Brain Research, 412 (1987) 68–72
Elsevier

BRE 12582

Endogenous opioid systems regulate cell proliferation in the developing rat brain

Ian S. Zagon and Patricia J. McLaughlin

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

(Accepted 14 October 1986)

Key words: Endogenous opioid; Opioid receptor; Cerebellum; Naltrexone; Methionine-enkephalin; Growth; Autoradiography; Cell proliferation

The role of endogenous opioid systems in modulating the proliferation of developing cerebellar cells was examined autoradiographically in 6-day-old rats. The blockade of endogenous opioid–opioid receptor interaction by naltrexone, a potent opioid antagonist, was accompanied within 1–2 h by an increased proportion of cells incorporating [^{3}H]thymidine. When high doses of naltrexone (50 mg/kg) were administered this index was still elevated 12 h later; however, when low doses of naltrexone (1 mg/kg) were administered the index of labeled cells was decreased markedly. Injection of methionine-enkephalin, an endogenous opioid peptide, also resulted in a decrease in the proportion of cells incorporating [^{3}H]thymidine. Concomitant injection of 1 mg/kg naloxone, however, blocked the inhibitory effects of methionine-enkephalin on cell division but did not itself affect cell generation. These studies demonstrate that endogenous opioid systems can regulate the proliferation of cell populations in the developing nervous system and do so through an inhibitory mechanism.

INTRODUCTION

The regulation of neurodevelopmental events by endogenous opioid systems (i.e. endogenous opioids and opioid receptors) is an important concept that has emerged during the past few years[18–22,24–26]. Components of the endogenous opioid systems are present in brain tissue during ontogeny, with the highest (and sometimes only) levels of opioid receptor binding and of tissue endorphins occurring during development[6,12–14]. The pivotal role of endogenous opioid systems in developmental neurobiology has been revealed, in large part, by experimental paradigms utilizing opioid antagonists to deliberately interrupt the interaction of endogenous opioids and opioid receptors. Opioid antagonists exert a marked, stereospecific influence on the growth of normal and abnormal neural tissues[23], dependent on the duration of opioid receptor blockade[20], and unrelated to β-funaltrexamine sensitive μ-receptors[26]. Continuous daily blockade of opioid receptors each day enhances growth, whereas intermittent opioid receptor blockade inhibits growth[19–22,24,25]. Recent studies[27] showing that enkephalin immunoreactivity occurs in germinative cells of the developing postnatal rat cerebellum, but not in adult neural cells, further underscores the importance of the special relationship of endogenous opioid systems to development.

Preweaning rats that receive daily injections of naltrexone, a potent opioid antagonist, at a dose (50 mg/kg) that blocks opioid receptors for 24 h each day, have increased body, brain and organ weights[8–20,22] and acceleration in the appearance of physical characteristics and maturation of spontaneous motor and sensorimotor behaviors[19,21]. In contrast, daily administration of 1 mg/kg naltrexone, a dose that blocks the opioid receptor for only 4–6 h each day, inhibited development. The morphological effects involved in opioid-antagonist modulation of neurobiological development have been documented[18, 24,25]. A prominent feature of these studies appears to be changes in cell populations which are mitotic dur-

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

ing postnatal life. For example, the number of internal granule neurons, as well as glial cells, in the cerebellum are increased significantly in 21-day-old rats that had a complete opioid receptor blockade during the preweaning period. These observations would suggest that endogenous opioids influence neuro-ontogeny, serving to inhibit cell proliferation. In the present study we explored this aspect further by examining the actions of opioid antagonists and endogenous opioids on cell division of external germinal cells in the developing rat cerebellum. Our results show that endogenous opioid systems play an important role in regulating cell proliferation in the developing brain.

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 described elsewhere[21].

Experimental protocol

On postnatal day 6, an equal number of males and females were selected at random and distributed into experimental groups. In Expt. I, rats were injected subcutaneously (s.c.) with naltrexone hydrochloride (1 or 50 mg/kg) or sterile water. Thirty minutes prior to sacrifice at 2 or 12 h following naltrexone administration, rats received an injection (intraperitoneal) of [^{3}H]thymidine (10 μCi/g b. wt.; 6.7 Ci/mmol, New England Nuclear, Boston, MA). In Expt. II, rats were injected (s.c.) with naltrexone (1 or 50 mg/kg) or sterile water. Thirty minutes prior to sacrifice at either 1, 2, 3, 4, 8, 10 or 12 h following naltrexone administration, rats received an injection of [^{3}H]thymidine as described earlier. In Expt. III, rats received an s.c. injection of 80 μg/kg methionine-enkephalin (Sigma, St. Louis, MO), 80 μg/kg methionine-enkephalin and 1 mg/kg naloxone hydrochloride (Sigma, St. Louis, MO), 1 mg/kg naloxone hydrochloride or sterile water. Thirty minutes prior to sacrifice at 2 h postdrug injection, rats received an injection of [^{3}H]thymidine as described previously. The dosage of naloxone chosen was capable of blocking opioid receptors (as determined by morphine challenge experiments and measuring nociception) but did not have any affects on cell proliferation (see Results).

Histology and autoradiography

Six to 18 animals at each time point and drug dosage were sacrificed by cardiac perfusion with 10% neutral buffered formalin. Brains were excised, fixed for 3 days, embedded in polyester wax and midsagittal sections (8 μm) collected. Tissue sections were coated with NTB-2 nuclear track emulsion (Kodak, Rochester, NY), exposed at 4 °C for 2 weeks, developed in D-19, and stained with hematoxylin. The labeling index (LI = number of labeled cells/total cells) for external germinal cells (EGL) was determined in the VIIIth lobule of the cerebellum at 630 ×. At least 200 cells were counted per section, 3 sections per animal. The LI was calculated for each section and averaged for the 3 sections per animal. Therefore, a minimum of 3600 cells/treatment/time point were evaluated.

Statistical analysis

Labeling indexes averaged for each animal were analyzed using the ANOVA program adapted for the Apple II computer. Subsequent planned comparisons were performed using Newman–Keuls test.

RESULTS

Two hours following administration of 1 or 50 mg/kg naltrexone, the LI was 38 and 42%, respectively (Fig. 1), and was significantly elevated in comparison to control values (32%). At 12 h, the 50 mg/kg naltrexone group had an LI of 43%; this was statistically different from controls. The 1 mg/kg naltrexone group had a marked reduction ($P < 0.01$) in their LI (18%) at 12 h.

In order to evaluate the extent of the changes encountered with naltrexone, autoradiographic assessment of material collected at various time points after drug injection was performed (Fig. 2). Throughout the 12-h period analyzed, the LI of rats receiving 50 mg/kg naltrexone averaged 39.5 ± 1.0%; this was elevated significantly from control values (mean ± S.E.M. = 31.1 ± 0.3%). The LI of rats in the 1 mg/kg showed a biphasic response over time. At 1 h follow- reduced relative to controls from 4 to 12 h postinjec-

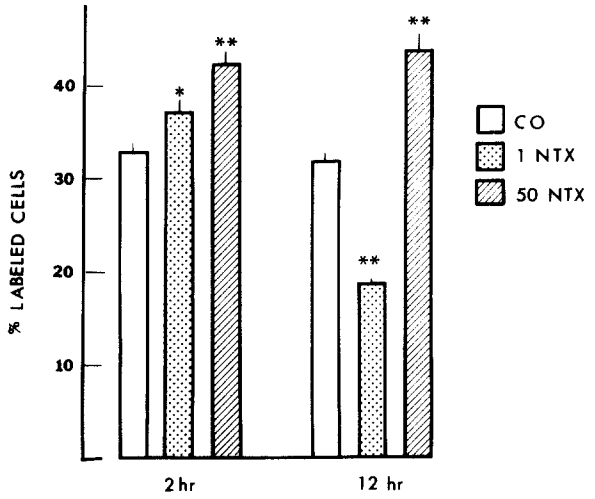

Fig. 1. The percentage of cerebellar external germinal cells incorporating [^{3}H]thymidine in 6-day-old rats injected with sterile water (CO) or naltrexone at dosages of 1 mg/kg (1 NTX) or 50 mg/kg (50 NTX). Bar indicates S.E.M. Significantly different from controls at $P < 0.05$ (*) or $P < 0.01$ (**).

ing drug administration, both the 1 mg/kg naltrexone and control groups were comparable in the number of labeled EGL cells recorded. However, at 2 and 3 h postinjection, the 1 mg/kg naltrexone group exhibited a significant elevation (37.8 ± 0.2%), but was

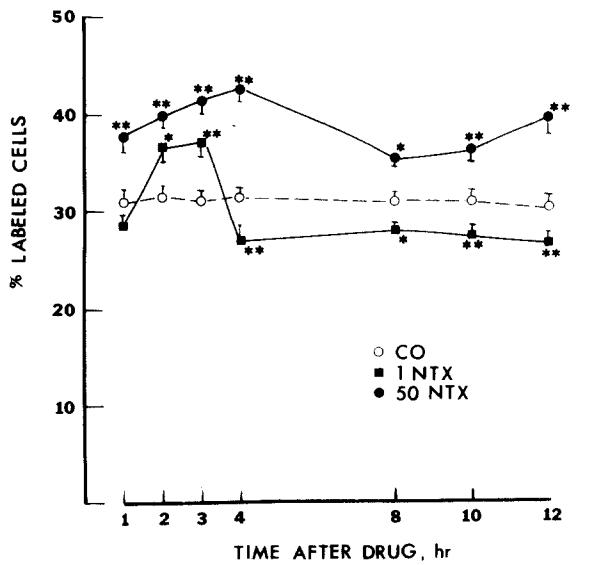

Fig. 2. The percentage of cerebellar external germinal cells incorporating [^{3}H]thymidine in 6-day-old rats injected with sterile water (CO) or naltrexone at dosages of 1 mg/kg (1 NTX) or 50 mg/kg (50 NTX). Bar indicates S.E.M. Significantly different from controls at $P < 0.05$ (*) or $P < 0.01$ (**).

tion (28.3 ± 0.2%). To confirm and extend these observations on the role of endogenous opioid systems in development, a third experiment was designed to ascertain directly the effects of endogenous opioids (i.e. methionine-enkephalin) on the LI of EGL cells. In comparison to control levels of 31.6%, the LI of methionine-enkephalin-injected animals was significantly ($P < 0.01$) subnormal (25.6%). The effects of methionine-enkephalin were blocked by concomitant administration of naloxone. Moreover, at the dosage utilized, naloxone alone did not alter the LI.

DISCUSSION

The results of this study support earlier reports[18–22,24–27] demonstrating the importance of endogenous opioid systems in neurobiological development and provide insight into the mechanisms of cellular action by neuropeptides. Our experiments with opioid antagonists show that a drug regimen (i.e. 50 mg/kg naltrexone) which blocks opioid receptors for the entire day[18,20,22] has a stimulatory influence on the proliferation of cerebellar EGL cells during the blockade. Opioid antagonist administration at a dosage (i.e. 1 mg/kg naltrexone) that only blocks opioid receptors temporarily each day[20,22] has a biphasic effect on cell division. Initially a marked increase in cell proliferation is observed that is consistent with the period of receptor blockade. This is followed by a notable decrease when the blockade is terminated. A detailed study of the temporal relationships involved with opioid antagonist-induced modulation revealed that control of proliferation in developing neural cells relies in part on endogenous opioid systems. Thus, within an hour of perturbing endogenous opioid–opioid receptor interaction by administration of opioid antagonists, marked changes occur in the number of cells incorporating [^{3}H]thymidine. From the time course experiments with opioid antagonists, cell proliferation is dependent on the duration of opioid receptor blockade. During opioid receptor blockade the rate of cell proliferation is higher than normal but, following blockade, cell division is depressed. Thus, multiplication of neural cells appears to be orchestrated carefully by endogenous opioid systems, and the delicate interaction of neuropeptides and receptors establishes the normal pace of cell proliferation. In essence, the opioid peptides accomplish this

modulatory role by serving as inhibitory factors. The decreased cell division subsequent to opioid receptor blockade could well be a heightened expression of this mechanism. Administration of opioid antagonists elevates endogenous opioid levels[8], increases opioid receptor number and produces a supersensitivity to opioids[3]. Therefore, in the present study opioid antagonist-induced increases in opioid receptors on developing cells could be envisioned to interact with basal or even elevated levels of endogenous opioids. This could result in a cellular supersensitivity manifested by a reduced rate of proliferation. In further support for this concept we have found that administration of endogenous opioids depressed incorporation of [^{3}H]thymidine, whereas concomitant injection of an opioid antagonist reversed this effect. These data lend additional support to the notion that the endogenous opioids mediate cell proliferation by way of the opioid receptor.

Earlier work with regenerating and developing tissues identified opioid-induced suppression of cell division[4,5,11,15], but failed to show tonic regulation by endogenous opioids. This could reflect the dilution of a small population of affected cells with a larger population of unaffected cells. The present investigation is the first to examine a specific population of proliferating neural cells and to utilize autoradiography and statistics to document opioid action during development. Autoradiographic procedures permit assessment of changes in a small population of dividing cells that may be undetected by incorporation studies utilizing scintillation counting. Furthermore, autoradiographic procedures circumvent the possibility that a drug may alter the quantity of radiolabeled thymidine incorporated per cell. The use of the opioid antagonist paradigm, in which the interactions of endogenous opioids and opioid receptors are functionally understood, provides a more complete picture of the relationship between opioids and cell proliferation. It appears that both exogenous and endogenous opioids have a similar influence on mitotic events, shaping this process by an inhibitory mechanism. Correlated with these observations, it seems that the timing of the cell cycle is dependent upon endogenous opioid–opioid receptor interactions. Without this interfacing, the rate of cell division is accelerated. Currently, we are identifying which aspect(s) of the cell cycle is(are) influenced by the opioids and how the endogenous opioids and opioid receptors effect their regulatory capabilities.

Given the importance of endogenous opioid systems in determining cell proliferative activities in the developing nervous system, it would be of considerable interest to examine the repercussions when this control goes askew. For example, one would predict that if growth-related opioids are present in abundance, cell production might be altered in such a way as to retard neuro-ontogeny and quite possibly lead to permanent reductions in neural cells. In fact, studies in which animals were exposed to exogenous opioids such as methadone and morphine in early life demonstrate perturbations in cellular content[3,7,16,17] and polyamine levels[2,9,20]. Alternatively, if growth-related opioids were in subnormal quantities, cell proliferative activities might be enhanced. Evidence in which opioid antagonists are administered during crucial stages of neural development shows that continual interference of endogenous opioids and opioid receptors results in a remarkable increase in those neural cells which replicate during the time of administration. Of course other scenarios could be postulated and require study. Certainly of paramount concern, is whether disturbances in endogenous opioid systems may be involved with neurobiological abnormalities occurring in humans? This question is deserving of further consideration by clinical and basic science research.

ACKNOWLEDGEMENTS

This work was supported in part by NIH Grants NS-20500 and NS-20623. We thank Ms. Doris Lineweaver for assistance in manuscript preparation.

REFERENCES

1 Bardo, M.T., Bhatnagar, R.K. and Gebhart, G.F., Age-related differences in the effect of chronic administration of naloxone on opiate binding in rat brain, *Neuropharmacology*, 22 (1983) 453–461.

2 Butler, S.R. and Schanberg, S.M., Effect of maternal morphine administration on neonatal rat brain ornithine decarboxylase (ODC), *Biochem. Pharmacol.*, 24 (1975) 1915–1918.

3 Ford, D.H. and Rhines, R.K., Prenatal exposure to methadone HCl in relationship to body and brain growth in the

rat, *Acta Neurol. Scand.*, 59 (1979) 248–262.
4 Hui, F.W., Krikun, E., Hirsh, E.M., Blaiklock, R.G. and Smith, A.A., Inhibition of nucleic acid synthesis in the regenerating limb of salamanders treated with a DL-methadone or narcotic antagonists, *Exp. Neurol.*, 53 (1976) 267–273.
5 Kornblum, H.I., Loughlin, S. and Leslie, F.M., Morphine effects on [^{3}H]thymidine incorporation into neonatal rat brain DNA, *Soc. Neurosci.*, 11 (1985) 555.
6 Maseda, C., Aguado, E.G., Mena, M.A. and De Yevenes, J.G., Ontogenetic development of β-endorphin immunoreactivity in rat brain regions, *Neurosci. Lett.*, Suppl., 14 (1983) S237.
7 Peters, M.A., The effect of maternally administered methadone on brain development in the offspring, *J. Pharmacol. Exp. Ther.*, 203 (1977) 340–346.
8 Recant, L., Voyles, N.R., Luciano, M. and Pert, C.B., Naltrexone reduces weight gain, alters 'β-endorphin', and reduces insulin output from pancreatic islets of genetically obese mice, *Peptides*, 1 (1980) 309–313.
9 Slotkin, T.A., Lau, C. and Bartolome, M., Effects of neonatal or maternal methadone administration on ornithine decarboxylase activity in brain and heart of developing rats, *J. Pharmacol. Exp. Ther.*, 199 (1976) 141–148.
10 Slotkin, T.A., Seidler, F.J. and Whitmore, W.L., Effects of maternal methadone administration on ornithine decarboxylase in brain and heart of the offspring: relationship of enzyme activity to dose and to growth impairment in the rat, *Life Sci.*, 26 (1980) 861–867.
11 Smith, A.A. and Hui, F.W., Inhibition of neurotrophic activity in salamanders treated with opioids, *Exp. Neurol.*, 39 (1973) 36–43.
12 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.
13 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.
14 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.
15 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.
16 Zagon, I.S. and McLaughlin, P.J., Perinatal methadone exposure and brain development: a biochemical study, *J. Neurochem.*, 31 (1978) 49–54.
17 Zagon, I.S. and McLaughlin, P.J., Neuronal cell deficits following maternal exposure to methadone in rats, *Experientia*, 38 (1982) 1214–1216.
18 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.
19 Zagon, I.S. and McLaughlin, P.J., Naloxone modulates growth in infant rats, *Life Sci.*, 33 (1983) 2449–2454.
20 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.
21 Zagon, I.S. and McLaughlin, P.J., Naltrexone's influence on neurobehavioral development, *Pharmacol. Biochem. Behav.*, 22 (1985) 441–448.
22 Zagon, I.S. and McLaughlin, P.J., Opioid antagonist-induced regulation of organ development, *Physiol. Behav.*, 34 (1985) 507–511.
23 Zagon, I.S. and McLaughlin, P.J., Stereospecific modulation of tumorigenic response by opioid antagonists, *Eur. J. Pharmacol.*, 113 (1985) 115–120.
24 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.
25 Zagon, I.S. and McLaughlin, P.J., Opioid antagonist (naltrexone) modulation of cerebellar development: histological and morphometric studies, *J. Neurosci.*, 6 (1986) 1424–1432.
26 Zagon, I.S. and McLaughlin, P.J., β-Funaltrexamine (β-FNA) and the regulation of body and brain development in rats, *Brain Res. Bull.*, 17 (1986) 5–9.
27 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.