Expression of \( \mu \)-Opioid receptors in developing rat spinal cord: An autoradiographic study

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Received 29 May 2003; revised 24 February 2004

The expression of \( \mu \)-opioid receptors in the developing rat spinal cord (Postnatal days 7, 14, 30) was studied by autoradiography using \([^{1}H]\)DAMGO. When compared to camera lucida drawings, the receptor was noted over the entire gray matter and dorsal root ganglia at postnatal days 7 and 14. At postnatal day 30, the receptor expression decreased over the gray matter except the superficial laminae (laminae I and II). At all age groups studied, a higher expression of the receptor was noted over the superficial laminae. The study shows that \( \mu \)-opioid receptors appears early in postnatal development and attains mature receptor distribution relatively late in ontogeny, suggesting a possible role in the normal development of nervous system. This is affirmed by an impairment of psychomotor development in babies born to mothers, addicted to opiates.

**Keywords**: Ontogeny, \( \mu \)-receptor, Spinal cord, \([^{1}H]\)DAMGO

The endogenous opioid system (endogenous opioid peptides like enkephalins, endorphins and dynorphins and opioid receptors like \( \mu \), \( \delta \) and \( \kappa \)) has been proposed to play a role in the normal development of the nervous system\(^{1,2}\). Exogenously administered opiates like morphine have been used for centuries to relieve pain. In utero exposure to morphine, which binds to \( \mu \)-opioid receptors, leads to significantly decreased neuronal cell counts and cell density in the brain\(^{3}\). More specifically, morphine has been shown to directly inhibit DNA synthesis in dividing neurons\(^{4}\). [Met\(^{5}\)]enkephalin, an endogenous opioid peptide derived from the polyprotein precursor Proenkephalin, has been reported to prevent proliferation and migration of cells in the developing nervous system\(^{5}\). Also, the appearance of proenkephalin mRNA and enkephalin in the external granular and Purkinje cell layers of the cerebellum, coincides with their maturation\(^{6}\).

Among the classical opioid receptors, \( \mu \)- and \( \kappa \)-opioid receptors appear earlier in the developing brain of the rat\(^{7}\) and human\(^{8}\) as compared to \( \delta \)-receptors\(^{9}\). In rat, \( \mu \)- and \( \kappa \)-opioid receptors are expressed from mid-gestation onwards, a period corresponding with an important stage in brain development\(^{10}\). The importance of this period stems from the observation that peak neurogenesis in the striatum occurs during the 14\(^{th}\) and 15\(^{th}\) day of gestation. Interestingly, activation of \( \mu \)- and \( \kappa \)-opioid receptors in the ventricular zone of embryonic neocortex delays progression of cell cycle in contrast to \( \delta \)-receptors, which accelerate it\(^{11}\). Continuous blockade of opioid receptors by naltrexone during gestation leads to increased body weight at birth and an earlier development of behavioural milestones in rat\(^{12}\). Other abnormalities noted were hypolocomotion and a decreased frequency of rearing, grooming and wet-dog shakes. Besides, increased dendritic and/or spine elaboration in pyramidal cells from both the frontoparietal cortex and CA1 region of hippocampus and Purkinje cells from cerebellum were also noted in the immediate postnatal period\(^{13}\). In the adult human cerebellum, opioid receptors (\( \mu \), \( \delta \) and \( \kappa \)) are expressed at much lower levels as compared to newborns\(^{14}\). However, its association with neurogenesis, if any, in the adult cerebellum, is not known. Glia too expresses \( \mu \)-opioid receptors during development and can be affected by opiate administration\(^{15}\).

In humans, prenatal exposure to opiates, leads to low birth weight babies, an increase in congenital malformations and a lower 5-min Apgar score\(^{16}\).
Additionally, there is impairment in the development of psychomotor skills as measured by the Griffiths developmental quotient (DQ) at one year of age. Autoradiographic studies are there on the ontogeny of opioid receptors in the spinal cord of the rat. However, ligands, specific for individual receptor subtypes—\( \mu \), \( \delta \), and \( \kappa \)—were used in only one study, which investigated the lumbar region of the spinal cord. It is possible that the development of opioid receptors vary in different parts of the spinal cord. Maturation in the spinal cord is supposed to be progressive and follows a rostro-caudal direction. Thus, it was presumed that the cervical region would be the first to mature. The present report is on the ontogeny of \( \mu \)-opioid receptor in the cervical region of the spinal cord using \([\text{H}]\text{D-ala}2,\text{N-methyl-phe}4,\text{-glyco}5\) enkephalin (DAMGO), a highly specific ligand for labeling \( \mu \)-receptor. We also observed expression of the receptor in the dorsal root ganglia corresponding with that in the spinal cord, not reported by the previous studies. Some of the age-groups studied (P7, P14, P30; P1 was reported earlier) were also different. The results are correlated with the development of \( \mu \)-receptor in the human fetal spinal cord, noted earlier.

A preliminary report on the present study was earlier presented in abstract form.

**Materials and Methods**

Albino wistar rat pups (n=5 for each age group) were sacrificed with an overdose of diethyl ether at postnatal days (P) 7, 14 and 30. The day of birth was taken as P 0 and the next day as P 1. The pups were separated from their mothers at the time of experiment. The cervical region of the vertebral column with the spinal cord within it, was removed and frozen in liquid nitrogen before being stored at -20°C. This was done to obtain the dorsal root ganglia along with the spinal cord. Cryostat sections (20 \( \mu \)m thickness) of the spinal cord were cut at -18°C and collected on subbed slides.

The autoradiographic procedure for labeling \( \mu \)-opioid receptor was standardized earlier. The sections were initially preincubated in 50 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl, 1mM EGTA and BSA (1 mg/ml) for 15 min. Later, the sections were incubated in the same buffer containing 2nM tritium labeled [D-ala2,N-methyl-phe4,-glyco5] enkephalin (DAMGO) and BSA (1 mg/ml) for 1 hr at room temperature. The sections were washed in Tris-HCl buffer at 4°C, twice for 1 min each, before being rapidly dried. Then, the sections were exposed to \([\text{H}]\) sensitive hyperfilms for 8 weeks at about 4°C. Non-specific binding was determined under the same experimental conditions using 1000 fold excess levorphanol (2\( \mu \)M) and the cryostat sections exposed to the autoradiographic film. At the end of this period, the films were developed in Kodak D19 developer. The autoradiographic images were photographed using a Nikon E600 microscope.

Every fifth section was stained with cresyl violet (0.5%) and camera lucida tracing was done to localize the boundaries of the gray and white matter and the relative extent of laminae I and II. \([\text{H}]\) DAMGO (specific activity 63 Ci/mmol) and Hyperfilms were from Amersham, U.K. Levorphanol was from Sigma, USA.

**Results**

At P 7, \( \mu \)-opioid receptor was observed over the entire gray matter and dorsal root ganglion when compared to camera lucida tracing of cresyl violet stained sections (Figs 1,2 a and 3a). A higher expression of the receptor was noted over the superficial part of the dorsal horn, which corresponds to the superficial laminae I-II. At P 14, an increased expression of the receptor was seen over the whole of the gray matter with respect to P 7 (Fig 2 b and 3b). Further increase in receptor expression was seen over the superficial part of dorsal horn. At P 30, the density of the receptor had decreased over the gray matter except at the region of lamine I-II (Fig. 2 c and 3c). There was re-organization of the receptor expression.
over the laminae I-II, which resembled the adult pattern of expression as reported earlier. The distinctive laminar arrangement of neurons in the spinal cord was noted at all age-groups (Fig. 2). Earlier studies also noted well-developed laminae at birth.

Receptor expression was also observed over the dorsal root ganglion at P7 and P14 (Fig. 2 a, b). Due to difficulty of cutting cryostat sections along with the vertebrae, the dorsal root ganglion at P30 could not be included. Non-specific binding was negligible and was at the level of background which indicated that the autoradiographic labeling was specific for µ-receptors (photomicrograph not given).

Discussion
The main observation of the present study was a changing pattern of µ-receptor expression in the cervical region of the developing rat spinal cord. As

Fig. 2—Camera lucida tracing of cresyl violet stained sections of spinal cord at P7(a), P14(b) & P30(c). The boundaries of gray and white matter and the changing profile of gray matter are demarcated at these age groups. The extent of laminae I and II is also shown. The dorsal root ganglia (Drg) are seen at P7 and 14. The bar represents 500µm.

Fig. 3—Transverse sections of cervical region of spinal cord labeled for µ-opioid receptors. A higher expression of receptors is noted over the superficial part of the dorsal horn at all ages (arrow head) (a) P7: µ-receptor is expressed over the dorsal horn ganglia and the gray matter, (b) P14: Increased receptor expression over the gray matter as compared to P7. Receptor expression is also noted over the dorsal root ganglia, (c) P30: Decrease in receptor expression over gray matter except laminae I-II. dh: dorsal horn, vh: ventral horn. arrow head: superficial part of dorsal horn. Drg: dorsal root ganglion. The bar in (a) represents 500µm.
reported earlier, in the P 1 rat spinal cord, receptor expression was noted over the entire spinal cord (both gray and white matter) with selective increase over the superficial part of dorsal horn and dorsal root ganglia 27 . Henceforth, receptor expression disappeared over the white matter of the spinal cord (P 7-30) as noted in the present study (Fig. 3 a-c). The transition from immature to a more mature distribution of the receptor could be observed between P 14-30 as the receptor expression decreases over the gray matter except laminae I-II (Fig. 3 b, c). The early and extensive expression of μ-opioid receptors which is replaced gradually by the adult pattern of expression suggests its role in the normal development of the nervous system 25 . In fact, continuous receptor blockade by naltrexone during prenatal life has been shown to disrupt somatic, physical and behavioural development in the rat 12 . In another study, maturation of corticostriatal pathway was observed to coincide with expression of μ-receptors in the caudate-putamen nucleus 26 . Abnormalities of the endogenous opioid system in children, born of mothers addicted to opioids, may contribute to sudden infant death syndrome, autism and self-destructive behaviour 27 . In mothers, opiates can augment the tonic inhibitory influence of endogenous opioids on ongoing maternal behaviour 28 .

An earlier study on the ontogeny of μ-opioid receptors in the developing rat spinal cord noted high density of receptors over the dorsal horn at P 1 (Ref. 22). A peak receptor expression was observed at P 4 followed by a global decrease till P 28 . In another study, μ-receptor expression was noted at the time of birth in rat 17 . Afterwards, it increased in density till P 7, though in a diffuse manner, before becoming localized to the superficial laminae of the dorsal horn (I-II) by P 56 .

μ-Receptor in the superficial dorsal horn is associated with modulation of pain. Majority of the receptors are supposed to be associated with the termination of Aδ and C fibres in the spinal cord 29 . However, the increased receptor density during development could be related to direct expression of the receptor by the developing neurons and glia 30 . In an elegant study, Rahman et al. have shown that all the μ-receptors during development are not functionally coupled to C-fibre evoked dorsal horn responses 31 . The same study also noted that morphine produced a greater inhibition of the C-fibre evoked responses between P14-P21, indicating a greater sensitivity to morphine at this age than in adults. It may be hypothesized that endogenous opioids could have a protective role with regard to preventing pain, during and after birth.

In the human fetal spinal cord, μ-opioid receptors could be detected by 12-13 week of gestation in the dorsal horn of the spinal cord with an increase over the superficial part of the dorsal horn 22 . This pattern persisted till 21-22 week, when receptor expression was also noted over the ventral horn and lateral funiculi. A highly selective increase over the superficial layers of the dorsal horn was noted at 24-25 week. In the rat, an identical selective μ-receptor expression was noted at P 30 .

The dorsal root ganglion showed increased expression of μ-receptor at P 1 as compared to other age-groups in the present study. A higher expression of μ-receptor between P 0-7 as compared to P 21 has been previously reported 31 . A shift in receptor expression to predominantly small and medium-diameter neurons by P 21 was also reported. It is also known that in adult, about 50% of the neurons in the dorsal root ganglia express the μ-opioid receptor 32 . These are mainly the small to medium sized neurons, which also contain calcitonin gene-related peptide (CGRP).

During development, the expression of μ-receptor in the spinal cord and dorsal root ganglia follows a specific spatio-temporal pattern. This, in turn, suggests that the receptor is functionally associated with the normal development of the nervous system.

Acknowledgement

The work was financially supported by the Department of Biotechnology (DBT), Government of India.

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