogenous opioids by cerebellar cells in developing rats (see ref. 32). Thus, explants were only taken from the lateral portions of crus I and II (i.e., between the posterior superior fissure and the ansoparamedian fissure\textsuperscript{24}). To measure the labeling index within the EGL, explants were trimmed parasagitally using a micro-surgery scalpel (4 mm blade) aided with 40 \(\times\) dissecting microscope and positioned so the EGL was at their peripheral border as previously described\textsuperscript{17,18}. Explants were maintained in organotypic culture in 12-well (22 mm diameter) plastic tissue culture chambers as previously described\textsuperscript{17,18}. Cultures were treated continuously for the last 24 h with media alone (untreated), media containing morphine sulfate (NIDA) (10 nM, 1 or 100 \(\mu M\)) alone or with \((-)\)-naloxone (DuPont, Wilmington, DE) (30 nM, 3 or 300 \(\mu M\), respectively), or media containing the above concentrations of naloxone alone. During the last 4 h of opiate treatment, 5-bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was added to the media to a final concentration of 50 mg/ml.

After 48 h in vitro, explants were fixed \textit{in situ} on the coverslips on which they were grown. It was important to optimize the BrdU signal-to-noise ratio while preserving cellular morphology (EGL cells are identified based on their unique size and morphology). Therefore, several previously described\textsuperscript{9,11,31,38} BrdU immunocytochemical procedures were compared in preliminary studies. In the present study, explants were fixed at 4°C in 2% paraformaldehyde\textsuperscript{38} for 30 min or in 70% ethanol overnight\textsuperscript{31}. Explants were later permeabilized by treatment in 50% ethanol in 0.1 M PBS (pH 7.4) (30 min), 70% ethanol in 0.1 M PBS (30 min) and 50% ethanol in 0.1 M PBS (30 min) as modified from Jaeger et al.\textsuperscript{20}. Permeabilization was followed by brief DNA denaturing (0.07 M NaOH or 2 N HCl for 0.5 or 1 h)\textsuperscript{35,38}. In some cases, DNA was denatured before alcohol-PBS permeabilization. Primary monoclonal antibodies against BrdU (Chemicon, Temecula, CA) diluted 1:50 or 1:100 (w/v) in PBS (phosphate buffered saline), pH 7.4, containing 0.1% Triton-X 100 and 1% crystalline grade bovine serum albumin (BSA; Calbiochem, CA). Tissues were incubated with antisera for 48 h at 4°C on an orbital shaker at 40–60 r.p.m.

Anti-BrdU antibodies were detected using a biotin-avidin-peroxidase detection system as directed (Vectorstain ABC kit, Vector Laboratories, Burlingame, CA). The reaction was visualized using a nickel-intensified DAB chromagen consisting of freshly made 2.5% Nickel ammonium sulfate, 0.35% diaminobenzidine and 0.012% \(H_2O_2\) in 0.1 M sodium acetate, pH 6.0 (ref. 14). Approximately 2,000 randomly selected EGL cells were sampled from explants from each rat. The labeling index [labeled cells/(labeled + unlabeled cells)] was determined using a 100 \(\times\) oil immersion objective with Hoffman modulation contrast optics as modified from Gao et al.\textsuperscript{9} who studied BrdU-labeled EGL cells in reaggregate culture. Labeling index was only determined when all cells (labeled and unlabeled) were visible within a defined EGL. Multiple explants from individual rats were distributed across experimental groups. Each value represents explants sampled from \(n = 4\) to \(n = 6\) rats. Data were analyzed using ANOVA and reported as the mean ± S.E. Post hoc comparisons were made using Newman–Keuls test. To estimate the concentration of morphine that would half-maximally suppress the labeling index in EGL neuroblasts, non-linear, least-squares regression analysis was performed (GraphPad; GraphPad Software).

For protein kinase C (PKC) II immunocytochemistry, explants were fixed with 4% paraformaldehyde in Sorenson’s phosphate buffer, pH 7.4. Explants were permeabilized and incubated in goat anti-PKC II antiserum (a gift from Dr. F.L. Huang, NIH) diluted 1:1,500 or 1:2,000 as modified slightly from Huang et al.\textsuperscript{19} for use with an ABC kit (Vector Labs). In some cases, second antibodies conjugated to alkaline phosphatase were visualized with a red reaction product (Vectastain...
Fig. 1. A–E: bromodeoxyuridine (BrdU) immunocytochemistry of organotypic explant cultures of 10-day-old rat cerebellum at 48 h in vitro that were incubated with BrdU for 4 h and then harvested. The cerebellar external granular layer (EGL) is a transient proliferative zone consisting of neuroblasts and postmitotic neurons which can be readily identified in organotypic explants. The explant culture in A was sectioned tangentially (horizontally) through several folia at the lateral edge of the cerebellum and shows how the histiotypic organization of the EGL is maintained in vitro. In the present study, explants were much thinner and sagittally sectioned so that the EGL was at the peripheral border of the explant where cells are easily counted (B). EGL cell bodies are typically 5–8 μm in diameter and have only a thin rim of cytoplasm (B). The nuclei of replicating EGL neuroblasts incorporate BrdU and dividing cells often form clusters (B,C). D,E: the outgrowth from cerebellar explants contains some BrdU-immunoreactive EGL progeny (arrow) that are positioned outside the EGL. Granule cells (EGL progeny) migrate from the EGL both inwardly into the explant and outwardly into the outgrowth. Larger non-neuronal cells (arrowheads) resemble astroblasts. Phase-contrast (D) and brightfield (E) micrographs of the same field. Scale bars: A, 100 μm; B, 10 μm; C, 25 μm; D,E, 25 μm.
III Kit). Immunoreactivity was not seen when either BrdU or PKC II primary antibodies were excluded from the reaction.

BrdU labeling indexes appeared comparable irrespective of whether the tissue was fixed in ethanol or paraformaldehyde. Ethanol fixation gave a better BrdU signal-to-noise ratio, but with slight loss in cellular morphology. However, because our past studies have identified EGL cells based on their morphology in paraformaldehyde fixed explant cultures, BrdU incorporation was also assessed in paraformaldehyde fixed tissue. Although more prolonged (1 h) DNA denaturing, as well as the use of a more concentrated (i.e., 1:50 dilution) of the BrdU primary antibody, improved the BrdU signal, paraformaldehyde fixation still resulted in increased non-specific staining compared to ethanol fixation. Therefore, the reported measurements were made on ethanol fixed tissue. To validate the BrdU procedure, [3H]thymidine autoradiography was performed in six untreated explants in parallel with the present BrdU procedure and yielded similar results (Hauser, unpublished results).

EGL neuroblasts and their progeny were readily identified in organotypic cultures based on criteria as previously noted by myself and others. These cells have a distinct size (approximately 5–8 μm cell body diameter) and morphology and are sometimes distributed in clusters at the edge of the explant. EGL progeny migrate both into and away from the explant. EGL cells are the only neuronal precursors to incorporate BrdU, since all other cerebellar neurons are postmitotic in the postnatal rat. Antisera against an isozyme of PKC II also labeled EGL cells and their progeny further confirmed their identity. Some immature glia, however, also expressed PKC II. Because PKC II was not an exclusive marker for granule cell progenitors in the present culture system, these results are not reported. Non-neuronal cells were distinguishable from EGL cells. Replicating astrocyte progenitors were larger (i.e., 11.8 ± 1.4 μm diameter cell body) than EGL cells. These immature astrocytes typically had multiple nucleoli within a cell with discernible cytoplasm, were GFAP immunoreactive and distributed at the periphery of the outgrowth.

Morphine caused a concentration-dependent reduction in BrdU incorporation by EGL cells (ANOVA; P < 0.01) (Fig. 2). Treatment with 1 or 100 μM morphine caused a significant reduction in the BrdU labeling index of EGL neuroblasts compared to untreated cultures (Fig. 2). It was predicted that 22.5 nM morphine would produce a half-maximal reduction in BrdU incorporation by EGL neuroblasts using regression analysis. The effects of 100 μM morphine were prevented by co-administration of 300 μM naloxone (ANOVA; P < 0.01). Naloxone treatment alone, at concentrations up to 300 μM, had no effect on EGL cell labeling index (Fig. 3).

The results indicate that morphine’s action on cellular proliferation was concentration dependent and prevented by the opiate antagonist naloxone and therefore mediated through opioid receptors. Morphine affected DNA synthesis in neuroblasts within isolated explants, suggesting that opiates can intrinsically inhibit neuroblast proliferation in the cerebellum.

It was not surprising that proliferating EGL cells would respond to opiates in vitro. First, [3H]thymidine
opioid receptors have been implicated in growth. Kephalin and increased by opioid antagonists in dosages cerebellum. Opioid receptors reported to be present in ment4,21,43,47,48. This suggests that opiates may affect trophic input from the axons of granule cells (EGL Purkinje cell development, in part dependent on incorporation by EGL cells is decreased by [Met5]-en- the developing cerebellum include the µ-, κ- and ζ- opioid receptors characterized in rat cerebellum (see refs. 41,42). Thus, morphine might be acting through one or more non-µ-sites. This notion is supported by studies of opiate-dependent growth in astrocytes41,42. Recently, [3H]naloxone binding sites have been localized within the EGL in humans21, suggesting that EGL cells themselves may respond directly to opioids.

In many brain regions, opioids per se are proposed to directly affect neural growth by inhibiting cell proliferation41,51. However, in some regions of the nervous system opioids may only indirectly affect cell numbers23,36. Kornblum et al.23 found that morphine did not affect [3H]thymidine incorporation in isolated nervous tissue in vitro. DiCiccio-Bloom et al.9 found that [Met5]enkephalin, at 0.1–10 µM concentrations, did not affect the proliferative rate of superior cervical ganglion neuroblasts from embryonic rats in vitro. Similarly, the growth of neurons in the hippocampus is profoundly affected by opioids in vivo15,16, yet the outgrowth of cultured hippocampal explants is unaffected by 10 µM morphine18. In vitro, there are regional and temporal differences in the ability of opiates to regulate the growth of type 1 astrocytes that are not evident in vivo16. Lastly, morphine increases neuronal numbers in the ciliary ganglion, not by affecting replication, but by increasing their rate of survival30. These findings show that opiates can affect cell numbers, but suggest that they can act both by direct and indirect mechanisms depending on the specific target18. Probably no single trophic factor acts alone to regulate any event during neural development28. Adrenal corticosteroids46 and thyroid hormone22 affect the rate of proliferation of EGL neuroblasts. Cell-cell interactions also affect EGL cell proliferation9. Opiates are likely to interact with other factors to modulate key maturational events23,28. Yet, because opiates per se can affect the growth in isolated explants; this suggests that, in the cerebellum, opioids might normally be an initiating signal that triggers later events. Opiates affect many aspects of cerebellar development in vitro. For example, Purkinje cell dendritic length and complexity (Hauser, in preparation), glial cell numbers18, and the rate of EGL cell replication are intrinsically modified by opiates in culture. In conclusion, cerebellar histogenesis appears to be directly affected by opiates with EGL neuroblasts being a principle cellular target of action. Additional studies are needed to assess whether opiates per se affect dividing neuroblasts in other brain regions.

It was interesting that naloxone treatment by itself had no effect on the labeling index of EGL neuroblasts. Continuous blockade of opioid receptors by opioid antagonists speeds the rate of [3H]thymidine incorporation by EGL51 and Purkinje cell dendritic growth in rats15,16. This and other findings support the hypothesis that opioids are normally present during cerebellar development17,32,40,43,52 and function by tonically inhibiting growth15–17,50–52. Cerbellar astrocytes17,40, Purkinje32 and EGL cells52 express proenkephalin mRNA and peptide products. Importantly, EGL neuroblasts in 10-day-old rats52 and in explant cultures derived from newborn rats33, respectively, contain enkephalin and proenkephalin immunoreactivity. Yet, if locally produced opioids can inhibit cerebellar growth and opioids are expressed in vitro, then why does naloxone alone have no effect in the present study? One explanation is that there are significant differences in amount of endogenous opioids that are available to developing cells in different regions of the cerebellum at different times. Osborne et al.32 found using in situ hybridization that proenkephalin mRNA is not expressed uniformly throughout the developing cerebellum in rats. Expression is greater in caudal32 and lateral regions (Osborne and Hauser, unpublished results). There may be more opioids in lobule VIII in 6-day-old rats (region examined in ref. 51) than in explants from the lateral hemispheres of 10-day-old rats. However, this explanation is inconsistent with some preliminary findings. Fewer cells in 6-day-old rats express proenkephalin products than in 10-day-old rats32 and fewer cells within the lobule VIII express proenkephalin mRNA than in the dorsal paraflocculus (Osborne and Hauser, in preparation) which is considered to be a lateral extension of lobule VIII24. The local expression of other opioid genes, i.e., prodynorphin and proopiomelanocortin (POMC) might be in-
volved in cerebellar development. POMC gene products are likely to have developmental effects in the cerebellum as well as in other brain regions. Alternative explanations might be that circulating opioids mediate the effect, or that opioids produced in vitro might be diluted and/or degraded differently from those in vivo. Lastly, the EGL is a dynamic structure and the response of dividing cells to opioids may differ with age. Vértés et al. found that 200 μg/100 g body weight of naloxone had no effect on [3H]thymidine incorporation at 1, 3, 6, 9 or 12 h after treatment in biochemical studies of 11-day-old rat cerebellum. In the same study, they found that [3H]thymidine incorporation was significantly increased at 1 and/or 3 h in the forebrain and hypothalamus. Lorber et al. similarly found that i.c. administration of 3 μg/g brain weight of naloxone had no effect on DNA synthesis after 1 h in the cerebellum of 10-day-old rats. Further studies are needed to assess the role of locally produced endogenous opioids in the developing cerebellum.

In summary, the results provide evidence that opiates per se can regulate the proliferation of EGL neuroblasts through a direct action on the developing cerebellum. The finding that opiates can intrinsically inhibit neurogenesis has implications regarding chronic licit or illicit opiate drug use during pregnancy and early childhood.

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3 Altman, J., Experimental reorganization of the cerebellar cortex. VII. Effects of late X-irradiation schedules that interfere with cell acquisition after stellate cells are formed. J. Comp. Neurol., 165 (1976) 65–76.
31 Miller, M.W. and Nowakowski, R.S., Use of bromodeoxyuridine-
immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system, Brain Res., 457 (1988) 44-52.


