Brain Research, 542 (1991) 318–323
Elsevier

BRES 16377

Identification of opioid peptides regulating proliferation of neurons and glia in the developing nervous system

Ian S. Zagon and Patricia J. McLaughlin

Department of Anatomy, The Pennsylvania State University, The M.S. Hershey Medical Center, Hershey, PA 17033 (U.S.A.)

(Accepted 18 September 1990)

Key words: Opioid receptor; Nervous system; Enkephalin; Development; Cerebellum; Rat; Opioid peptide; Endogenous opioid; Growth; Neuron; Glia

Endogenous opioid systems (i.e. opioids and opioid receptors) play a role in regulating neural development. Using the cerebellar cortex of 6-day-old rats, the most potent opioid peptides involved with cell proliferation were assessed. In both the external germinal (granule) layer (EGL), a germinative matrix giving rise to neurons, and the medullary layer (MED), a pool of cells that are the precursors of glia (astrocytes and oligodendrocytes), [Met^5]enkephalin and peptide F were extremely potent in depressing the labeling index (LI) using [^{3}H]thymidine and autoradiographic techniques; concentrations as low as 100 μg/kg reduced the LI of EGL cells by 24% and MED cells by 43%. This inhibition of DNA synthesis by opioid peptides was blocked by concomitant exposure to naloxone, an opioid antagonist. Peptide action was apparent 2 h following drug administration, and concentrations of 80 μg/kg but not 1 or 10 μg/kg [Met^5]enkephalin depressed the LI. These results identify a selective group of opioid peptides, derived from proenkephalin A, as the potent, natural, inhibitory factors targeted to cell proliferation of cells destined to be neurons and glia in the developing nervous system.

INTRODUCTION

Development of the nervous system is governed, in part, by endogenous opioid systems (i.e. endogenous opioids and opioid receptors)[4–6,22–27]. Endogenous opioids serve as inhibitory growth factors, acting on cell proliferative events[28]. Evidence for the role of endogenous opioid control of neural cell replication comes from a number of avenues. Studies investigating the effects of opioid antagonist (i.e. naltrexone) interruption of opioid–opioid receptor interaction revealed that complete receptor blockade during preweaning cerebellar development resulted in an increased number of postnatally derived neurons and glial cells in this region of the brain[22,27]. These reports suggested that endogenous opioids are inhibitory to cell proliferation, and that this influence is tonically governed. Subsequent studies[28] using [^{3}H]thymidine and autoradiography showed that DNA synthesis was increased in the nervous system as a result of opioid receptor blockade. Moreover, rats subjected to an injection of an opioid peptide, [Met^5]-enkephalin, had a pronounced decrease in the number of cells incorporating [^{3}H]thymidine. This effect on cell replication by opioids was blocked by concomitant injection of the opioid antagonist naloxone, indicating that endogenous opioids governed cell proliferation at the level of the opioid receptor.

In order to understand which opioid(s) is(are) involved with growth, a comprehensive profile of the effects of opioid peptides on cell proliferation in the developing nervous system is required. The present study was designed to investigate systematically the response of proliferating cells to opioid peptides. Since the postnatal rat cerebellum has been employed as a model in earlier inquiries on endogenous opioid systems and neural embryogenesis[22,27,28], this system was used to assess the effects of a wide variety of opioid peptides on neurogenesis and gliogenesis in the external germinal (granule) layer (EGL) and medullary (MED) layer, respectively.

MATERIALS AND METHODS

Animals

Male and female Sprague–Dawley rats (Charles River Labs, Wilmington, MA) were mated and their offspring used in this study. At birth, litters were culled to 8 pups per mother. All animals were housed under standard laboratory conditions as described elsewhere[25].

Experimental protocol

In Expt. I the effects of a wide variety of opioids on the proliferation of neuronal and glial precursors in the developing

Correspondence: I.S. Zagon, Department of Anatomy, The M.S. Hershey Medical Center, Hershey, PA 17033, U.S.A.

cerebellum were examined. Six-day-old rats were injected subcutaneously with opioid compounds at a dosage of either 0.1, 1, or 10 mg/kg; controls received an equal volume of vehicle (sterile water). Thirty minutes prior to sacrifice at 4 h following drug administration, rats received an intraperitoneal injection of [^{3}H]thymidine (10 μCi/g body weight; 20 Ci/mmol, DuPont-New England Nuclear, Boston, MA). Three to nine animals per drug were utilized at concentrations of 0.1 mg/kg, whereas 1 to 4 rats per drug were employed at concentrations of 1 and 10 mg/kg. In Expt. II the effects of different dosages of a potent growth-related opioid peptide, [Met^5]enkephalin, on cell replication were assessed. Six-day-old rats were injected with various concentrations of [Met^5]-enkephalin including 0.1, 10, 80, 100, 200, 500, and 1000 μg/kg; control subjects received an equal volume of vehicle (sterile water). In addition, to evaluate whether the action of opioids on mitogenesis was at the locus of the opioid receptor, some animals were injected with 100 μg/kg [Met^5]enkephalin and 1 mg/kg naloxone hydrochloride; evaluation of the effect of 1 μg/kg naloxone alone on cell replication was included. Thirty min prior to sacrifice at 4 h following drug administration, rats received an injection of [^{3}H]thymidine as described earlier. At least 2 animals per drug concentration were utilized. In Expt. III the temporal course of opioid activity on cell replication was examined. Six-day-old animals received an injection of 100 μg/kg [Met^5]enkephalin and were sacrificed at 1, 2, 3, 4, 6, 8, 10, and 24 h after drug injection; 30 min prior to sacrifice the rats received an injection of [^{3}H]thymidine as described above. At least 2 animals/treatment per time point were employed.

Histology and autoradiography

Animals were anesthetized, killed, and brains rapidly removed and fixed in 10% neutral buffered formalin for 3 days. Tissues were embedded in polyester wax and midsagittal sections (8 μm) collected. Sections were coated with NTB-2 nuclear track emulsion (Kodak, Rochester, NY), exposed at 4 °C for 10–14 days, developed in D-19, and stained with hematoxylin.

The labeling index (LI = number of labeled cells/total cells) for EGL cells was determined in matched sections of lobule VIII of the cerebellum at 630× . At least 600 cells were counted per section, 2 sections per animal.

The LI of dividing cells in the MED of the cerebellum was determined in matched midsagittal sections where the deep cerebellar nuclei were observed. At least 400 cells were counted from 8 or more sections per animal. Dividing cells were considered to be related to the ontogeny of astrocytes and oligodendrocytes; deep cerebellar neurons and endothelial cells were excluded.

Chemicals

The following compounds were obtained from the indicated sources: [Met^5]enkephalin, [Met^5,Arg^6,Phe^7]enkephalin, [Met^5, Arg^6,Gly^7,Leu^8]enkephalin, [Leu^5]enkephalin, [Met^5(O)]enkephalin (sulfoxide), [Des-Tyr^1,Met^5]enkephalin, Dynorphin A(1–13) (porcine), Sigma (St. Louis, MO); β-funaltrexamine (β-FNA), Research Biochemicals (Wayland, MA); morphine sulfate, Mallinkrodt (St. Louis, MO); U50,488, Upjohn Diagnostics (Kalamazoo, MI); [D-Ala^2,MePhe^4,Glyol^5]enkephalin (DAGO), peptide F, Peninsula (Belmont, CA); ICI 174,864, Cambridge Research Biochemicals (Valley Stream, NY); ethylketocyclazocine (EKC), Sterling Winthrop (Rensselaer, NY), levorphanol tartrate, Hoffmann-La Roche (Nutley, NJ); (—)naloxone hydrochloride, heroin, SKF-10,047, National Institute on Drug Abuse (Rockville, MD); [D-Ala^2, D-Leu^5]enkephalin (DADLE), [D-Pen^{2,5}]enkephalin (DPDPE), β-endorphin (β-lipotropin(61–91), human), [Des-Met^5]enkephalin (β-lipotropin(61–64)), BAM12P (bovine), Bachem (Torrence, CA).

Statistical analysis

The three experiments were analyzed independently. Labeling indexes for each treatment were analyzed using analysis of variance with ANOVA software adapted for the Apple IIe computer. Subsequent planned comparisons were performed using the Newman–Keuls test.

TABLE I

Opioid peptides and analogs influencing the labeling index of EGL cells in the 6-day-old rat cerebellum

Values represent means ± S.E.M.

Compound	*100 µg/kg*	*1 mg/kg*	*10 mg/kg*
[Met^5]Enkephalin	20.9 ± 0.8**	22.1 ± 0.4**	22.3 ± 0.3**
[Des-Met^5]Enkephalin	23.6 ± 0.9**	23.8 ± 0.7**	25.5 ± 0.2**
Peptide F	24.2 ± 0.6*	24.9 ± 0.6**	25.4 ± 0.2**
BAM12P	27.7 ± 0.4	27.1 ± 0.3	26.3 ± 0.4*
Control	27.4 ± 0.4	28.9 ± 0.4	29.8 ± 0.4

Significantly different from controls at *$P < 0.05$ or **$P < 0.01$.

RESULTS

The effect of various opioids on the proliferation of EGL cells in 6-day-old rat cerebellum is given in Tables I and II. At drug concentrations as little as 100 μg/kg both [Met^5]enkephalin and peptide F had a profound action on DNA synthesis, whereas at 10 mg/kg BAM12P also depressed cell replication. The synthetic opioid, [Des-Met^5]enkephalin, was found to have an inhibitory action on the LI with all 3 drug concentrations. No marked differences in the magnitude of inhibitory effects of [Met^5]enkephalin or peptide F over a 100-fold range were detected. Thus, [Met^5]enkephalin depressed the LI by 23.5 to 25.2% when concentrations of 100 μg/kg, 1 mg/kg, and 10 mg/kg were employed. A wide variety of opioids (synthetic and natural, exogenous and endogenous), some extremely selective for certain opioid receptors, had no effect on cell proliferation at concentrations as high as 10 mg/kg (Table II).

The effect of opioids on the replication of dividing cells in the MED of the 6-day-old rat cerebellum revealed an inhibitory action of some opioids (Table III), as well as

TABLE II

Opioid compounds having no effect on cell proliferation in the EGL or MED of the 6-day-old rat cerebellum at drug concentrations up to 10 mg/kg

When possible, the compounds have been arranged with regard to receptor selectivity but this does not indicate exclusivity.

µ-receptor	*ε-receptor*
β-FNA	β-Endorphin
DAGO	*σ-receptor*
Morphine	SKF-10,047
δ-receptor	*Others*
DADLE	Heroin
DPDPE	Levorphanol
ICI 174,864	[Met^5,Arg^6,Phe^7] Enkephalin
κ-receptor	[Met^5,Arg^6,Gly^7,Leu^8]Enkephalin
Dynorphin A(1–13)	[Des-Tyr^1,Met^5]Enkephalin
U50,488	[Met^5(O)]Enkephalin
EKC	[Leu^5]Enkephalin

TABLE III

Opioid peptides and analogs influencing the labeling index of cells in the MED of the 6-day-old rat cerebellum

Values represent means ± S.E.M.

Compound	*100 μg/kg*	*1 mg/kg*	*10 mg/kg*
[Met^5]Enkephalin	8.4 ± 0.2**	7.9 ± 0.1**	8.1 ± 0.1**
[Des-Met^5]Enkephalin	10.8 ± 1.3*	6.6 ± 1.7**	7.9 ± 1.2**
Peptide F	8.4 ± 0.9**	6.9 ± 0.8**	8.4 ± 1.4**
BAM12P	13.1 ± 0.6	10.5 ± 0.4**	9.5 ± 1.4**
Control	14.8 ± 0.9	14.8 ± 1.3	12.9 ± 1.5

Significantly different from controls at $*P < 0.05$ or $**P < 0.01$.

a profile of growth-selective opioids (Tables II and III), similar to that recorded with EGL cells (Tables I and II). Both [Met^5]enkephalin and peptide F were extremely potent inhibitory agents with respect to the proliferation of glial precursors, depressing the LI by 43% of control values at drug concentrations as little as 100 μg/kg. No prominent differences in the magnitude of inhibitory action of [Met^5]enkephalin or peptide F were recorded within a 100-fold concentration range. BAM12P was noted to influence the LI at concentrations greater than 1 mg/kg. A variety of opioid peptides, some selective for other opioid receptor types, had no effect on cell proliferation at concentrations as high as 10 mg/kg (Table II).

In order to evaluate the temporal changes occurring with opioid inhibition of cell proliferation, the effects of [Met^5]enkephalin on the replication of EGL cells were monitored over a 24-h period. Administration of 100 μg/kg [Met^5]enkephalin had no influence on the LI of EGL cells within 1 h of injection, but a marked depression in the LI of this germinative matrix was noted beginning at 2 h and extending to 24 h postinjection (Fig. 1). At 2 h, [Met^5]enkephalin treated animals exhibited decreases in the LI of 21.3% from control values, and at 3 h postinjection the opioid group was 15.5% less than controls; even at 24 h following drug administration, the LI of EGL cells of opioid-treated rats was decreased 24.5% from control values. The LI analyzed across time (2-factor, Time × Treatment, analysis of variance) did not reveal any significant differences for the [Met^5]-enkephalin-exposed animals. Control rats did have sig-

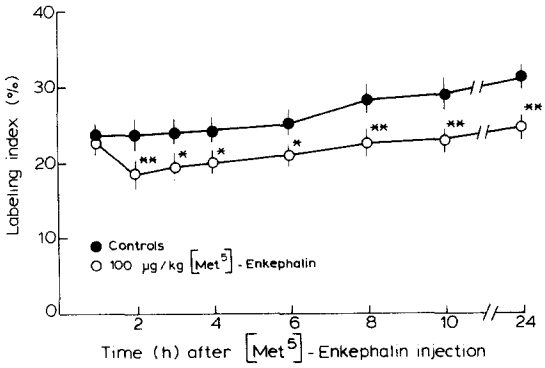

Fig. 1. The effect of [Met^5]enkephalin on the number of radiolabeled cells (labeling index) in the EGL of the developing cerebellar cortex of 6-day-old rats as a function of time following injection of the peptide. Rats were injected (i.p.) with 100 μg/kg [Met^5]-enkephalin and given an injection of [^{3}H]thymidine 30 min before harvesting tissue. Controls were injected with sterile water. Bars = S.E.M. Significantly different from controls at $*P < 0.05$ or $**P < 0.01$.

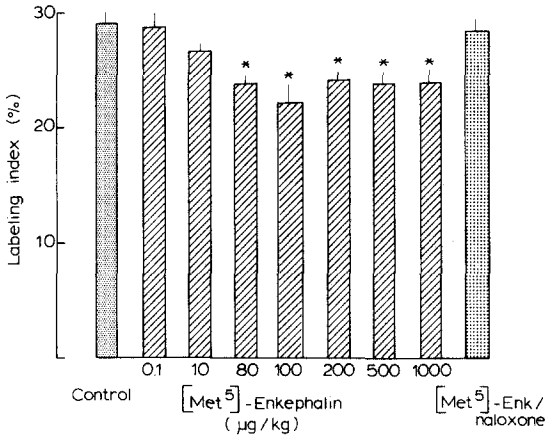

Fig. 2. The effects of [Met^5]enkephalin on the number of radiolabeled cells (labeling index) in the EGL of the developing cerebellar cortex of 6-day-old rats as a function of drug concentration. Rats were injected (i.p.) with various concentrations of [Met^5]enkephalin and given an injection of [^{3}H]thymidine 30 min before sacrifice. Tissues were harvested at 4 h following injection of the opioid peptide. Controls received sterile water. Bars = S.E.M. Significantly different from controls at $*P < 0.05$.

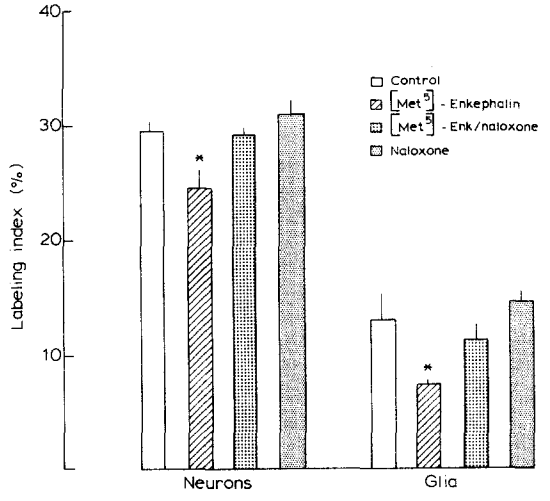

Fig. 3. The effects of [Met^5]enkephalin (0.1 μg/kg) or [Met^5]-enkephalin (0.1 μg/kg) and naloxone (1 mg/kg) on the labeling index (LI) of neural cells in the EGL and MED of the 6-day-old rat cerebellum. Rats were injected (i.p.) with drugs and given an injection of [^{3}H]thymidine 30 min before sacrifice. Tissues were harvested at 4 h following injection of the opioid. Controls received sterile water. Bars = S.E.M. Significantly different from controls at $*P < 0.05$.

nificantly elevated LI's at 8 and 24 h relative to their 1 h time point; the 2, 3, 4, and 6 h time points were comparable to values at 1 h.

Analysis of drug concentrations that altered growth was conducted with [Met^5]enkephalin and the EGL of the developing rat cerebellum (Fig. 2). Dosages of 0.1 and 10 μg/kg had no effect on cell replication. However, concentrations above 80 μg/kg sigificantly depressed the LI of EGL cells. There was no marked difference in the magnitude of effects on cell replication over a 12.5-fold range of [Met^5]enkephalin concentrations (i.e. 80 to 1000 μg/kg).

To ascertain whether opioid action on cell proliferation was mediated by opioid receptors, the ability of an opioid antagonist to block opioid agonist action was examined (Fig. 3). Assessment of EGL cells and MED cells in the 6-day-old rat cerebellum revealed that the inhibitory influence of [Met^5]enkephalin (0.1 mg/kg) on cell proliferation was blocked by concomitant administration of the opioid antagonist, naloxone (1 mg/kg). Naloxone (1 mg/kg) alone had no effect on neurogenesis or gliogenesis in the rat cerebellum (Fig. 3).

DISCUSSION

The present results demonstrate, through the use of structure-activity studies, that some opioids can profoundly influence the proliferation of both neuronal and glial precursors in the developing brain. The data identify three opioid peptides: [Met^5]enkephalin, peptide F, and BAM12P, as potent inhibitors in regard to neural cell replication. Both [Met^5]enkephalin and peptide F were effective in influencing neurogenesis and gliogenesis at concentrations as low as 100 μg/kg. Experiments with a wide variety of opioid peptides, natural and synthetic, endogenous and exogenous, showed the exquisite selectivity of compounds such as [Met^5]enkephalin on cell replication. For example, deletion of the Tyr^1 at the N-terminus and extension of the C-terminus (with the exception of peptide F and BAM12P) eliminated growth-inhibitory activity. Interestingly, the tetrapeptide [Des-Met^5]enkephalin altered cell replication. However, this flexibility was extremely limited, since [Leu^5]enkephalin and [Met^5(O)]enkephalin did not influence cell proliferation. Further studies with [Met^5]enkephalin revealed that the effects on cell replication were dependent on dosage, with concentrations of 80 μg/kg and greater required to alter proliferation. However, no differences in DNA synthesis were noted at concentrations of [Met^5]enkephalin ranging from 80 to 1000 μg/kg. Temporal studies with [Met^5]enkephalin demonstrated that the effects on DNA synthesis initially appeared at 2 h, with optimal effects noted 3 h following drug administration. The effect of [Met^5]enkephalin on cell replication was observed at least 24 h later. No difference in the magnitude of peptide action on DNA synthesis could be discerned from 3 to 24 h. Finally, these data show that the regulation of neural cell proliferation by opioid peptides is related to mechanisms involving the opioid receptor, since the opioid antagonist, naloxone, blocked the effects of opioid agonists such as [Met^5]enkephalin. However, naloxone alone at a concentration of 1 mg/kg had no effect on the genesis of neurons and glia.

The three opioid peptides, [Met^5]enkephalin, peptide F, and BAM12P, modulating cell replication are derived from proenkephalin A[1,2,10]. Complete proteolytic processing of proenkephalin is known to yield [Met^5]-enkephalin, [Leu^5]enkephalin, [Met^5,Arg^6,Phe^7]enkephalin, and [Met^5,Arg^6,Gly^7,Leu^8]enkephalin[1,2,10]. Our data reveal that of these peptides only [Met^5]-enkephalin influenced mitogenesis, demonstrating the exquisite functional selectivity of this opioid. Larger enkephalin-containing polypeptides of proenkephalin such as peptide F and BAM12P, which also alter cell proliferation, have been identified as intermediates in the biosynthesis of enkephalins[7,9]. Whether peptide F and BAM12P remain intact to influence growth, or if these neuropeptides undergo further proteolytic cleavage (at double basic amino acid residues present in both fragments) to yield [Met^5]enkephalin needs clarification. However, in tissue culture studies using neural tumor cells [Met^5]enkephalin, but neither peptide F nor BAM12P, influenced cell proliferation[29]. These results may suggest that systemic application of peptide F and BAM12P in the present study could liberate [Met^5]-enkephalin as a result of proteolysis, and that this pentapeptide serves as the principal regulator of neuronal and glial cell proliferation.

The present results confirm and extend previous evidence[8,11,12,28] suggesting that opioid systems modulate cell replication in the developing nervous system, as well as in neural tumor tissue and cells[29,30], and serve as the first identification of the opioid peptides governing neural ontogenetic events. Studies on the temporal appearance and distribution of [Met^5]enkephalin during neural development are also consistent with the role of this neuropeptide as a growth modulator. [Met^5]enkephalin-like immunoreactivity has been detected by light[5,6,31,32] and electron microscopy[31], and radioimmunoassay[15] in developing neural tissues. For example, this opioid peptide is found in proliferating and differentiating cells of the cerebellum[31,32] and retina[5,6] during the developmental period, but not in adulthood. This peptide is specifically localized in replicating and maturing neural cells, being present in the cortical cytoplasm as a mesh-work throughout the cell but not in the cell nucleus.

The source of this opioid peptide in the cerebellum, as well as the retina, needs to be elucidated. Using cDNA probes to preproenkephalin (PPE) we have found in preliminary studies that mRNA PPE is expressed in the developing cerebellum (e.g. day 6) but not in adult cerebellum using Northern analysis (Zagon, McLaughlin and LaGamma, in preparation). Although Golgi type II cells have been found to express enkephalin in the adult cerebellum, our data may suggest a transient expression of enkephalin peptides during development of the cerebellum presumably associated with replicating cells; in situ hybridization studies will be necessary to clarify whether proliferating and differentiating neural cells produce this peptide.

The present data, along with previous information[28], provide some valuable insights into the regulation and plasticity of cell proliferation during neural ontogeny. We know from experiments using a paradigm of continuous opioid receptor blockade that the 'full capacity' of proliferative neural cells such as those in the EGL extends to approximately 25% beyond control values. As to lower limits, the present data demonstrate that exposure to an extremely low concentration (i.e. 100 μg/kg) of a potent growth-related opioid peptide such as [Met^5]enkephalin can depress cell division by 25%; proliferating glial cells appear to be even somewhat more sensitive to [Met^5]enkephalin regulation, with cell replication being reduced by as much as 40%. Although opioids such as [Met^5]enkephalin have a half-life in plasma of about 2 min[3], acute exposure to this compound obviously has a marked and long-lasting action on cell proliferation. Whether continuous exposure (e.g. delivery by osmotic minipumps) would completely depress the genesis of all neurons and glia needs to be addressed in future studies. Thus, it appears that cell proliferation in the developing nervous system has the capability of increasing or decreasing by at least 25%; presumably this plasticity plays a role in the ability of the developing nervous system to compensate for insults or other irregularities in regulatory capabilities. Finally, with regard to manipulation of the endogenous opioid systems during development (i.e. using the opioid antagonist paradigm described earlier), we know that modulation of opioids and receptors during this critical period has a notable influence on the number, structure, and biochemistry of brain cells, as well as on neurobehavioral ontogeny[4–6,11,16,22–28]. However, studies in our laboratory suggest that animals exposed to potent opioid antagonists early in life have brains similar to controls when examined at adulthood (Zagon and McLaughlin, unpublished observations). Therefore, although obviously very important, the regulation of brain development by endogenous opioid systems is only one of many factors (e.g. cell death, selectivity of targets, synapse and process elimination) involved in the orchestration of neural ontogeny.

Evidence gathered in this and other studies[6,13–15,28] suggest that the effects of [Met^5]enkephalin on cell proliferation are mediated by opioid receptors. Using radiolabeled [Met^5]enkephalin and binding assays, a specific, saturable, and high-affinity opioid receptor termed zeta (ζ) has been discovered to be associated with the growth of normal[17,19] and abnormal[18,20,21] neural cells. In tissue culture models of murine neural cells[29], the median effective concentration of [Met^5]enkephalin found to depress cell proliferation was 10^{-10} M and the binding affinity (K_d) was 1.6 nM, indicating that the K_d and EC_{50} in tissue culture cells were similar[21,29]. Studies[17] using homogenates of the developing rat cerebellum in binding assays with radiolabeled [Met^5]enkephalin also reveal the presence of the ζ receptor[17]. It is interesting to note that the K_d of this receptor is approximately 2.0 nM and the binding capacity (B_{max}) is about 23 fmol/mg protein[17]. Although difficult to extrapolate a nM drug concentration from in vivo experiments, our findings showed that extraordinarily low (80 μg/kg) peptide concentrations had a marked effect on cell replication. Thus, physiologically relevant concentrations of peptide appear to modulate developmental events.

A critical question raised by these studies is whether [Met^5]enkephalin is important in human development. Tissue culture studies show that this opioid peptide can depress the growth of a variety of neural and non-neural cell types of human origin[29], and that enkephalin immunoreactivity is associated with proliferating cells of the developing human cerebellum (Zagon and McLaughlin, unpublished observations). Using radiolabeled [Met^5]-enkephalin, we[19] have discovered that the ζ-receptor is in abundance in the human cerebellum during the first postnatal weeks, but is not detected in adulthood. These results suggest that, as in the development of the nervous system of animals, human neural ontogeny with respect to growth is also governed by endogenous opioid systems. The implications of these findings with regard to developmental problems of the human nervous system are important. Evidence presented earlier concerning perturbations of endogenous opioid systems during animal development and resultant repercussions has already been discussed and may prove relevant to the human condition. It is interesting to note that expression of the ζ-receptor has been discovered in the cerebellum of a human adult male with metastatic adenocarcinoma[18], but not in the cerebellum of normal subjects . Thus, diseases of the nervous system that involve cell proliferation appear to be associated with the re-expression of a growth-related opioid receptor normally found only

during the developmental period. This remarkable finding emphasizes the importance of further exploration of endogenous opioid systems and normal and abnormal development.

Acknowlegements. This work was supported by NIH Grant NS-20500. We thank Kristen Cook for technical support throughout the course of this project.

REFERENCES

1 Comb, M., Seeburg, P., Adelman, J., Eiden, L. and Herbert, E., Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA, *Nature,* 295 (1982) 663–666.
2 Gubler, U., Seeburg, P., Hoffman, B.J., Gage, L.P. and Udenfriend, S., Molecular cloning establishes proenkephalin as precursor of enkephalin-containing peptides, *Nature,* 295 (1982) 206–209.
3 Hambrook, J.M., Morgan, B.A., Rance, M.J. and Smith, F.C., Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates, *Nature,* 262 (1976) 782–783.
4 Hauser, K.F., McLaughlin, P.J. and Zagon, I.S., Endogenous opioid systems and the regulation of dendritic growth and spine formation, *J. Comp. Neurol.,* 281 (1989) 13–22.
5 Isayama, T., McLaughlin, P.J. and Zagon, I.S., Opioid antagonist regulation of cell proliferation during retinal development in the rat, *FASEB J.,* 4 (1990) A1001.
6 Isayama, T., McLaughlin, P.J. and Zagon, I.S., Endogenous opioids regulate cell proliferation in the retina of developing rat, *Brain Research,* in press.
7 Jones, B.N., Stern, A.S., Lewis, R.V., Kimura, S., Stein, S., Udenfriend, S. and Shively, J.E., Structure of two adrenal polypeptides containing multiple enkephalin sequences, *Arch. Biochem. Biophys.,* 204 (1980) 392–395.
8 Kornblum, H.I., Loughlin, S.E. and Leslie, F.M., Effects of morphine on DNA synthesis in neonatal rat brain, *Dev. Brain Res.,* 31 (1987) 45–52.
9 Mizuno, K., Minamino, N., Kangana, K. and Matsuo, H., A new endogenous opioid peptide from bovine adrenal medulla: isolation and amino acid sequence of a dodecapeptide (BAM-12P), *Biochem. Biophys. Res. Comm.,* 95 (1980) 1482–1488.
10 Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. and Numa, S., Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin, *Nature,* 295 (1982) 202–206.
11 Schmal, W., Funk, R., Miaskowski, U. and Plendl, J., Long-lasting effects of naltrexone, an opioid receptor antagonist, on cell proliferation in developing rat forebrain, *Brain Research,* 486 (1989) 297–300.
12 Stiene-Martin, A. and Hauser, K.F., Opioid-dependent growth of glial cultures: suppression of astrocyte DNA synthesis by Met-enkephalin, *Life Sci.,* 46 (1990) 91–98.
13 Tsang, D. and Ng, S.C., Effect of antenatal exposure to opiates on the development of opiate receptors in rat brain, *Brain Research,* 188 (1980) 199–206.
14 Tsang, D., Ng, S.C. and Ho, K.P., Development of methionine-enkephalin and naloxone binding sites in regions of rat brain, *Dev. Brain Res.,* 3 (1982) 637–644.
15 Tsang, D., Ng, S.C., Ho, K.P., and Ho, W.K.K., Ontogenesis of opiate binding sites and radioimmunoassayable β-endorphin and enkephalin in regions of rat brain, *Dev. Brain Res.,* 5 (1982) 257–261.
16 Vertes, Z., Melegh, G., Vertes, M. and Kovacs, S., Effect of naloxone and D-Met^2-pro^5-enkephalinamide treatment on the DNA synthesis in the developing rat brain, *Life Sci.,* 31 (1982) 119–126.
17 Zagon, I.S., Gibo, D. and McLaughlin, P.J., Zeta (ζ), a growth-related opioid receptor in developing rat cerebellum identification and characterization, *Brain Research,* in press.
18 Zagon, I.S., Gibo, D. and McLaughlin, P.J., Expression of zeta (ζ), a growth-related opioid receptor, in metastatic adenocarcinoma of the human cerebellum, *J. Natl. Cancer Inst.,* 82 (1990) 325–327.
19 Zagon, I.S., Gibo, D. and McLaughlin, P.J., Adult and developing human cerebella exhibit different profiles of opioid binding sites, *Brain Research,* 523 (1990) 62–68.
20 Zagon, I.S., Goodman, S.R. and McLaughlin, P.J., Characterization of zeta (ζ): a new opioid receptor involved in growth, *Brain Research,* 482 (1989) 297–305.
21 Zagon, I.S., Goodman, S.R. and McLaughlin, P.J., Demonstration and characterization of zeta (ζ), a growth-related opioid receptor, in a neuroblastoma cell line, *Brain Research,* 511 (1990) 181–186.
22 Zagon, I.S. and McLaughlin, P.J., Increased brain size and cellular content in infant rats treated with an opiate antagonist, *Science,* 221 (1983) 1179–1180.
23 Zagon, I.S. and McLaughlin, P.J., Naloxone modulates growth in infant rats, *Life Sci.,* 33 (1983) 2449–2454.
24 Zagon, I.S. and McLaughlin, P.J., Naltrexone modulates body and brain development in rats: a role for endogenous opioids in growth, *Life Sci.,* 35 (1984) 2057–2064.
25 Zagon, I.S. and McLaughlin, P.J., Naltrexone's influence on neurobehavioral development, *Pharmacol. Biochem. Behav.,* 22 (1985) 441–448.
26 Zagon, I.S. and McLaughlin, P.J., Opioid antagonist-induced modulation of cerebral and hippocampal development: histological and morphometric studies, *Dev. Brain Res.,* 28 (1986) 233–246.
27 Zagon, I.S. and McLaughlin, P.J., Opioid antagonist (naltrexone) modulation of cerebellar development: histological and morphometric studies, *J. Neurosci.* 6 (1986) 1424–1432.
28 Zagon, I.S. and McLaughlin, P.J., Endogenous opioid systems regulate cell proliferation in the developing rat brain, *Brain Research,* 412 (1987) 68–72.
29 Zagon, I.S. and McLaughlin, P.J., Endogenous opioid systems regulate growth of neural tumor cells in culture, *Brain Research,* 490 (1989) 14–25.
30 Zagon, I.S. and McLaughlin, P.J., Opioid antagonist modulation of murine neuroblastoma: a profile of cell proliferation and opioid peptides and receptors, *Brain Research,* 482 (1989) 16–28.
31 Zagon, I.S. and McLaughlin, P.J., Ultrastructural localization of enkephalin-like immunoreactivity in developing rat cerebellum, *Neuroscience,* 34 (1990) 479–489.
32 Zagon, I.S., Rhodes, R.E. and McLaughlin, P.J., Localization of enkephalin immunoreactivity in germinative cells of developing rat cerebellum, *Science,* 227 (1985) 1049–1051.