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Developmental Expression of the μ, κ, and δ Opioid Receptor mRNAs in Mouse

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To characterize further the establishment of the opioid system during prenatal mouse development, we have examined the spatial and temporal expression patterns of μ, κ, and δ opioid receptor mRNAs and find that the expression patterns of these mRNAs are distinct at all ages. Within the embryo, κ is the first opioid receptor expressed, with transcripts detected in the gut epithelium as early as embryonic day 9.5 (E9.5). By E10.5, μ receptor expression is first detected in the facial–vestibulocochlear preganglion complex, whereas δ receptor mRNA is first detected at E12.5 in several peripheral tissues, including the olfactory epithelium, heart, limb bud, and tooth. In the brain, both μ and κ mRNAs are first detected at E11.5 in the basal ganglia and midbrain, respectively. During mid-gestation and late gestation, the expression of both μ and κ receptors extends to other brain regions that exhibit high expression in the adult, including the medial habenula, hypothalamus, pons, and medulla for μ and the basal ganglia, thalamus, hypothalamus, raphe, and ventral tegmental area for κ. Thus by E17.5, many aspects of the adult expression patterns of μ and κ receptors already have been established. Compared with μ and κ, δ receptor mRNA expression in the brain begins relatively late, and the expression levels remain very low even at E19.5. In contrast to its late appearance in the brain, however, δ is the first opioid receptor expressed in the dorsal root ganglion, at E12.5, before its expression in the spinal cord begins at E15.5. μ receptor is the first opioid receptor expressed in the spinal cord, at E11.5. These results extend previous ligand-binding data to significantly earlier ages and suggest that early developmental events in both neural and non-neural tissues may be modulated by opioid receptors. Several examples of possible autocrine and paracrine loops of opioid peptide and receptor expression have been identified, suggesting a role for these local circuits in developmental processes.

Key words: opioid receptor; in situ *hybridization; ontogeny; development; embryo; CNS*

On the basis of radioligand binding and pharmacological experiments, receptors recognizing both exogenous opiate drugs and endogenous opioid peptides classically have been defined into three types: the δ, μ, and κ opioid receptors (Martin et al., 1976; Lord et al., 1977). Classic studies show that each of these receptors has a specific pharmacological profile (Goldstein and Naidu, 1989) and a unique distribution in the adult CNS (Mansour et al., 1988), and each could be associated with specific functions (Herz, 1993).

Recently, the opioid receptor cDNAs encoding μ, κ, and δ receptor activities have been cloned (Kieffer, 1995). The mRNA expression patterns of the μ and κ receptors in adult rat and the δ receptor in adult rat and mouse already have been characterized (Mansour et al., 1993, 1994). The distributions of the receptor mRNAs have been compared with previously identified sites of ligand binding; generally, there is a good correlation between mRNA and binding site distributions.

Previously, radioligand binding has been the primary method used to study opioid receptor ontogeny during prenatal development, albeit with limited sensitivity and cellular resolution. For example, tissue homogenates generally have been used because of small tissue size, and the CNS usually has been the only tissue examined. In the mouse, μ receptor binding activity can be detected in homogenates as early as embryonic day 12.5 (E12.5) (Rius et al., 1991), whereas κ receptor binding is first detected at E14.5. In contrast, CNS δ receptor binding sites cannot be detected at all prenatally (Kornblum et al., 1987; Rius et al., 1991). In the rat spinal cord, μ and κ binding sites first appear at E15, whereas δ sites are not detected until postnatal day 1 (P1) (Attali et al., 1990).

Numerous studies have demonstrated that neurotransmitters expressed early are involved in regulating neuronal outgrowth and survival (Mattson, 1988). *In vivo* and *in vitro* studies have shown that opioid antagonists, opioid peptides, and opiate drugs can all affect certain developmental processes, including regulation of neuronal and glial proliferation, differentiation (Zagon and McLaughlin, 1983; Zagon, 1987; Stiene-Martin et al., 1993), and cell death (Meriney et al., 1985, 1991). More recently, it has been suggested that opioids regulate cell division via μ and κ receptors in astroglial culture established from postnatal mouse brain (Gurwell et al., 1996; Hauser et al., 1996) as well as fetal rat brain cell aggregates in culture (Barg et al., 1993; Gorodinsky et al., 1995).

The cloning of the μ, κ, and δ opioid receptors has provided a unique opportunity to use *in situ* hybridization to analyze the spatial and temporal patterns of opioid receptor gene expression during development. Our results show that the expression of μ, κ,

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and δ receptor mRNAs begins significantly earlier than previously believed and is distinct at all ages. In addition, several examples of possible autocrine and paracrine loops of opioid peptide and receptor expression have been identified, suggesting a role for these local circuits in developmental processes.

MATERIALS AND METHODS

Tissue preparation. All studies were conducted in accordance with the principles and procedures outlined in National Institutes of Health Guidelines for Care and Use of Experimental Animals. Fresh-frozen, post-fixed sections were used for all experiments. At least one litter for each age group from which data are reported (E7.5, E8.5, E9.5, E10.5, E11.5, E12.5, E13.5, E15.5, E17.5, and E19.5) was used, with at least two embryos examined at each age. Pregnant C57Bl/6J mice were decapitated. For embryos of E7.5, E8.5, and E9.5 the whole uteri were freshly frozen and embedded in OCT compound for cryostat sectioning. Mouse embryos older than E10 (E10.5–E19.5) were dissected quickly and staged according to their limb bud morphology, using the criteria described (Wanek et al., 1989), before they were frozen and embedded in OCT. Embryonic trunk sagittal and transverse sections as well as embryonic brain horizontal sections were prepared to detail the expression pattern. Sections were mounted onto 3-aminopropyltriethoxy-silane-coated microscope slides and stored at −70°C until their use in the hybridization procedure.

Probes. [^{33}P]-uridine 5′-triphosphate-labeled single-stranded RNA probes were synthesized and purified *in vitro* from the plasmid vectors harboring the appropriate cDNA sequences. Transcription templates were selected with care to ensure that hybridizations distinguished distinct expression patterns for each of the three opioid receptors without cross-hybridization. For δ opioid receptor cRNA probe, plasmid DK1B [a gift from Dr. Christopher Evans, University of California, Los Angeles (UCLA)] containing the whole-mouse cDNA sequence of DOR-1 (Evans et al., 1992) was cut with *Sac*I and transcribed with T3 RNA polymerase to produce a 630 base pair (bp) antisense cRNA probe corresponding to nucleotides (nt) 1206–1835. The same plasmid was linearized with *Bgl*II, transcribed with T7 RNA polymerase to make a sense probe (669 bp, corresponding to nt 1–668 of the cDNA). For the μ opioid receptor, a 346 bp PCR fragment (generated from 5′ primer "aaa gcg cct ccg tgt act tc" and 3′ primer "g ctc aac ttg tcc cac gtt gat") corresponding to nt −237 to 108 of the mouse μ receptor cDNA (a gift from Dr. Christopher Evans, UCLA) was subcloned into pCRII vector (TA Cloning kit, Invitrogen, San Diego, CA). To make antisense probe, we linearized the vector with *Hin*dIII (at the 5′ end of the insert) and then transcribed it with T7 RNA polymerase. To produce sense probe, we linearized the vector with *Xho*I (at the 3′ end of the insert) and transcribed it with SP6 RNA polymerase. For the κ opioid receptor we subcloned a 376 bp *Pst*I–*Eco*RI fragment of λmsl-1 corresponding to nt 172–548 of the mouse κ receptor cDNA into pGEM-3Z (a gift from Dr. Graeme I. Bell, University of Chicago, Chicago, IL) (Yasuda et al., 1993). The κ receptor antisense probe synthesis involved linearizing with *Hin*dIII at the 5′ end of the insert and transcribing with T7 RNA polymerase. The κ sense probe synthesis involved linearizing with *Eco*RI at the 3′ end of the insert and transcribing with SP6 polymerase. All probes were run on formaldehyde gels, dried, and exposed to autoradiographic film to confirm the full-length transcription of a single fragment.

In situ *hybridization. In situ* hybridizations were performed according to the protocols described (Zheng and Pintar, 1995). Autoradiography was performed at 4°C with a 1:1 dilution of Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY) for 4–8 weeks. In all cases hybridization with control (sense) RNA in adjacent sections yielded only low background.

RESULTS

No opioid receptor mRNA expression was detected within the embryo at E7.5 and E8.5, although unexpected expression of all three receptor genes was observed in the surrounding uterus or placenta at these ages (Y. Zhu and J. Pintar, unpublished data). The following results demonstrate that cells expressing μ, κ, and δ opioid receptor mRNAs are differentially distributed in both the CNS and periphery of the mouse embryo as early as E9.5 with changing expression patterns through E19.5. Consistent with previous ligand-binding results, expression of the δ receptor lags behind that of μ and κ receptors in the brain. However, in peripheral tissues, including peripheral ganglia, the first appearance of δ receptor occurs concurrently with that of μ and κ receptors. The expression patterns of the three receptor mRNAs in the brain, spinal cord, peripheral ganglia, and other peripheral tissues are presented below. The anatomical results are illustrated as low-magnification dark-field micrographs as well as high-resolution cellular images of μ, κ, and δ receptor mRNAs in selected regions after emulsion autoradiography.

Expression of the μ and κ genes in peripheral tissues precedes expression in CNS

The κ receptor gene is the first of the opioid receptor gene family to be expressed in the embryo proper (Fig. 1). Transcripts of this receptor are first detected at E9.5 throughout the gut epithelium (Fig. 1*A*), and the expression continues until late gestation (see Fig. 8*O*). By E10.5, κ receptor mRNA also is detected in the primitive pia-arachnoid (McLone and Bondareff, 1975) outside the hindbrain (Fig. 1*B,D*). κ expression in these cells is transient, with the expression continuing until E13.5 (Figs. 1*L*, 2*N,O*), and is restricted spatially to the region from the diencephalon through the hindbrain.

Also at E10.5, μ receptor mRNA is first detected in cells of the facial and vestibulocochlear preganglion complex at the time these ganglia are beginning to form (Fig. 1*C*). This expression is illustrated at E11.5 (Fig. 1*E*) and E13.5 (see Fig. 7*A*) and continues through development.

Expression of both μ and κ in the brain precedes δ expression

The expression of the μ, κ, and δ opioid receptor mRNAs in the brain begins in specific regions at discrete developmental stages. The following paragraphs summarize major features of the expression patterns.

E11.5–E12.5

Both μ and κ receptor mRNAs are first detected in the CNS at E11.5 but in different regions. Newly differentiated neurons of the basal ganglia express μ receptor mRNA (Fig. 1*E*), whereas κ receptor mRNA is first detected in the midbrain at this age (Fig. 1*F*).

By E12.5, expression for both μ and κ has expanded. μ receptor expression in the midbrain and hindbrain regions (Fig. 1*I*) begins while the expression in the basal ganglia continues (Fig. 1*G,K*). Low levels of κ receptor mRNA in the midbrain continue to be detected, although κ expression is located in the caudal region (Fig. 1*J*), whereas μ expressing cells are located more rostrally (Fig. 1*I*). In addition, κ is first detected in the basal ganglia (Fig. 1*H*), but expressing cells are fewer in number and generally located more rostrally as compared with μ expression (Fig. 1*G*). κ expression also begins in the rostral hindbrain (Fig. 1*J*) and raphe nuclei (Fig. 1*L*).

E13.5

The extent of μ and κ receptor expression in the brain continues to widen at E13.5 while δ receptor expression first appears (Fig. 2). μ continues to be expressed in the basal ganglia (Fig. 2*F,H*) and midbrain, which includes tectum (Fig. 2*B*) and tegmentum (Fig. 2*C*), while its expression in the medial habenula (Fig. 2*D*), hypothalamus (Fig. 2*D–G*), pons (Fig. 2*F,G*), trigeminal nucleus (Fig. 2*D*), and medulla (Fig. 2*G,H*) begins.

κ receptor expression persists in the dorsal raphe (Fig. 2*O*) and primitive pia-arachnoid (Fig. 2*N,O*). Like μ, κ mRNA expression

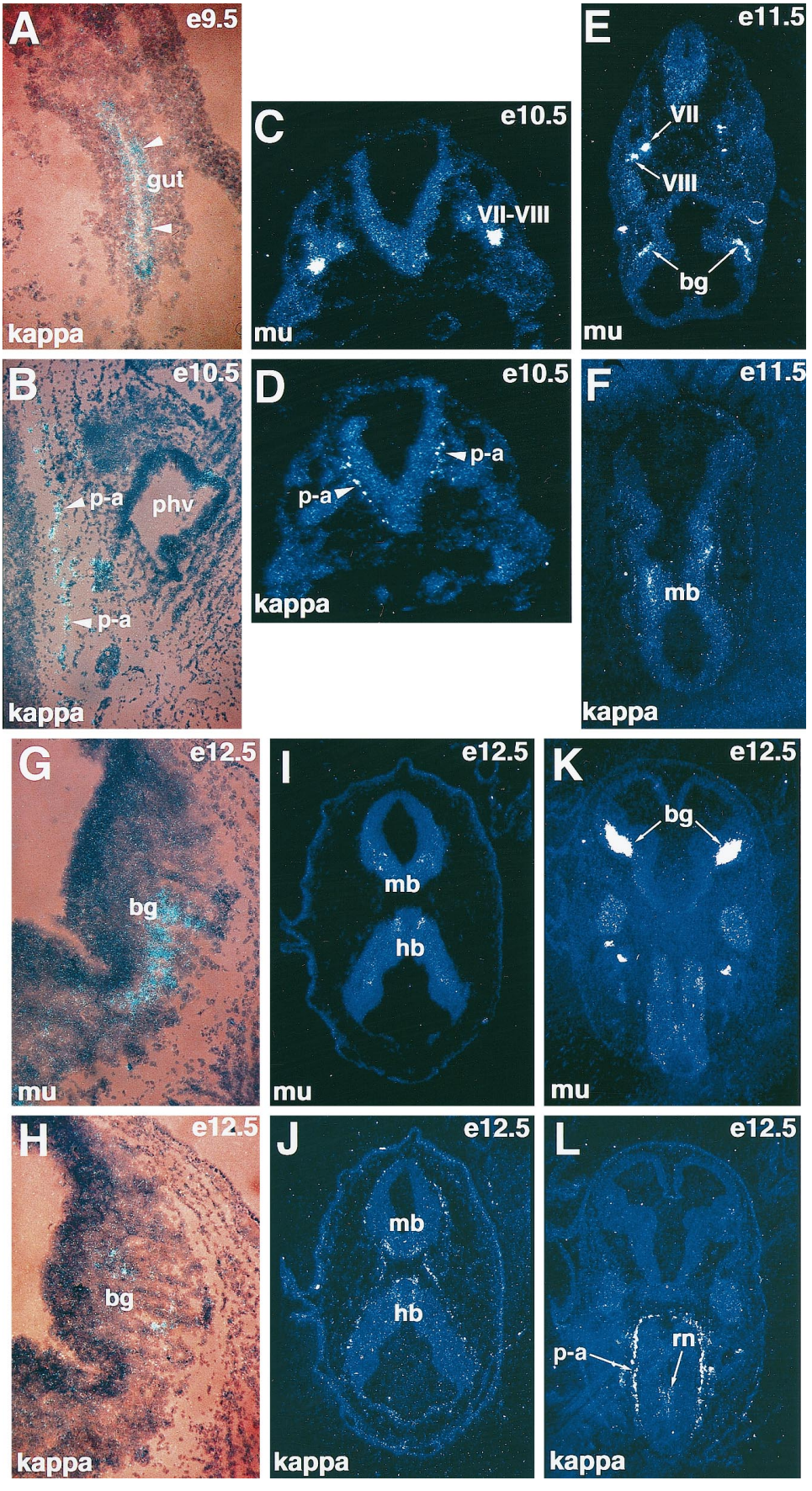

Figure 1. Dark-field low-power (*C–F, I–L*) and polarized high-power (*A, B, G, H*) photographs illustrating the cellular expression of the μ and κ receptor mRNAs before E12.5. The earliest expression among the three opioid receptors can be detected at E9.5, which is the κ receptor mRNA in the gut mucosa (*gut*, *arrowheads* in *A*). Shortly after, the κ receptor mRNA is expressed in the primitive pia-arachnoid (*p-a*, *arrowheads*) outside the hindbrain (*B*, *D*). *C* shows that the μ receptor is first detected at E10.5 in the facial (*VII*) and vestibulocochlear (*VIII*) preganglion complex. In the CNS, both μ and κ receptor mRNAs can be first detected at E11.5 (*E*, *F*). *H* shows that, at E12.5, κ also is detected in the basal ganglia (*bg*), but the labeled cells are fewer in number and appear to be more rostral as compared with μ (*G*). *hb*, Hindbrain; *mb*, midbrain; *phv*, primary head vein; *rn*, raphe nucleus.

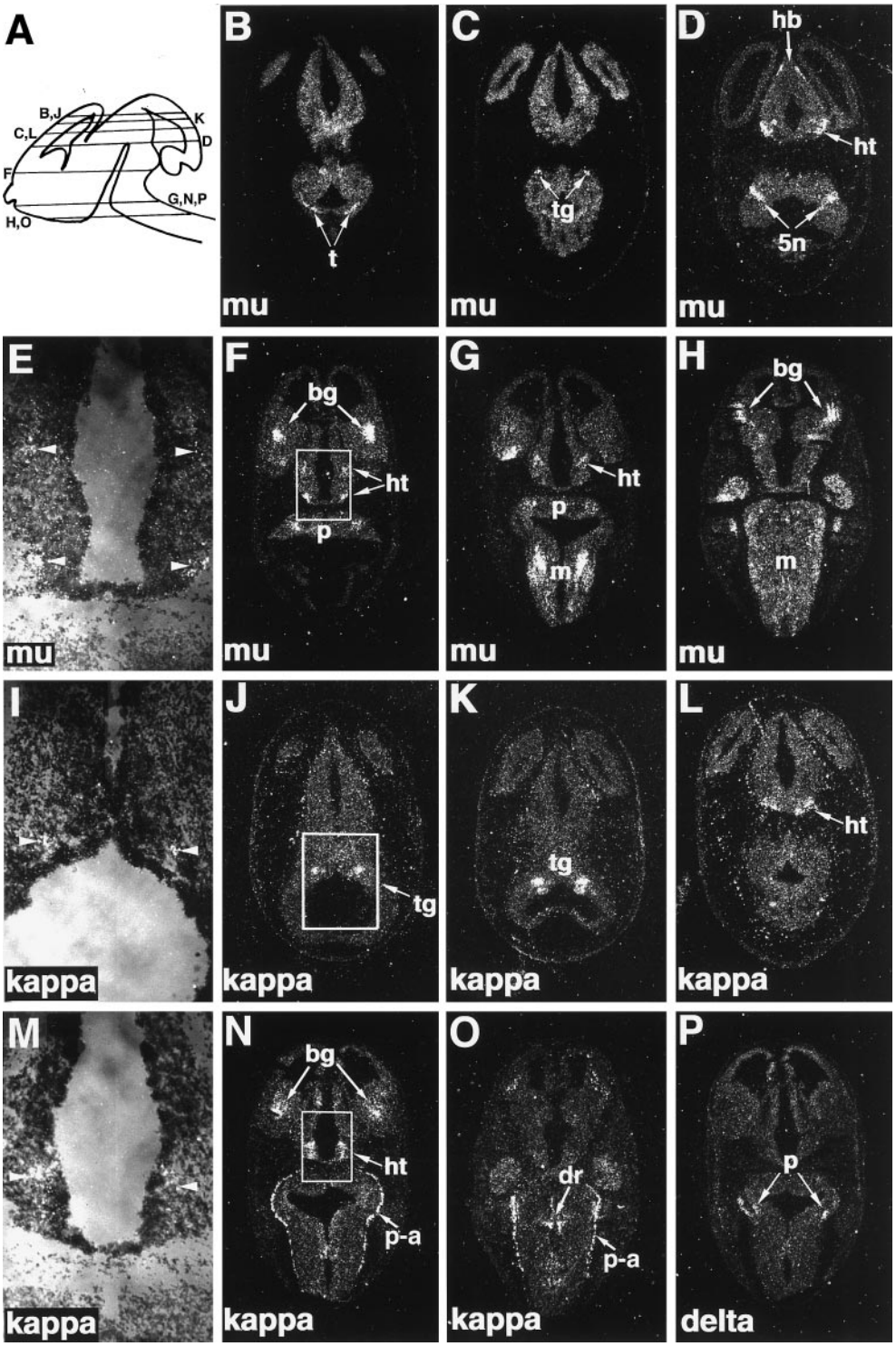

Figure 2. Dark-field low-power (*B–D*, *F–H*, *J–L*, *N–P*) photographs illustrating the mRNA distribution of the μ, κ, and δ opioid receptor genes in the brain of E13.5 embryos, with the approximate planes of horizontal sections indicated in the schematic drawing (*A*). *E*, *I*, and *M* are high-power photographs corresponding to the regions outlined in the adjacent low-power photographs *F*, *J*, and *N*, respectively. The extent of μ and κ receptor expression in the brain widens at E13.5. *P*, δ receptor is first detected in the brain at this age, in the pons (*p*). Note that the expression of the κ receptor is detected in the caudal part of the hypothalamus (*arrow*, *ht* in *L, N*) whereas μ receptor is expressed in both the caudal and rostral parts of the hypothalamus (*D–G*). *bg*, Basal ganglia; *dr*, dorsal raphe; *hb*, medial habenula; *m*, medulla; *p-a*, primitive pia-arachnoid; *t*, tectum; *tg*, tegmentum; *5n*, trigeminal nucleus.

also begins in the hypothalamus at this age (Fig. 2*L–N*), but it is restricted to the caudal region, whereas μ receptor mRNA is present in both caudal and rostral hypothalamus (Fig. 2*D–G*). Positive hybridization for κ mRNA also is detected in the tegmentum (Fig. 2*I–K*).

The first evidence of δ receptor mRNA expression in the brain is observed at this age, with low levels seen in the caudal hypothalamus (see Fig. 7*C*) and pons (Fig. 2*P*). The caudal hypothalamus is continuous with the prospective posterior lobe of pituitary (infundibulum), which is a prominent δ expression site at this age (see Fig. 8*I*).

E15.5

Compared with E13.5, a significant increase in the extent and level of μ receptor expression is observed at E15.5 (Fig. 3). For example, the intensity of the μ hybridization signal has increased greatly in the caudate putamen (Fig. 3*E–G*), medial habenula (Fig. 3*B*), and pons (Fig. 3*D–G*) while the expression

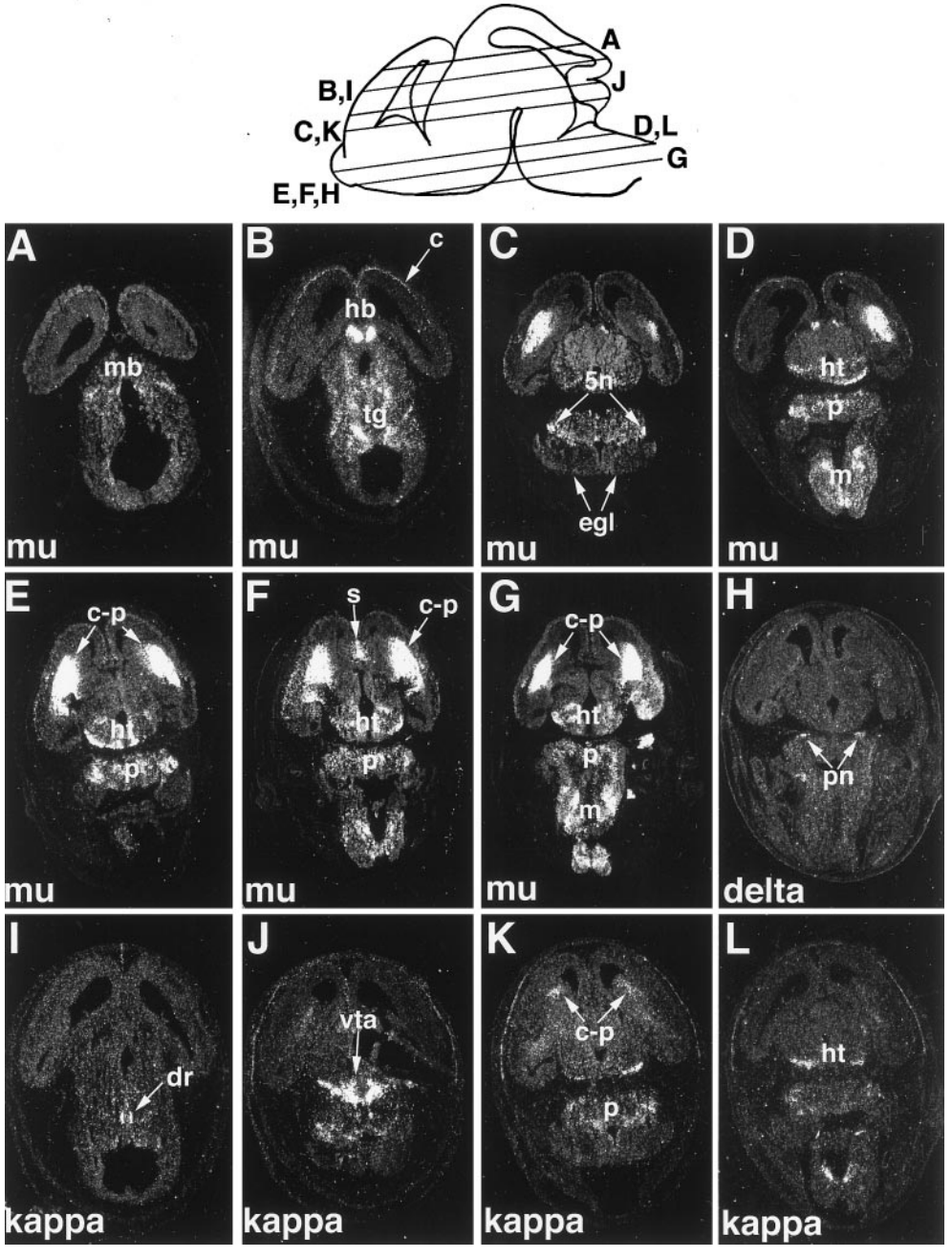

Figure 3. Dark-field photographs illustrating the cellular localization of the μ (*A–G*), κ (*I–L*), and δ (*H*) opioid receptor mRNAs in the brain of E15.5 embryos, with the approximate planes of horizontal sections indicated in the schematic drawing *above* the panels. Note the increased expression of the μ receptor in the caudate putamen (*c-p*), medial habenula (*hb*), and pons (*p*). Also note the relatively high levels of κ expression in the ventral tegmental area (*vta*). See Results for more details. *c*, Subplate of cortex; *dr*, dorsal raphe; *egl*, external granular layer; *ht*, hypothalamus; *m*, medulla; *mb*, midbrain; *pn*, parabrachial nuclei; *s*, septum; *tg*, tegmentum; *5n*, trigeminal nucleus.

in the hypothalamus (Fig. 3*D–G*), midbrain (Fig. 3*A*), tegmentum (Fig. 3*B*), trigeminal nucleus (Fig. 3*C*), and medulla (Fig. 3*D,G*) continues. In the cortex, cells expressing μ receptor mRNA appear in the subplate (Fig. 3*B*). μ also is first detected in the septum (Fig. 3*F*).

In contrast to the broad distribution of the μ receptor at this age, κ receptor expression remains restricted to a few discrete regions, including the caudate putamen (Fig. 3*K*), hypothalamus (Fig. 3*L*), dorsal raphe (Fig. 3*I*), and pons (Fig. 3*K*). Fairly high levels of κ expression also are detected in the ventral tegmental area (Fig. 3*J*).

δ receptor transcripts are still detected only at low levels, such as those observed in the parabrachial nucleus (Fig. 3*H*).

E17.5

Representative sections illustrating expression patterns of the three opioid receptors at E17.5 are shown in Figure 4. For μ and κ, the major expression pattern characteristic of the adult brain (Mansour et al., 1994) already has been established by this time.

Expression of the μ receptor in the septum (Fig. 4*D*), caudate putamen (Fig. 4*D,G,J,M*), medial habenula (Fig. 4*A,D*), hypothalamus (Fig. 4*P,S,V*), midbrain (Fig. 4*A*), pons (Fig. 4*M,P*), and trigeminal nucleus (Fig. 4*M*) as well as the subplate of the cortex (Fig. 4*A*) remains, while the expression in the olfactory bulb (Fig. 4*D,G*) and dorsal tegmental nucleus (Fig. 4*J*) is first detected.

In contrast to the μ receptor, fairly high levels of κ transcripts are observed in the centromedial and centrolateral thalamus (Fig. 4*E,H*), whereas κ expression in the caudate putamen (Fig. 4*E*) and hypothalamus (Fig. 4*W*) remains very low, with the expression still primarily located in the rostral part of the caudate putamen (Fig. 4*E*). Additional new sites of κ receptor expression

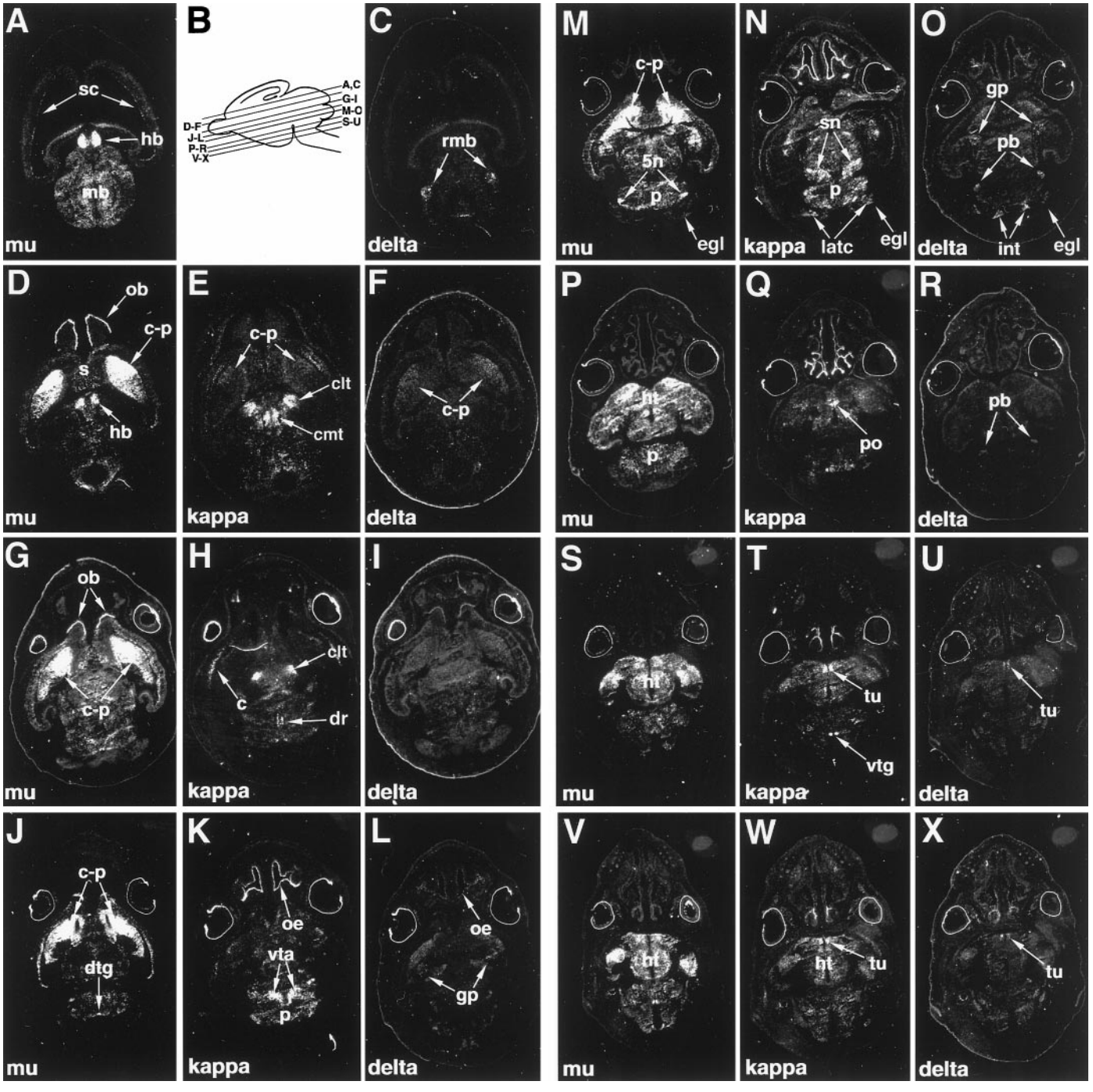

Figure 4. Dark-field photographs comparing mRNA distributions of the μ, κ, and δ opioid receptors in the brain of E17.5 embryos. Adjacent horizontal brain sections were hybridized separately with cRNAs for the μ (*A, D, G, J, M, P, S, V*), κ (*E, H, K, N, Q, T, W*), and δ (*C, F, I, L, O, R, U, X*) opioid receptors. The schematic drawing in *B* indicates the approximate planes of the horizontal sections. See Results for details (note that the pigment of the eye is not a real signal). *c*, Cortex; *clt*, centrolateral thalamus; *cmt*, centromedial thalamus; *c-p*, caudate putamen; *dr*, dorsal raphe; *dtg*, dorsal tegmental nucleus; *egl*, external granular layer; *gp*, globus pallidus; *hb*, medial habenula; *ht*, hypothalamus; *int*, interposed cerebellar nucleus; *latc*, lateral cerebellar nucleus; *m*, medulla; *mb*, midbrain; *ob*, olfactory bulb; *oe*, olfactory epithelium; *p*, pons; *pb*, parabrachial nuclei; *po*, preoptic area; *rmb*, roof of midbrain; *s*, septum; *sc*, subplate of cortex; *sn*, substantia nigra; *tu*, olfactory tubercle; *vta*, ventral tegmental area; *vtg*, ventral tegmental nucleus; *5n*, trigeminal nucleus.

are identified also, including the cortex (Fig. 4*H*), olfactory tubercle (Fig. 4*T,W*), preoptic area (Fig. 4*Q*), substantia nigra (Fig. 4*N*), ventral tegmental nucleus (Fig. 4*T*), and lateral cerebellar nucleus (Fig. 4*N*). The expression of κ receptor in the ventral tegmental area (Fig. 4*K*), dorsal raphe (Fig. 4*H*), and pons (Fig. 4*K,N*) continues.

Compared with μ and κ, δ receptor mRNA expression levels still remain very low at this age. However, in the caudate putamen (Fig. 4*F*) and roof of midbrain (Fig. 4*C*), δ receptor labeling cells are clearly detectable. Interestingly, the pattern that higher numbers of cells expressing δ receptor mRNA are observed in the lateral caudate putamen in the adult mouse (Mansour et al., 1994)

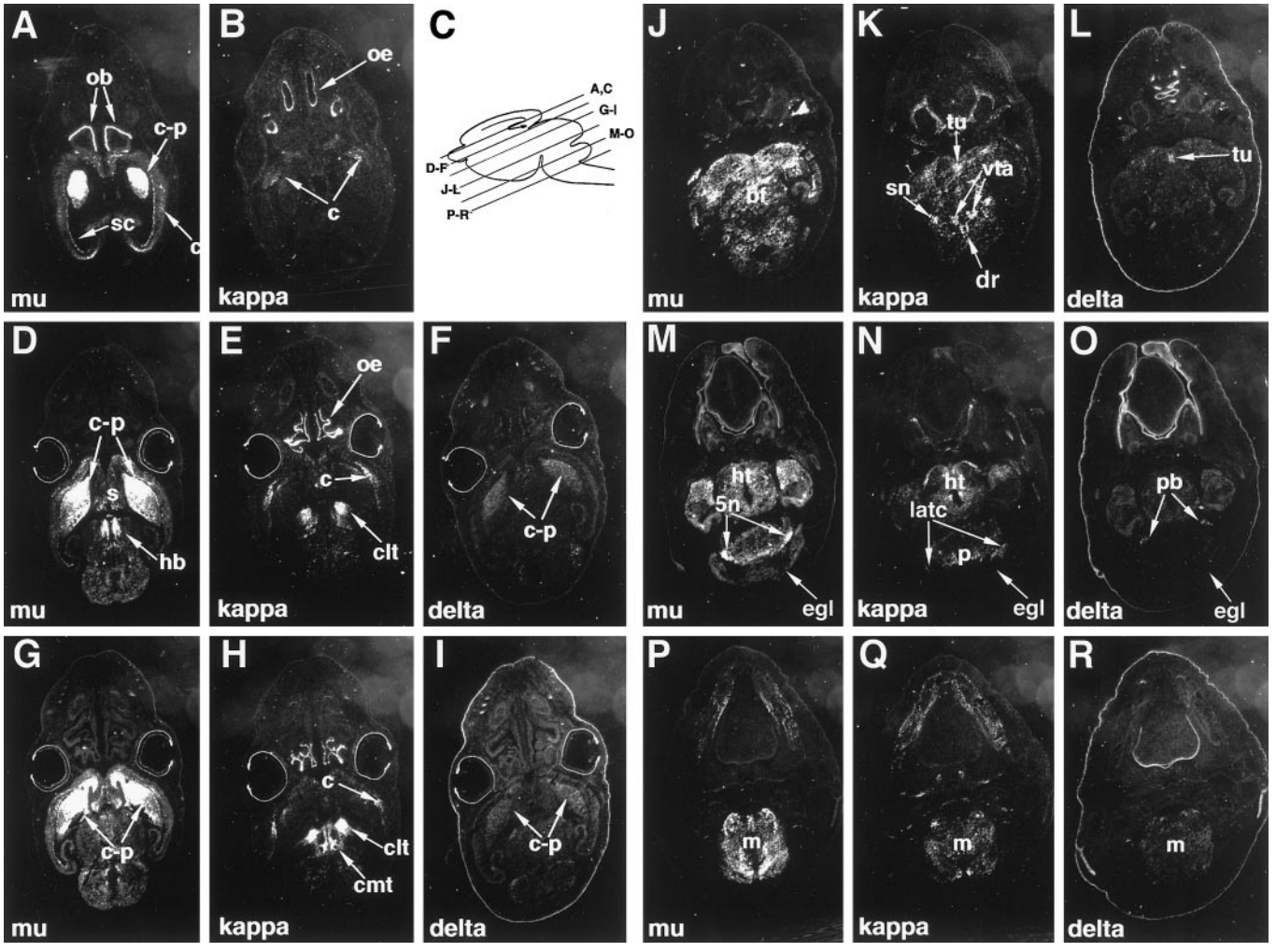

Figure 5. Dark-field photographs comparing mRNA distributions of the μ, κ, and δ opioid receptors in the brain of E19.5 embryos. Adjacent horizontal brain sections were hybridized separately with cRNAs for the μ (*A, D, G, J, M, P*), κ (*B, E, H, K, N, Q*), and δ (*F, I, L, O, R*) opioid receptors. The schematic drawing in *C* indicates the approximate planes of the horizontal sections. See Results for details (note that the pigment of the eye is not a real signal). *bf*, Basal forebrain; *c*, cortex; *clt*, centrolateral thalamus; *cmt*, centromedial thalamus; *c-p*, caudate putamen; *dr*, dorsal raphe; *egl*, external granular layer; *hb*, medial habenula; *ht*, hypothalamus; *latc*, lateral cerebellar nucleus; *m*, medulla; *ob*, olfactory bulb; *oe*, olfactory epithelium; *p*, pons; *pb*, parabrachial nuclei; *s*, septum; *sc*, subplate of cortex; *sn*, substantia nigra; *tu*, olfactory tubercle; *vta*, ventral tegmental area; *5n*, trigeminal nucleus.

already is established at this age (Fig. 4*F*). Low levels of δ receptor expression also are detected in the globus pallidus (Fig. 4*L,O*), olfactory tubercle (Fig. 4*U,X*), parabrachial nucleus (Fig. 4*O,R*), and interposed cerebellar nucleus (Fig. 4*O*).

E19.5

At this age the expression of all three receptors has a rather similar distribution to that of E17.5, with the addition of a few new sites. μ receptor mRNA continues to be detected in the olfactory bulb (Fig. 5*A*), subplate of the cortex (Fig. 5*A*), septum (Fig. 5*D*), caudate putamen (Fig. 5*A,D,G*), medial habenula (Fig. 5*D*), hypothalamus (Fig. 5*M*), basal forebrain (Fig. 5*J*), trigeminal nucleus (Fig. 5*M*), and medulla (Fig. 5*P*). In addition, low levels of μ transcripts appear in the cortex (Fig. 5*A*).

The centromedial and centrolateral thalamus continue to express the highest level of κ receptor transcripts in the brain at this age (Fig. 5*E,H*). In addition, cells expressing κ receptor mRNA are still located in the cortex (Fig. 5*B,E,H*), olfactory tubercle (Fig. 5*K*), hypothalamus (Fig. 5*N*), ventral tegmental area (Fig. 5*K*), substantia nigra (Fig. 5*K*), dorsal raphe (Fig. 5*K*), pons (Fig. 5*N*), lateral cerebellar nucleus (Fig. 5*N*), and medulla (Fig. 5*Q*).

Again, δ receptor expression is confined to regions that include caudate putamen (Fig. 5*F,I*), olfactory tubercle (Fig. 5*L*) and parabrachial nucleus (Fig. 5*O*).

No labeling above background has been detected for any of the three receptor genes in the external granular layer of the cerebellum at E15.5 (see Fig. 3*C*), E17.5 (see Fig. 4*M–O*), and E19.5 (Fig. 5*M–O*).

Spinal cord and dorsal root ganglia (DRG)

Spinal analgesia can be mediated by all three types of opioid receptors. Therefore, we also have examined opioid receptor expression in the developing spinal cord and dorsal root ganglion (DRG).

Among the three opioid receptors, μ is the first to be detected in the spinal cord and appears at E11.5 (Fig. 6*A*). By E13.5, μ transcripts begin to be detected at a low level in the DRG (Fig. 6*B*). At this age and at E15.5, the hybridization signals of μ receptor are still restricted to the ventral aspect of the spinal cord (Fig. 6*B,D*). At E17.5, μ expression has expanded to cover both the dorsal and ventral spinal cord regions as well as the DRG (Fig. 6*G*).

The δ receptor, in contrast to its low expression in the brain, is

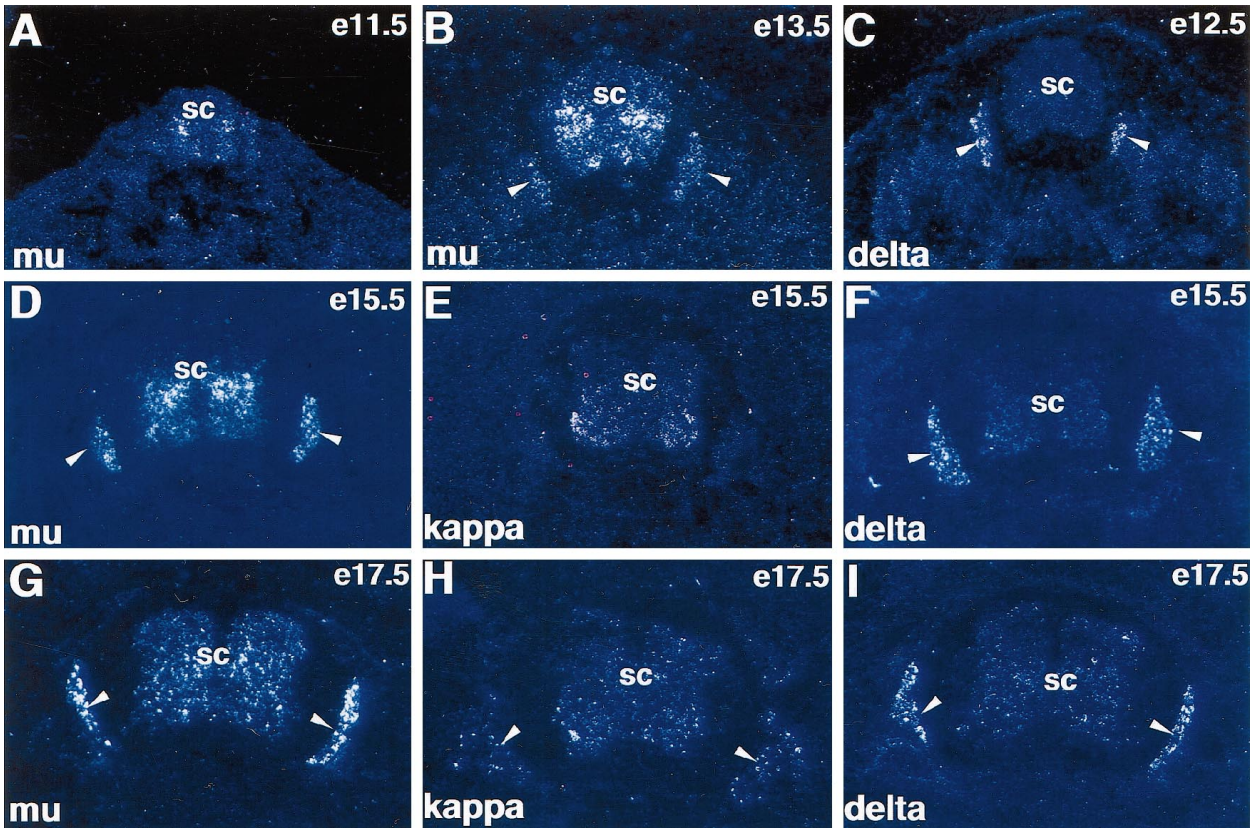

Figure 6. Dark-field photographs demonstrating mRNA expression of the μ, κ, and δ receptors in the spinal cord (*sc*) and DRG (*arrowheads*). μ receptor mRNA can be detected in the spinal cord as early as E11.5 (*A*). At E15.5, μ expression in the DRG is apparent, and the expression in the spinal cord is restricted to the ventral region (*D*). δ receptor is the first one that can be detected in the DRG, at E12.5 (*C*), before its expression in the spinal cord starts at E15.5 (*F*). At E17.5, the expression of all three receptors has expanded to cover both the dorsal and ventral regions of the spinal cord as well as the DRG (*G–I*).

the first opioid receptor expressed in the DRG, at E12.5 (Fig. 6*C*). δ expression in the spinal cord begins significantly later, at E15.5, and the expression is restricted to the ventral part of spinal cord (Fig. 6*F*), similar to the μ receptor. By 17.5, δ expression has expanded to the dorsal region of the spinal cord (Fig. 6*I*).

Compared with μ and δ, κ receptor expression in both spinal cord and DRG begins relatively late. κ expression first appears in the ventral spinal cord at E15.5 (Fig. 6*E*) and extends to the dorsal spinal cord by E17.5, when expression in the DRG also begins (Fig. 6*H*).

Peripheral ganglia

Figure 7 illustrates mRNA expression patterns of the μ, κ, and δ opioid receptors in peripheral ganglia. As discussed earlier, μ receptor expression in the facial (VII) and vestibulocochlear (VIII) ganglia begins as early as E10.5 (see Fig. 1*C*) and continues through E13.5 (Fig. 7*A*). In the vagus ganglia, μ receptor-expressing sites are first detected at E11.5 (Fig. 7*D*), whereas in the trigeminal (Fig. 7*A*) and sympathetic ganglia (Fig. 7*E*), μ expression begins at E13.5. Interestingly, a complementary expression pattern of μ receptor and proenkephalin mRNAs is found at E12.5. Whereas μ is expressed in the facial and vestibulocochlear ganglia (Fig. 7*F*), proenkephalin is expressed in the adjacent mesenchyme (Fig. 7*G*) (M. Zheng and J. Pintar, unpublished data), suggesting a local opioid circuit in this region.

In addition to the trigeminal ganglia (Fig. 7*C*), δ receptors are expressed in the facial ganglia (Fig. 7*C*) at E13.5. κ receptor gene also is detected in the trigeminal ganglia at E13.5, but at a much lower level as compared with μ and δ receptors (Fig. 7*B*), and is absent from all other peripheral ganglia, as mentioned above. Cells in these peripheral ganglia continue to express each of the opioid receptor genes through late gestation (E19.5; data not shown).

The expression of δ, κ, and μ characterizes discrete fetal peripheral tissues

Additional fetal expression sites of the opioid receptor genes are illustrated in Figure 8. These sites include, for κ and δ, expression in several non-neural peripheral tissues, whereas additional sites of μ expression include neural cells of the retina and gut.

By mid-gestation, before its expression in the CNS begins, δ receptor mRNA is already present in several peripheral tissues, such as the tooth (Fig. 8*B,F*), mesenchyme of the limb bud (Fig. 8*C,G*), heart (Fig. 8*M*), and the olfactory epithelium (Fig. 8*A,E*). Surprisingly, δ receptor also is detected in the infundibulum (Fig. 8*I*), which develops into the posterior lobe of the pituitary (Fig. 8*J,K*).

Cells expressing κ mRNA are located in the outflow track of the heart (Fig. 8*D,L*) and mesenchyme just beneath the dorsal limb ectoderm (Fig. 8*H,L*), which is also a site of prodynorphin gene expression (M. Zheng and J. Pintar, unpublished data). Additional κ receptor-expressing sites are detected in the physiological umbilical hernia (Fig. 8*Q*), limb (Fig. 8*U*), cartilage (Fig. 8*V*), ossification (Fig. 8*V,W*), and tongue (Fig. 8*W,X*). There is also an overlapping but distinct expression pattern of κ and δ receptors in the olfactory epithelium (Fig. 8*N,R*).

Compared with its wide expression in the CNS, the distribution

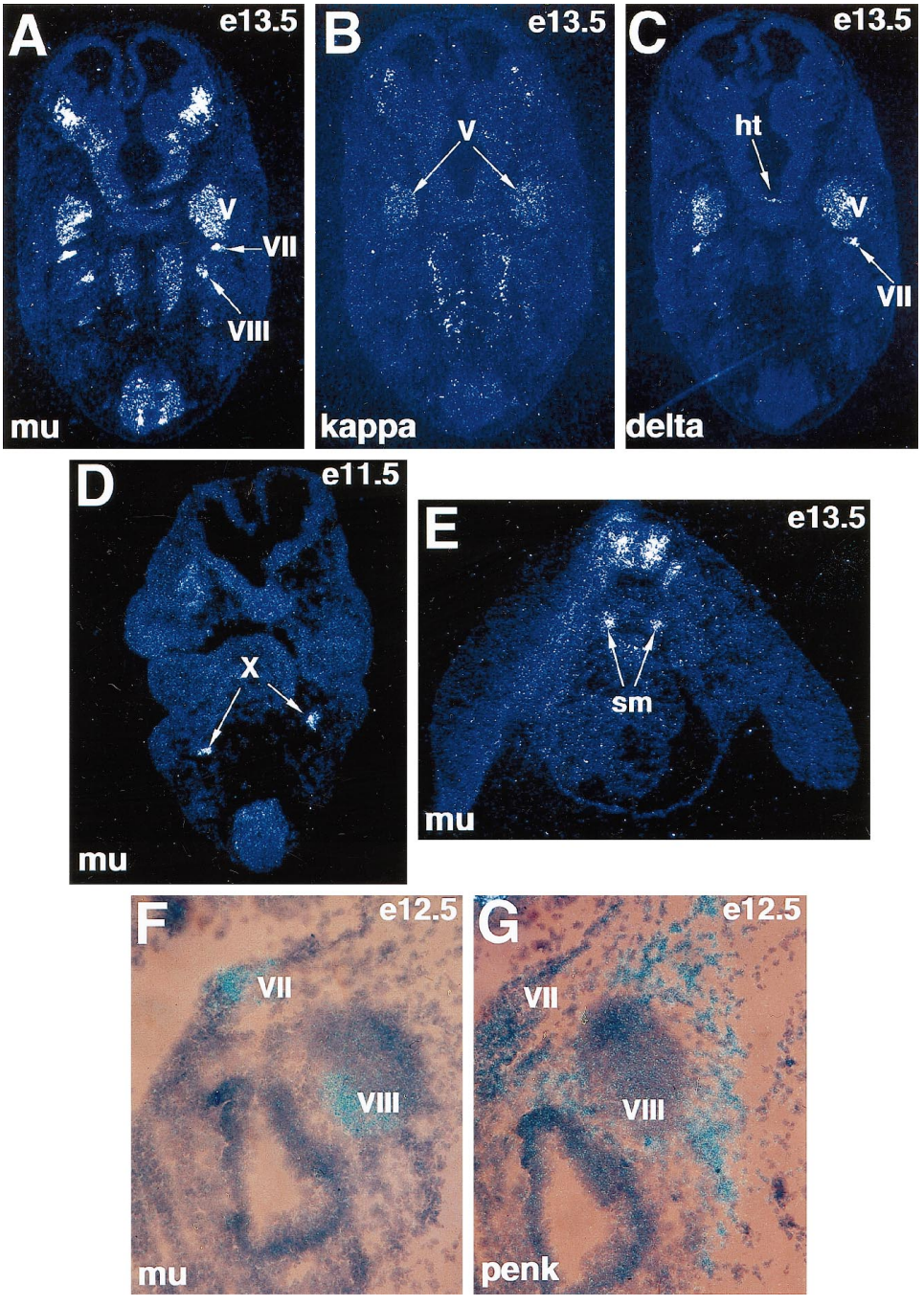

Figure 7. Illustration of the mRNA expression pattern of the μ, κ, and δ opioid receptors in peripheral ganglia. Note the labeling of the μ receptor gene in the trigeminal (*V*), facial (*arrow*, *VII*), vestibulocochlear (*arrow*, *VIII* in *A*), vagus (*arrows*, *X* in *D*), and sympathetic (*arrows*, *sm* in *E*) ganglia. κ receptor also is expressed in the trigeminal ganglia (*B*), whereas δ receptor gene is expressed not only in the trigeminal but also in facial ganglia and ventral hypothalamus (*arrow*, *ht* in *C*). Also note the complementary expression pattern of the μ receptor and proenkephalin gene. Although μ is expressed in the facial and vestibulocochlear ganglia (*F*), proenkephalin is expressed in the adjacent mesenchyme (*G*).

of μ receptor mRNA in peripheral tissues is much more restricted, being found in the myenteric plexus of the intestine (Fig. 8*S*), stomach (Fig. 8*P*), and inner nuclear layer of the retina (Fig. 8*T*). In the intestine, although μ is expressed in the myenteric plexus (Fig. 8*S*), κ receptor mRNA is expressed in the mucosal epithelium (Fig. 8*O*).

DISCUSSION

In this study we have provided the first comparative analysis of cellular localization of the μ, κ, and δ opioid receptor mRNAs during prenatal mouse development. The expression patterns of the δ, μ, and κ receptor genes begin significantly earlier than previously suggested by ligand binding and are distinct at all ages. κ receptor is the first to be detected at E9.5, in the gut mucosa. By E11.5, κ receptor expression begins in the midbrain, extends to many other regions by late gestation, and also is detected in several peripheral tissues, including the olfactory epithelium, heart, limb, and tongue. Significant levels of μ mRNA are detected in the facial and vestibulocochlear preganglion complex as early as E10.5, before CNS expression begins at E11.5 in the basal ganglia. μ is also the first opioid receptor to be detected in the spinal cord, at E11.5, as well as several peripheral ganglia and enteric neurons in the intestine and stomach. In contrast, δ receptor mRNA is expressed in the prenatal brain at a much lower level than μ and κ. Although δ expression is first detected in the pons at E13.5 and then extends to several regions, including the caudate putamen and olfactory tubercle, the expression levels remain low even near term, except for high but transient expression in the infundibulum. In contrast to its late appearance in the

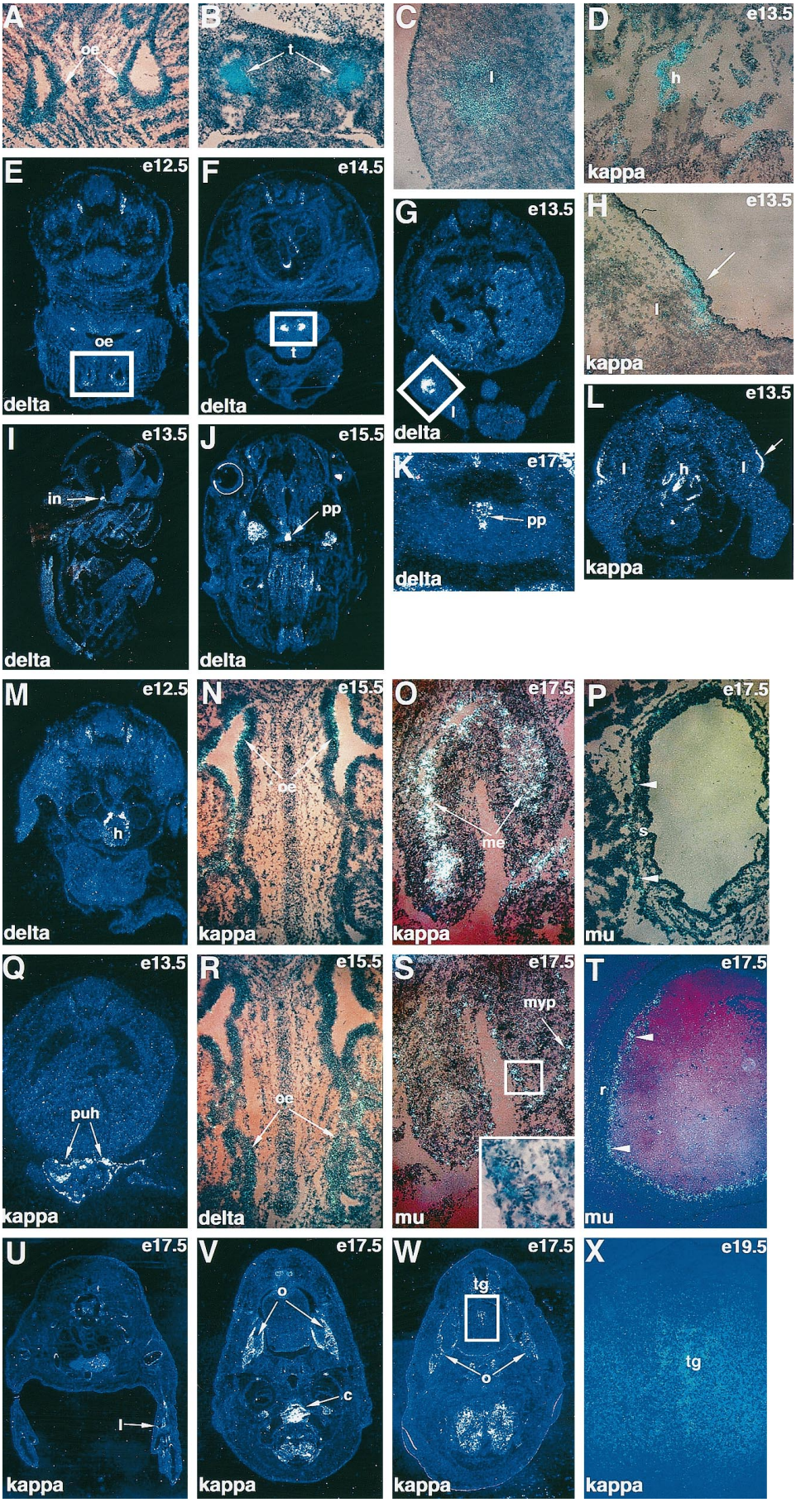

Figure 8. Additional opioid receptor expression sites in peripheral tissues. *A, B, C,* and *X* are high-power photographs corresponding to the regions outlined in the adjacent dark-field low-power photographs *E, F, G,* and *W*, respectively. *Inset* in *S* shows the region outlined in the same panel with higher magnification. δ receptor mRNA is detected in the infundibulum (*arrow* in *I*), which develops into posterior lobe of the pituitary (*arrow*, *pp* in *J*, *K*). Cells expressing κ receptor are located in the mesenchyme just beneath the dorsal limb ectoderm (*arrows* in *H*, *L*) and outflow track of the heart (*h* in *D*, *L*). Note the overlap but distinct expression pattern of κ and δ receptors in the olfactory epithelium (*arrows*, *oe* in *N*, *R*). In the intestine, κ receptor is expressed mainly in the mucosal epithelium (*arrows*, *me* in *O*), whereas μ is expressed in the myenteric plexus (*arrows*, *myp* in *S*). μ receptor mRNA also is detected in the inner nuclear layer of the retina (*arrowheads* in *T*) and stomach (*arrowheads* in *P*). *c*, Cartilage; *l*, limb; *o*, ossification; *puh*, physiological umbilical hernia; *r*, retina; *s*, stomach; *t*, tooth; *tg*, tongue.

brain, the δ receptor is the first opioid receptor expressed in the DRG, at E12.5. In addition, δ is expressed in the trigeminal and facial ganglia as well as several peripheral tissues, including olfactory epithelium, heart, limb, and tooth. Although the distribution of prenatal opioid receptor immunoreactivity has not been mapped in detail for any of the opioid receptors, the μ receptor protein has been detected in the striatum soon after neurons first differentiate (data not shown).

Taken together, the above results show early receptor gene expression that significantly extends inferences from previous ligand-binding studies showing initial receptor binding activities for μ and κ ligands at E12.5 and E14.5, respectively (Rius et al., 1991). The detection of the δ receptor mRNA in the DRG at E12.5, the pons at E13.5, and the caudate putamen at E17.5 also significantly extends binding data, which have been unable to detect δ binding activity until P1 (Kornblum et al., 1987; Rius et al., 1991). Some anatomical information on the postnatal development of the opioid receptor expression in rodent brain has been provided via ligand autoradiographic analysis (Xia and Haddad, 1991). Generally, the late gestation expression patterns presented here are consistent with the binding distribution reported at P1, but additional mRNA-containing sites (e.g., κ in hypothalamus and thalamus and δ in globus pallidus) do not show labeling until several days later (Xia and Haddad, 1991).

Our results show that, during late prenatal stages, many aspects of the adult receptor expression pattern (Mansour et al., 1994) are already present, but with some exceptions. For example, the expression levels of μ receptor are extremely low in the thalamus, which is a major site of expression in the adult (Mansour et al., 1994). Cells expressing μ receptors in the caudate putamen remain homogeneous in distribution even until E19.5, lacking the patch-like pattern seen in the adult (Mansour et al., 1994). Finally, δ is not detected prenatally in the neocortex at all, whereas in the adult the neocortex is one of the regions with the highest δ expression levels (Mansour et al., 1994). Even at late prenatal stages (E17.5 and E19.5), δ mRNA expression in the brain is restricted to only a few regions, with expression levels significantly lower than that in adult brain. By early P4, δ receptor expression has expanded to many other areas of the brain, and the expression levels have increased dramatically (Y. Zhu and J. Pintar, unpublished data), indicating a rapid maturation process for the δ receptor system in early postnatal development. Examples of transient expression were limited. Although in adult mouse the δ receptor mRNA is detected in the anterior, but not posterior, lobe of pituitary (Bzdega et al., 1993), our results demonstrate transient prenatal expression of the δ receptor gene instead in the posterior pituitary. This expression, along with the transient κ expression in mesenchyme just outside the neural tube, represents the only major sites of transient opioid receptor expression noted in neural-related structures.

In many instances, opioid receptor gene expression is detected at the earliest stages of neurogenesis in both the CNS and periphery. For example, the detection of the μ receptor mRNA in the facial and vestibulocochlear preganglion complex, as well as its expression at early ages in differentiating peripheral ganglia and striatum, suggests that opioid receptors may be involved in early postmitotic processes accompanying neuronal maturation and differentiation. The expression of the κ receptor in the gut mucosa at E9.5 and pia-arachnoid progenitor cells (McLone and Bondareff, 1975) outside the neural tube at E10.5 suggests that early developmental events in non-neural tissues also may be mediated by the opioid receptors. In contrast, at the prenatal stages examined here, we found no evidence for expression of any opioid receptor in any germinal center for CNS cells, including in ventricular or subventricular zone cells of the neural tube or in the proliferative cells of the external granular layer of the prenatal cerebellum. Several previous studies have suggested that postnatal neurogenesis, particularly in the external granule layer of the cerebellum as well as gliogenesis throughout the brain, may be modulated by opioid receptor agonists and antagonists (Zagon and McLaughlin, 1983, 1986, 1987; Hauser et al., 1987; Knapp and Hauser, 1996). The data presented here suggest that opioid receptor expression in these responsive populations is not initiated until postnatal ages.

Previous ligand-binding study (Attali et al., 1990) with rat spinal cord homogenates has shown that δ receptor binding could not be detected prenatally, although κ and μ binding sites first appeared at E15 and the number of κ sites predominated at all ages. However, our results show that, in mouse spinal cord, μ transcripts are the first to be detected and have the highest mRNA expression levels among the three receptors, whereas developments of the δ and κ parallel each other at similar transcription levels. This discrepancy may attributable to species differences or mismatches between the mRNA transcription and actual receptor binding. The observed ventral-to-dorsal gradient in the temporal appearance of all three opioid receptor genes in the spinal cord is in accord with the neurogenetic axis (Nornes and Das, 1972). Interestingly, proenkephalin also exhibits a similar ventral-to-dorsal gradient in its expression in the spinal cord during mouse prenatal development (M. Zheng and J. Pintar, unpublished data). Therefore, it is possible that the embryonic expression of the opioid receptors and ligands is closely regulated in this tissue.

The δ receptor, in contrast to its low expression in the brain, is the first opioid receptor expressed in the DRG, at E12.5. μ appears later at E13.5, and κ is expressed by E17.5. In the rat DRG, larger ganglion cells are produced (at ~E12) before the smaller ganglion cells (at ~E15) (Altman and Bayer, 1984). Therefore, our data support previous results showing that, in adult DRG, δ receptor mRNA is expressed predominantly in large-diameter neurons, μ receptor is localized in medium- and large-diameter neurons, whereas κ receptor is localized in smaller diameter neurons (Mansour et al., 1994).

We also have compared the mRNA expression patterns of the opioid receptors with those of their ligands (i.e., proopiomelanocortin, proenkephalin, and prodynorphin), with several examples of possible local opioid circuits identified. For example, whereas μ is expressed in the facial and vestibulocochlear ganglia, the proenkephalin gene is expressed in the adjacent mesenchyme. Also, the expression of proenkephalin in the trigeminal ganglia at E12, interposed cerebellar nucleus at E17.5, and heart at E12 (M. Zheng and J. Pintar, unpublished data) overlaps with δ receptor mRNA expression spatially and temporally. Expression of the κ receptor gene in the mesenchyme just beneath the dorsal limb ectoderm also overlaps with prodynorphin expression in the rat at a comparable age (M. Zheng and J. Pintar, unpublished data). These results all suggest a role for these local circuits in developmental processes that is supported in part by recent genetic evidence. For example, in μ opioid receptor knock-out mice, specific aspects of analgesia thought to be mediated by both δ receptor (Sora et al., 1997) and κ receptor (A. Schuller, M. King, G. Pasternak, and J. Pintar, unpublished data) agonists were reduced dramatically, suggesting that the μ receptor may regulate the development of δ and κ receptors;

alternatively, selective δ and κ receptor drugs may require μ receptor occupancies for full efficiency.

In conclusion, our results show that mRNAs for the μ, κ, and δ opioid receptors are expressed earlier than previous ligand-binding studies suggested and exhibit distinct temporal and spatial patterns of distribution in both the nervous system and peripheral structures during prenatal mouse development. Although no evidence for significant transient expression of any of the three receptors that were examined has been found, the presence of the opioid receptors on multiple populations of neurons soon after their differentiation suggests participation in early developmental events that now can be tested genetically.

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