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Mu opioid receptors in developing human spinal cord

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ABSTRACT

The distribution of mu opioid receptors was studied in human fetal spinal cords between 12–13 and 24–25 wk gestational ages. Autoradiographic localisation using [^{3}H] DAMGO revealed the presence of mu receptors in the dorsal horn at all age groups with a higher density in the superficial laminae (I–II). A biphasic expression was noted. Receptor density increased in the dorsal horn, including the superficial laminae, between 12–13 and 16–17 wk. This could be associated with a spurt in neurogenesis. The density increased again at 24–25 wk in laminae I–II which resembled the adult pattern of distribution. A dramatic proliferation of cells was noted from the region of the ventricular zone between 16–17 and 24–25 wk. These were considered to be glial cells from their histological features. Mu receptor expression was noted over a large area of the spinal cord including the lateral funiculus at 24–25 wk. This may be due to receptor expression by glial cells. The study presents evidence of mu receptor expression by both neurons and glia during early development of human spinal cord.

Key words: Neurogenesis; spinal cord; dorsal horn laminae; glial development.

INTRODUCTION

Among the factors controlling the development of the central nervous system (CNS), neurotransmitters play an important role. Gamma-aminobutyric acid (GABA) is one of the earliest neurotransmitters to appear in the mammalian brain (Lauder et al. 1986) even before the formation of synapses (Taylor et al. 1990). Growth cones have been demonstrated to contain significantly elevated levels of aspartate, glutamate, GABA and taurine as compared with the mature brain (Taylor et al. 1990). An isoform of GABA, different from that in adults, is transiently expressed in the differentiating neurons of the rat spinal cord (Behar et al. 1993). Acetylcholine, a ubiquitous neurotransmitter, seems to control cortical differentiation (Hohmann et al. 1988). Interruption of cholinergic input to the developing cortex results in loss of laminar boundaries and an initial absence of pyramidal cells. Glutamate, the most common excitatory neurotransmitter in the CNS, significantly inhibits neurite outgrowth and growth cone motility (Owen & Bird, 1997). Many of the major neurotransmitters of the monoamine system have also been shown to regulate the development of nervous system (Whitaker-Azmitia et al. 1991).

Many of the studies have also focused on the related developmental profile of neurotransmitter receptors in the nervous system. Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors show a transient peak during the 3rd postnatal week in the CA3 region of the rat hippocampus (Insel et al. 1990). The same receptor shows a temporary increase in the rat visual cortex at postnatal day (PND) 6 (Erdo & Wolff, 1990). Fluctuations in different neurotransmitter receptor densities in the cat visual cortex during development have also been reported (Shaw et al. 1986). The density of mu receptors peaks at PND 4 in the rat spinal cord before declining gradually (Kar & Quirion, 1995). The transient presence of neurotransmitters or their receptors suggests a specific functional role during particular stages of development (Shaw, 1996).

Endogenous opioids modulate the development of the nervous system by inhibiting growth (Zagon & McLaughlin, 1982, 1987). It has been suggested that

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this effect is receptor mediated as continuous opioid receptor blockade results in significantly increased dendritic and spine elaboration (Hauser et al. 1989). The mu opioid receptor is the most commonly distributed opioid receptor subtype in the adult human brain (Quirion & Pilapil, 1991). Autoradiographic studies on adult human spinal cord demonstrate opioid receptor in laminae I–III of the dorsal horn (Faull & Villiger, 1987). The ontogeny of the different opioid receptors has been well characterised in lower mammals (Kornblum et al. 1987; Xia & Haddad, 1991) as well as in the rhesus monkey (Bachevalier et al. 1986). However, to our knowledge, only one study has been performed on the autoradiographic localisation of mu receptors in the developing human spinal cord (Sales et al. 1989). The spinal cord is important in the present context as radioligand assays have revealed significantly higher opioid receptor sites in the spinal cord as compared with other parts of CNS (Magnan et al. 1988). An earlier study in our laboratory detected enkephalin immunoreactivity by 12 wk of gestation in human spinal cord (Bijlani et al. 1988). The present study is a report on the corresponding mu receptor development. A preliminary report has been presented in abstract form (Ray et al. 1997).

MATERIALS AND METHODS

In vitro tissue autoradiography, the mu opioid receptor was localised by incubating cryostat sections of the tissue with tritium labelled [D-ala^2, N-methyl-phe^4, -glycol^5] enkephalin (DAMGO). DAMGO is a highly specific ligand for the mu receptor (Handa et al. 1981).

Detailed methodology

The technique was initially standardised on stock bred adult Wistar rats (n = 3), PND 1 rat pups (n = 3) and PND 1 Leghorn (*Gallus domesticus*) chick (n = 3) spinal cords. Human fetal specimens(16) of gestational ages 12–13(3), 14–15(4), 16–17(4), 21–22(4) and 24–25(1) wk were obtained shortly after therapeutic abortions (performed for medical reasons). Prior consent was obtained from the ethical committee and the patient. The fetal age was determined by measurement of crown-rump length (Hamilton et al. 1962). The upper part of the cervical enlargement (C4–C8) of the spinal cord was used throughout the study. The tissue was briefly frozen in liquid nitrogen before being stored at −70 °C. Later, cryostat sections (20 μm) were cut at −20 °C and mounted on 1% gelatin subbed slides. Every 10th–12th section was briefly fixed in 10% formalin and processed for thionin staining.

The autoradiographic labelling was performed as follows (modified from Hammer, 1990). The cryostat sections were preincubated in 50 mM Tris-HCl buffer, pH 7.4 containing 150 mM sodium chloride and 1 mg/ml of bovine serum albumin (BSA) at 4 °C for 15 min. This was done to remove endogenous opioid peptides present in the tissue. The sections were rinsed in 50 mM Tris-HCl buffer at 4 °C, twice for 1 min each. The sections were then incubated in Tris-HCl buffer containing 2 nM [^{3}H] DAMGO (twice the K_d) and 1 mg/ml of BSA for 1 h at room temperature. A concentration of radioligand equivalent to twice the k_d gives a higher specific to nonspecific binding. The sections were rinsed in 50 mM Tris-HCl buffer twice for 1 min each at 4 °C before being rapidly dried. The dried sections along with tritiated plastic standards were exposed to tritium sensitive hyperfilm for 8 wk at 4 °C. The films were developed in Kodak D19 developer. The autoradiographic density over the superficial laminae of the dorsal horn was quantitated using computerised image analysis (Leica). Nonspecific binding was determined on every 3rd section under the same experimental conditions with 1000 fold excess of levorphanol.

[^{3}H]DAMGO (specific activity 63.0 Ci/mmol), hyperfilms-^{3}H and plastic standards were purchased from Amersham, UK. Other chemicals were from Sigma, USA.

RESULTS

Standardisation

In adult rats, the mu receptor was localised over laminae I and II of the spinal cord (Ray, 1996). In PND 1 rats, receptor expression was observed in the superficial part of the dorsal horn and in the dorsal root ganglia (Fig. 1*a*). A lesser degree of receptor expression was seen over remaining parts of the spinal cord. Mu receptor labelling was seen over the whole grey matter in PND 1 chick spinal cord (Fig. 1*b*). However, there was increased labelling over the superficial part of the dorsal horn. The surrounding white matter demonstrated lower receptor binding as compared with grey matter. Nonspecific binding ascertained by using 1000-fold excess levorphanol was negligible.

Histological features of human spinal cord

At 12–13 wk, the dorsal horn is composed of sparsely scattered small cells, almost devoid of Nissl substance.

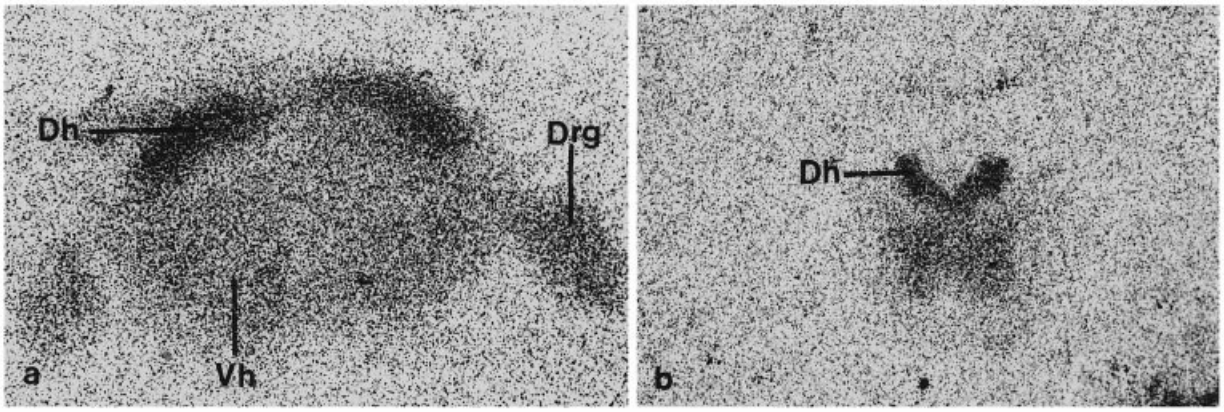

Fig. 1. Autoradiographic localisation of mu receptors in spinal cord. (*a*) Superficial part of the dorsal horn and dorsal root ganglia express the receptor in PND 1 rat. (*b*) The whole of the grey matter showing presence of the receptor in PND 1 chick. Higher concentration of receptor is present over the superficial aspect of the dorsal horn. Vh, ventral horn; Dh, dorsal horn; Drg, dorsal root ganglion. (*a*) ×36.5, (*b*) ×26.

The cells of the ventral horn appear to be more mature with small amounts of Nissl material (Fig. 2*a*). At 16–17 wk, the grey matter has differentiated further, with the ventral horn at a more advanced stage of maturation (Fig. 2*b*). A band of cells could be seen migrating out from the ventricular zone in both ventral and dorsal directions close to the midline. These cells, presumably glial, were also migrating into grey matter. A lesser degree of glial migration was seen at 12–13 wk. At 21–22 wk, the migrating cells were more specifically delineated with extensive migration into the lateral funiculus (Fig. 2*c*). Further growth and maturation of the spinal cord was seen at 24–25 wk (Fig. 2*d*). The anterior and posterior funiculi were larger. Cellular migration into the anterolateral and dorsal funiculi and also into grey matter was more pronounced. Viewed at higher magnification, the cells could be seen invading both grey and white matter (Fig. 2*e*, *f*).

Autoradiographic localisation

At 12–13 wk, mu receptor expression was seen over the dorsal horn with greater intensity over the superficial laminae (Fig. 3*a*). At 14–15 wk, the whole of the dorsal horn was found to express mu receptor with a selective increase over superficial laminae (Fig. 3*b*). At 16–17 wk, the pattern seen earlier was further intensified (Fig. 3*c*). A persistent problem encountered was detachment/breaking of sections during incubation. This may have been due to a low protein content in fetal tissues (R. P. Hammer Jr, personal communication). At 21–22 wk, the overall intensity of expression of the receptor had decreased. Radiolabelled newly formed cells were seen streaming out from the vicinity of the central canal. A faint but definite presence of receptor was seen over the ventral horn and lateral funiculus. At 24–25 wk, a dense zone of mu receptors was evident, covering the superficial aspect of the dorsal horn (Fig. 3*d*). Less dense labelling was seen over the remaining part of the grey matter and the lateral funiculus. Computerised image analysis of the superficial laminae of the dorsal horn revealed a progressively increasing concentration of mu receptors until 16–17 wk. At 21–22 wk, receptor density declined before rising at 24–25 wk (Fig. 4). Competitive binding with 1000-fold excess levorphanol exhibited negligible binding.

DISCUSSION

The present study demonstrates the presence of mu receptors by 12–13 wk, the earliest age group studied. The endogenous opioid system (endogenous opioids and opioid receptors) is said to critically regulate maturation of the nervous system (Hauser et al. 1996). In utero exposure to morphine, which binds to mu receptors, leads to significantly decreased neuronal counts and cell packing density in the cerebral cortex. (Seatrix & Hammer, 1993). Morphine has also been shown to reduce glial cell proliferation in cultures (Stein-Martin et al. 1991) and also DNA synthesis in neonatal rat brain (Kornblum et al. 1987). Conversely, mu receptor blockade results in a significant increase in the dendritic arborisation of pyramidal cells from frontoparietal cortex and the CA1 region of the hippocampus and of Purkinje cells in cerebellar cortex (Hauser et al. 1989).

The early appearance of the receptor is not surprising considering that enkephalin-positive

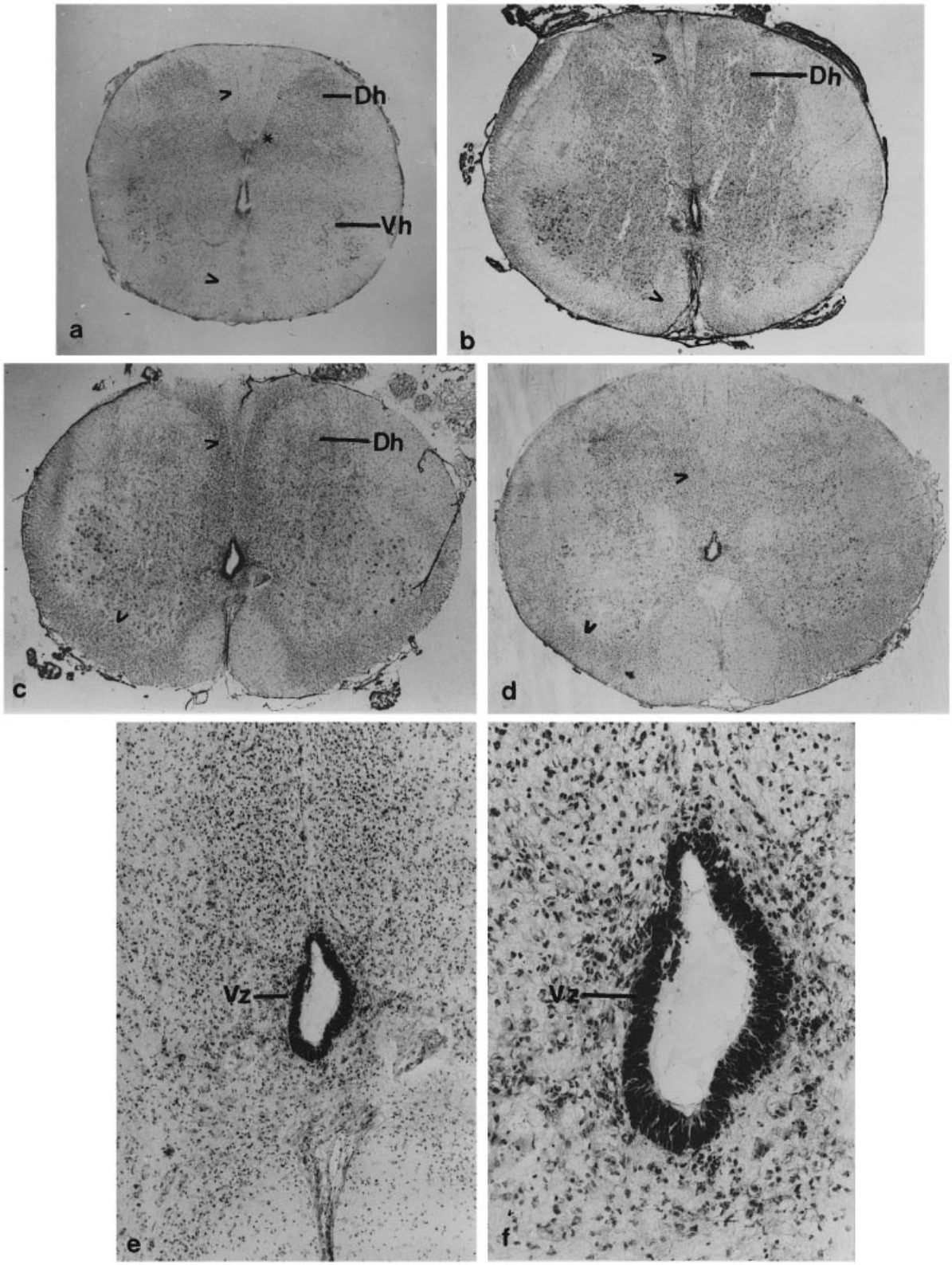

Fig. 2. Thionin stained sections of cervical region of human spinal cord. (*a*) 12–13 wk. The ventral horn is more mature than the dorsal horn. Cells can be seen migrating into the dorsal funiculi (indicated by arrowhead) on either side of the midline from the dorsal detached part of the central canal. Similar proliferation is also seen in the ventral funiculi (arrowhead). From the lateral sides of the central canal, cells are seen migrating into dorsal grey matter (asterix). (*b*) 16–17 wk. Grey matter has matured further with a large number of cells migrating into dorsal and ventral funiculi (arrowheads) (*c*) 21–22 wk, showing more extensive migration of cells into the ventrolateral funiculi as compared with the dorsal funiculi. (*d*) 24–25 wk showing similar appearances to *c*. (*e*, *f*) At higher magnification, the ventricular zone in the 21–22 wk specimen shows marked proliferation and migration of cells. The migrating cells are presumably glial considering their small size and their migration into both white and grey matter. Vh, ventral horn; Dh, dorsal horn; Vz, ventricular zone. (*a–d*) $\times 26$; (*e*) $\times 54$; (*f*) $\times 135$.

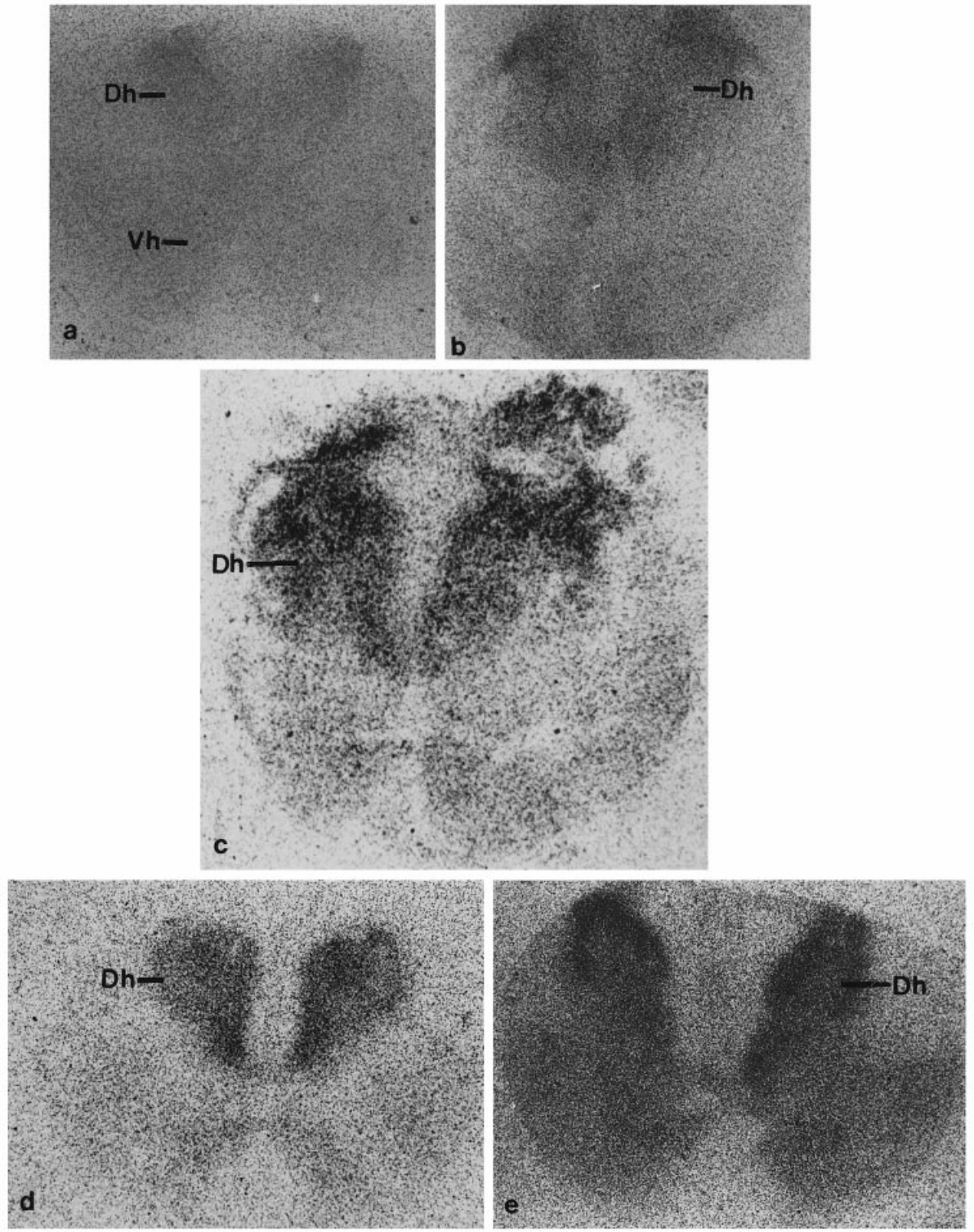

Fig. 3. Autoradiographic localisation of mu receptors in human spinal cord. (*a*) 12–13 wk showing expression of receptor over the dorsal part of the dorsal horn. (*b*) 14–15 wk showing increase in receptor expression in cells of the whole dorsal horn and a further increase over superficial laminae. (*c*) 16–17 wk showing increased receptor density over the dorsal horn with a higher concentration of receptors over the superficial part. Expression of receptors is higher as compared with *b*. (*d*) 21–22 wk Receptor expression over the dorsal horn has decreased. (*e*) 24–25 wk showing a selective increase of mu receptors over the superficial laminae and diffuse expression of receptors over the remaining grey matter and ventrolateral funiculi. Dh, dorsal horn; Vh, ventral horn. (*a*, *b*, *d*, *e*) $\times 26$; (*c*) $\times 34$.

immunoreactivity could be detected in the superficial layers of dorsal horn by 12–14 wk of gestation. Immunoreactivity was subsequently seen to increase with gestational age (Charney et al. 1984; Bijlani et al. 1988). Increased expression of the receptor was noted between 14–15 and 16–17 wk. This may be

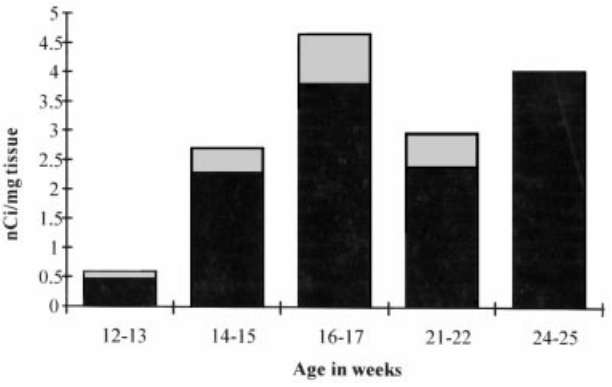

Fig. 4. Quantitative analysis of mu receptors in the superficial laminae of the dorsal horn of spinal cord at different gestational ages. The unshaded areas represent standard deviations.

related to a presumed role of opioid receptors during normal neurogenesis. Receptor density increased again at 24–25 wk in the superficial laminae. This pattern was reminiscent of opioid receptor distribution in the mature human spinal cord (Faull & Villiger, 1987). Mu receptor distribution over the superficial laminae is functionally associated with modulation of nociceptive stimuli (Fields et al. 1991). It is now known that the majority of the receptors are associated with primary afferent fibres in the dorsal horn (Gouarderes et al. 1991). The remainder of the receptors are located on a population of small dorsal horn neurons which are predominantly excitatory (Kemp et al. 1996).

Receptor density decreased at 21–22 wk. Immunohistochemical localisation of enkephalin in fetal spinal cord does not reflect a corresponding decrease during this time period (Bijlani et al. 1988). It may be hypothesised that the decrease could be related to dilution of receptor containing cells by elaboration of neuronal processes and positioning of glia. At 24–25 wk, a lower receptor density was also seen over the remaining grey matter and lateral funiculus. This diffuse pattern may be associated with mu receptor expression by newly formed glial cells, observed in the thionin sections. Both astrocytes and oligodendrocytes have been shown to express mu receptors (Hauser et al. 1996; Knapp & Hauser, 1996).

The present study was limited to the mu receptor as it is the predominant opioid receptor in the nervous system. It comprises $70 \pm 4\%$ of the total opioid receptors in the adult rat spinal cord as compared with delta ($23 \pm 2\%$) and kappa ($7 \pm 1\%$) receptors (Besse et al. 1990). During postnatal development, the relative proportions of mu and delta receptors were 16.5:1 in the PND 3 rat brain (Kornblum et al. 1987). However, no corresponding study is available for mature human spinal cord using specific ligands for individual receptor subtypes. In adult primate spinal cord dense mu opioid receptor immunoreactivity was detected in lamina II of the dorsal horn (Honda & Arvidsson, 1995). Using radioligand assay, both mu and kappa receptors were shown to be present in human brain at 18 wk of gestation and thereafter while delta receptors could not be detected until 38 wk of gestation (Tiberi & Magnan, 1989). An earlier study on the ontogeny of the mu receptor in human fetal spinal cord visualised the receptor by 14 wk of gestation (Sales et al. 1989). Conversely, the delta receptor was also noted to appear at an early stage of development. The receptors were mainly localised over the superficial layers of the dorsal horn, a finding similar to that of our study. However, the present observations clearly define the change in pattern of receptor distribution with increasing gestational age. Tritium quenching was not considered to be an important factor in these specimens of early gestational age period as myelination of tracts in the spinal cord starts at about the 4th month of gestation and is not completed until 1 y after birth (Sadler, 1995).

During development of the nervous system, neurogenesis occurs earlier as compared with that for glia (Levison & Goldman, 1993) with the exception of radial glia (Levitt et al. 1981). Radial glia has been shown to further differentiate into astrocytes and oligodendrocytes between E18 and birth in the rat spinal cord (Hirano & Goldman, 1988). A similar mechanism has been suggested for the human fetal CNS (Choi & Kim, 1984). Between 16–17 and 24–25 wk, massive proliferation of cells was noted from the ventricular zone. These were presumed to be predominantly glial cells considering their small size and dark cytoplasm with widespread migration into both grey and white matter. Further work is being undertaken to establish the subtype to which these belong.

The ventral horn was seen to be more mature as compared with the dorsal horn. This is in accordance with a previous report where maturation in the spinal cord was observed to follow a ventrodorsal and rostrocaudal gradient (Nornes & Das, 1974). Even the anterolateral funiculus had more advanced glial proliferation with respect to the dorsal funiculus at 24–25 wk.

During standardisation, a higher degree of mu receptor labelling was noted over the superficial part of the dorsal horn and dorsal root ganglion (DRG) in the PND 1 rat. A number of reports have documented high and selective concentrations of mu receptor over

laminae I–II of the dorsal horn in the mature rat spinal cord (see Besse et al. 1990 for references). The majority of these receptors are located at the pre-synaptic junction on thin primary afferent fibres (Gouarderes et al. 1991), which are the central processes of the DRG neurons. It was suggested by Dickenson (1996) that the mu receptor may probably be derived from cell-bodies of DRG neurons, which appears to be so, from the localisation of the mu receptor over the neonatal rat DRG in our study. An earlier study on the developing rat spinal cord (PND 1) reported high to moderately high expression of the receptor over the dorsal and ventral horns respectively (Kar & Quirion, 1995).

Regarding PND 1 chick spinal cord, the whole of the grey matter demonstrated mu receptor. Although no previous autoradiographic study is available on the spinal cord, high levels of opiate binding sites have been demonstrated in the chick brain between d 7 of incubation to PND 3 by radioreceptor assay (Gibson & Vernadakis, 1982). This period corresponds to a phase of active neonatal proliferation, differentiation and synaptogenesis in the chick brain.

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