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Differential Ontogeny of Multiple Opioid Receptors (μ, δ, and κ)[1]

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Abstract

We investigated the postnatal ontogeny of opioid receptors in rat brain under assay conditions which, when combined with computerized analysis, effectively reflect the developmental profile of high affinity binding to μ, δ, and κ subpopulations. Concentrations of μ sites were assessed with the selective ligand ^{3}H-[D-ala^2,mePhe^4,gly-ol^5]enkephalin (DAGO). The other two sites were analyzed in binding assays with less selective radioligands but in the presence of specific unlabeled ligands which suppress cross-reactivity. We utilized ^{3}H-[D-ala^2,D-leu^5]enkephalin (DADL) in the presence of 10 nM DAGO to label δ sites and ^{3}H-ethylketocyclazocine (EKC) in the presence of 100 nM DADL + 100 nM [D-ala^2,mePhe^4,Met(0)ol^5]enkephalin to detect κ receptors. After birth, the density (femtomoles per milligram of wet weight) of μ sites declined for several days and then rose sharply over the next 2 weeks, increasing 2-fold by adulthood. Delta (δ) sites appeared in the second week postnatal and increased more than 8-fold in the next 2 weeks. Levels of κ receptors were relatively low at birth and increased slowly (2-fold, overall). Computerized analyses of binding data revealed that DAGO and DADL were binding to single populations of sites throughout the postnatal period. DAGO and EKC affinities did not fluctuate in this period, whereas DADL affinities were low for the first week and then rose to adult levels. In summary, μ, κ, and δ receptors exhibit differential postnatal developmental profiles. The former two are present at birth, whereas the latter appears in the second week. The postnatal increase for all three sites appear to be preceded by the previously demonstrated emergence of opioid peptides.

Opioid receptors in the central nervous system of the rat, as detected by the specific binding of ^{3}H-naloxone or ^{3}H-naltrexone, are present at embryonic day 14, and their density increases until adulthood (Clendeninn et al., 1976; Coyle and Pert, 1976; Garcin and Coyle, 1976; Kent et al., 1982; Wohltmann et al., 1982). In addition, the development of enkephalin-binding sites has been examined in attempts to demonstrate a correlation between the ontogeny of opioid-binding sites and the developmental patterns of endogenous opioid peptides (Coyle and Pert, 1976; Bayon et al., 1979; Patey et al., 1980; Tsang et al., 1982). However, radioligands used in all of these studies were not entirely site specific. Considering the multiplicity of both opioid peptides and receptors, attempts to correlate their postnatal appearance require more specific binding assays. Recently, highly specific binding assays have been developed that appear to eliminate the problem of cross-reactivity. For example, μ sites appear to be labeled almost exclusively with ^{3}H-[D-ala^2,mePhe^4,gly-ol^5]enkephalin (DAGO) (Kosterlitz et al., 1981). Furthermore, site-specific cold ligands can be added to incubation mixtures containing less specific radioligands to improve selectivity for the target site (Chang et al., 1981; Gillan and Kosterlitz, 1982). By using ^{3}H-ethylketocyclazocine (EKC) in the presence of excess amounts of unlabeled μ- and δ-specific ligands, putative κ sites have been labeled. Likewise, ^{3}H-[D-ala^2,D-leu^5]enkephalin (DADL) will effectively label only δ sites in the presence of an excess of unlabeled μ-selective ligand.

The objective of the present study was to re-examine the development of opioid receptors in rat brain by utilizing combinations of ligands cited above and analyzing the binding data with a weighted, nonlinear regression, model-fitting computer program. By this approach, highly site-specific data can be obtained that more closely reflect the "true" developmental profile of these three opioid receptor types. The results reveal a differential development of μ, δ, and κ sites with the δ receptors not appearing until the second week postnatal.

Materials and Methods

Membrane preparation. Timed-pregnant Sprague-Dawley rats were obtained from Sasco Laboratories (St. Louis, MO) and maintained as previously described (Roth et al., 1980). Birth-dated pups (<12 hr) were sacrificed by decapitation at days 1, 4, 5, 7, 10, 14, 21, 28, or 35, and the brain was rapidly removed. Adult controls were sacrificed in a like manner. Whole brain was dissected into fore- and hindbrain by a coronal cut between the cerebrum and cerebellum, and the tissue for each age group (from one to eight animals) was pooled. Tissue was homogenized with a Polytron in 60 ml of 50 mM Tris-HCl buffer, pH 7.4, at 4°C, and was centrifuged at 20,000 × *g* for 25 min. The membranes were resuspended in fresh buffer (Dounce homogenizer) and incubated at 25°C for 20 min to remove endogenous opioid peptides, and the centrifugation was repeated. The pellet was taken up in sufficient Tris buffer (pH 7.4) to yield a final concentration of approximately 1 mg of protein/ml.

Binding assay. The final membrane homogenate was incubated with radioligand, a constant concentration of cold suppressor (if appropriate), and 12 varying concentrations of cold ligand, producing a Scatchard analysis by the method of isotopic dilution. The isotopic dilutions of either 0.1 nM ^{3}H-DAGO (60 Ci/mmol; Amersham, Arlington Heights, IL), 1 nM DADL (19.4 Ci/mmol; Amersham) in the presence of 10 nM DAGO, or 1 nM ^{3}H-EKC (16.5 Ci/mmol, New England Nuclear, Boston, MA) in the presence of 100 nM DADL and 100 nM [D-ala^2,mePhe^4,Met(0)ol^5]enkephalin (FK33824) were determined. The final volume of the incubation mixture for ^{3}H-DAGO and ^{3}H-DADL was 0.5 ml, and that for ^{3}H-EKC was 1 ml. Each assay was incubated

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for 1 hr at 25°C prior to rapid filtration and collection of membrane-bound ligand on glass fiber filters (Whatman GF/B) as previously described (Roth et al., 1981). Protein concentations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Computerized analysis. The curves produced by the isotopic dilution of radioligand were analyzed using the weighted, nonlinear, least squares regression LIGAND program (Munson and Rodbard, 1980). This iterative procedure constructs models of binding according to the laws of mass action for the interaction of multiple ligands with multiple binding sites. After determination of the binding affinity and capacity for each individual experiment with the LIGAND program, the results of the three or more experiments were then averaged to provide a weighted mean and SEM. All values, except where noted, represent the mean of three experiments performed in duplicate and calculated as follows:

$$\text{weighted mean} = \sum(WiXi)/\sum(Wi)$$

$$\text{where } Wi = 1/\text{SEi}^2; \text{ weighted SE} = 1/\sqrt{\sum Wi}.$$

Results

Binding affinities. Preliminary association experiments were performed using the assay conditions described above to ascertain that equilibrium binding was achieved within 1 hr for each radioligand. Isotopic dilution binding assays were then conducted at each age (Fig. 1), and K_d's were estimated (Table I). Computer analyses of ^{3}H-DAGO and ^{3}H-DADL binding revealed that a two-site model did not fit better than a one-site paradigm for all ages studied. Although, at times, the two- and one-site models fit equally well, the law of parsimony was invoked and the simpler model was accepted.

In almost all cases for ^{3}H-EKC binding, we observed that the two-site model fit better than a one-site model. However, there were a few experiments in which a one-site model fit the data as well. Since at all ages at least two of the three experiments fit the two-site model, we assume the anomalous data were due to experimental error that by chance resulted in a better fit for the one-site model. The high affinity EKC binding is designated as the κ site on the basis of displacement studies performed in the adult with 1 nM ^{3}H-EKC (+ 100 nM DAGO + 100 nM DADL) in the presence of dynorphin (1–13) (IC_{50} = 7 nM), SKF 10047 (IC_{50} = 60 nM), or phencyclidine (PCP) (<10% displacement by 1 to 1000 nM PCP) (data not shown). Also, the K_d values for ^{3}H-EKC binding to the second site were approximately two orders of magnitude lower than for the high affinity binding site.

The apparent K_d for ^{3}H-DAGO and ^{3}H-EKC did not change (within experimental error as determined by analysis of variance) throughout the postnatal period. With respect to the binding of ^{3}H-DADL before day 10 postnatal, we observed a small number of low affinity binding sites whereas, thereafter, we found only a high affinity form under our assay conditions. Because of the low affinity exhibited for ^{3}H-DADL during early development, it appears that few high affinity δ sites are present in the first 10 days after birth.

Binding capacities. All three opioid receptor types increased in density (femtomoles per milligram of wet weight) during postnatal development of the forebrain. From day 1 to adult, the number of both ^{3}H-DAGO and ^{3}H-EKC binding sites doubled, and ^{3}H-DADL receptor levels increased more than 8-fold (Fig. 2). Interestingly, levels of ^{3}H-DAGO binding sites initially declined after birth, resulting in a 32% reduction by day 4. This was followed by a rapid increase from day 7 through day 14, and adult levels were reached by day 21. This result is similar to the Scatchard analyses by Pasternak et al. (1980), showing a 2.8-fold increase in ^{3}H-morphine binding density for brain membranes of 2- and 14-day-old rats. ^{3}H-DADL binding sites increased linearly from day 4 through day 28, when adult levels were reached. ^{3}H-EKC binding revealed relatively sparse but measurable amounts of κ sites in rat brain that rose slightly, peaking at day 35 and declining to adult levels.

When the data are expressed as femtomoles per milligram of protein, the developmental profiles are altered somewhat (Fig. 3), reflecting a concurrent shift in the ratio of protein to wet weight. During the postnatal period this ratio displays a shallow sigmoidal curve, increasing from day 7 to day 21 and then leveling off (data not shown). Upon expressing the data in femtomoles per milligram of protein, a shift in the nadir of the μ sites is seen, and only the concentration of ^{3}H-DADL binding sites increased over the entire time period. A statistically significant 32% reduction in μ sites and no change in κ sites were observed.

Binding of ^{3}H-EKC was also determined in the developing hindbrain (brainstem and cerebellum). On day 1 both fore- and hindbrain possessed equal amounts of these receptors (femtomoles per milligram of wet weight) (Fig. 2). However, by day 5, receptor levels changed, with the concentration of κ sites in the hindbrain being about 2-fold higher. There appears to be no further fluctuation in density until day 14, whereupon hindbrain levels decrease and forebrain sites increase.

Discussion

Using site-specific assays and computer modeling, we demonstrated a distinct difference in timing and magnitude of development for the three types of opioid receptors examined (Figs. 2 and 3).

Mu (μ) sites. ^{3}H-DAGO is thought to interact at the μ site of adult rat brain membranes with a high degree of selectivity (Kosterlitz et al., 1981). Computerized analyses revealed that, in our hands, this ligand bound to a single high affinity population of receptors throughout the postnatal period. This was reinforced by the constancy of the estimated K_d for DAGO binding throughout postnatal development (Table I). These μ receptors are present at birth in rat forebrain, and, after a decline in concentration during the first few days, they undergo a subsequent rapid increase. A similar pattern has been observed for striatal naloxone-binding sites (Kent et al., 1982).

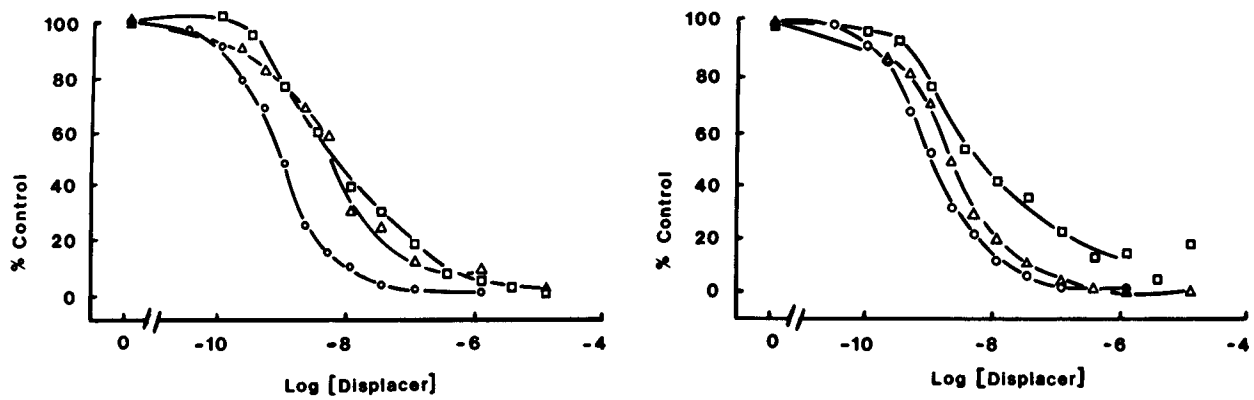

Figure 1. Representative displacement curves for the isotopic dilution of 0.1 nM ^{3}H-DAGO (O), 1 nM ^{3}H-DADL (Δ) (in the presence of 10 nM DAGO), and 1 nM ^{3}H-EKC (□) (in the presence of 100 nM DADL and 100 nM FK33824) determined for day 7 (*left*) and adult (*right*) crude forebrain homogenates. At each age, similar 12-point displacement curves were generated in three separate experiments performed in duplicate and were analyzed to determine affinities and binding capacities.

TABLE I

Estimated K_d values for DAGO, DADL, and EKC binding to rat brain membranes during postnatal development[a]

Age	DAGO	DADL	EKC			
			Forebrain		Hindbrain	
			Site 1	Site 2	Site 1	Site 2
1	0.557 ± 0.031	14.12 ± 2.29	3.83 ± 1.33	382 ± 151	2.16 ± 0.52	1160 ± 610
4	0.629 ± 0.021	4.01 ± 1.22				
5			4.62 ± 0.65[b]	357 ± 78[b]	3.82 ± 0.33[b]	941 ± 254[b]
7	0.569 ± 0.026	5.64 ± 1.02	2.31 ± 0.59	276 ± 73	1.86 ± 0.25	303 ± 68
10	0.530 ± 0.016	3.57 ± 0.56	4.54 ± 0.69	268 ± 151	4.41 ± 0.52	2170 ± 2080
14	0.665 ± 0.035	1.45 ± 0.16	1.30 ± 0.40	125 ± 27	3.50 ± 0.50	649 ± 189
21	0.615 ± 0.029	1.47 ± 0.24	6.59 ± 0.93		4.86 ± 1.30	456 ± 306
28	0.687 ± 0.021	2.19 ± 0.27	3.55 ± 0.76[b]	390 ± 83[b]	9.94 ± 3.4[b]	838 ± 226[b]
35			5.44 ± 0.93	634 ± 113	6.03 ± 1.10	618 ± 76
Adult	0.665 ± 0.016	1.09 ± 0.10	1.58 ± 0.83[c]	772 ± 440[c]	1.94 ± 0.46[c]	316 ± 78[c]

[a] Data were obtained from displacement curves as described in Figure 1. Values are nM ± SEM. $N = 3$ except where indicated.
[b] $N = 4$.
[c] $N = 7$.

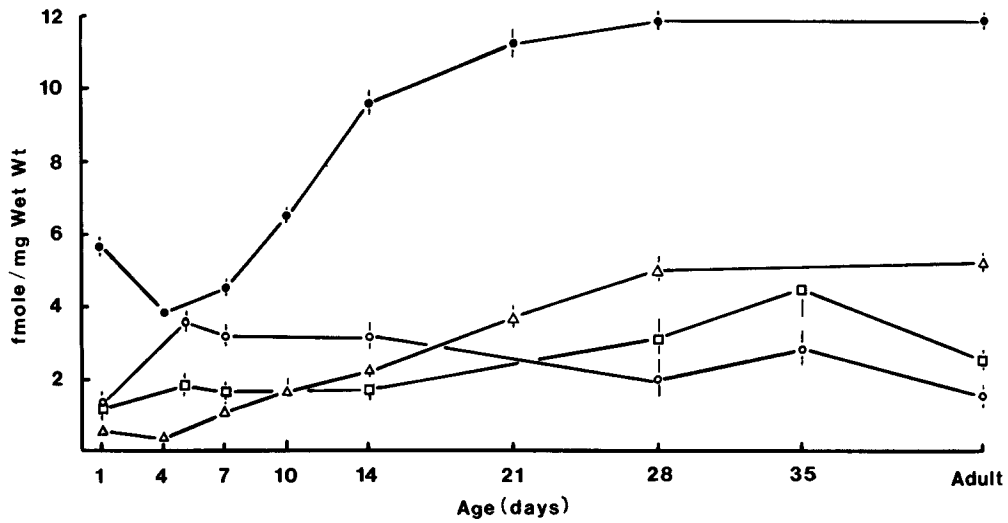

Figure 2. The ontogeny of ^{3}H-DAGO (●), ^{3}H-DADL (△), and high affinity ^{3}H-EKC (□) binding capacity in rat forebrain expressed as femtomoles bound per milligram of wet weight. The binding of ^{3}H-EKC in the rat hindbrain (brainstem and cerebellum) is also presented (○). Binding capacity was determined by isotopic dilution (as described in Fig. 1), and values reported are the weighted mean and SEM of three experiments (except where noted in Table I).

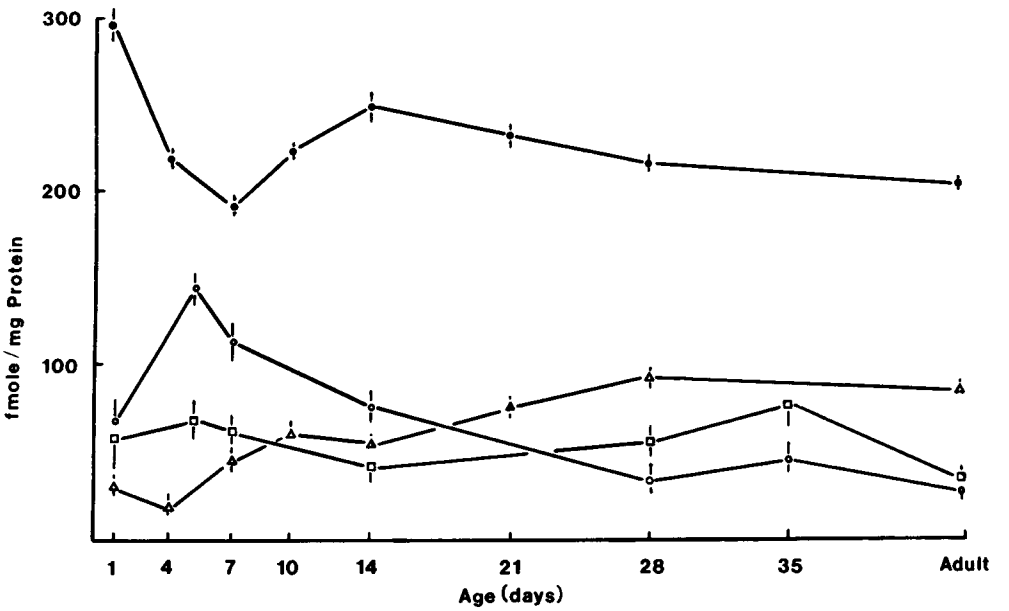

Figure 3. The ontogeny of ^{3}H-DAGO (●)m ^{3}H-DADL (△), and high affinity ^{3}H-EKC (forebrain, □; hindbrain, ○) binding capacity. The results presented in Figure 2 are here expressed as femtomoles per milligram of protein with the protein per assay measured by the method of Lowry et al. (1951).

Despite the decline in density, the number of μ sites per brain continues to rise during the first week after birth. The most pronounced rate of increase in their levels occurred during the second and third weeks postnatal.

Delta (δ) sites. Delta sites, as labeled by ^{3}H-DADL in the presence of 10 nM DAGO, are present in only very low levels, if at all, during the first week postnatal. From the second through the fourth week a nearly linear increase in their concentration occurs. The subsequent appearance in δ sites correlates well with the rise of μ site levels. In addition, we had previously demonstrated that naloxone-reversible effects of morphine on striatal catecholamine metabolism were not detectable until the end of the first week postnatal (Roth et al., 1980). By day 6, a statistically significant rise in striatal, 3,4-dihydroxyphenylacetic acid levels was elicited by intraperitoneal injection of morphine (10 and 20 mg/kg). Hence, the appearance of this effect parallels the rise in μ and δ receptors observed in forebrain in this study (Fig. 2) as well as the elevation of naloxone- and enkephalin-binding sites seen in neonatal rat striatum by Kent et al. (1982). Indeed, the second week postnatal appears to be one of significance to rat brain maturation. Cholinergic (Coyle and Yamamura, 1976) and dopaminergic (Pardo et al., 1977; Morris et al., 1980; Pittman et al., 1980) receptors also rise sharply at this time.

Similar to ^{3}H-DAGO, the binding of ^{3}H-DADL did not exhibit an improved fit for the two-site model in the time period examined. In contrast to ^{3}H-DAGO binding, the estimated K_d for ^{3}H-DADL was not constant but decreased significantly from day 1 to day 10, whereupon high affinity binding prevailed until adulthood. In a previous investigation (Wohltmann et al., 1982), we demonstrated that with DADL as radioligand, the IC_{50} for morphine increased with age after birth. During early development, morphine displaced ^{3}H-DADL with high affinitiy, whereas later in this period, morphine only weakly competed with this radioligand. Since the IC_{50} for morphine in the first week was relatively low in the initial study, it was clear that ^{3}H-DADL was binding to μ sites. In the present investigation a μ site suppressor was used with ^{3}H-DADL, essentially eliminating binding to this site. On the basis of binding capacities (Fig. 2), one might speculate that in the present study DADL is binding to κ sites during the first week after birth. However, DADL has a much lower affinity for κ sites in adult rat brain (Pfeiffer and Herz, 1982). It is also possible that 1-week-old rat forebrain contains newly synthesized, incompletely processed δ receptors (Roth et al., 1981). This hypothesis may be linked to the observation that rat striatal adenylate cyclase activity is not detectable until the second postnatal week (Pardo et al., 1977). Perhaps the δ receptor may require guanine nucleotide-binding protein and cyclase for high affinity binding (Kent et al., 1982).

Kappa (κ) sites. EKC binding affinity remained unchanged (within experimental error) during development, reflecting its binding to the same type of opioid receptors. However, the binding data fit a two-site better than a one-site model. Since the affinity for the second site was very low (Table I), limited binding to other types of opioid receptors may have contributed to this value despite the presence of the unlabeled suppressors, 100 nM DAGO and 100 nM DADL. However, the possibility that two-site binding is brought about by the use of a racemic ligand cannot be excluded.

The κ receptors displayed a differential pattern in fore- and hindbrain (Fig. 3). The initial densities at day 1 for both fore- and hindbrain were about the same, whereupon hindbrain levels rose substantially. After day 14, the density of forebrain κ sites increases and hindbrain levels appear to decline (Fig. 2). The decrease in hindbrain density may be caused by the growth of the cerebellum, a region containing fewer κ sites (data not shown). These results accentuate the desirability of careful regional studies in the future. Finally, the caudal-to-rostral development seen for these κ sites has been observed repeatedly for other types of opioid receptors (Coyle and Pert, 1976; Tsang and Ng, 1980; Bardo et al., 1982; Tsang et al., 1982) and neurotransmitters (Tsang et al., 1982).

Ontogenic variations in opioid receptors are often expressed in femtomoles per milligram of protein (Clendeninn et al., 1976; Tsang and Ng, 1980; Tsang et al., 1982), but rarely has the developmental pattern been compared with that of brain protein synthesis. In agreement with previous data (Pardo et al., 1977), we found the postnatal developmental profile of forebrain protein (milligrams of protein per milligram of wet weight) to be sigmoidal, with the most pronounced increase occurring between days 7 and 28. This time interval correlates well with the period of rapid development of μ and δ sites. It is then of interest to compare changes in the numbers of opioid receptor types with respect to protein concentration. Of the three sites investigated, the fluctuations of the δ receptors parallel the changes in protein content most closely. Conversely, by day 14, the concentration of μ sites (femtomoles per milligram of protein) levels off, while protein synthesis continues to increase.

These results take on added significance in light of recent discoveries of trophic effects upon administration of opiates to neonates. Agonists inhibited neurobiological development whereas antagonists increased brain size and the numbers of naloxone-binding sites, neuronal cells, and glial cells (Slotkin et al., 1980; Zagon and McLaughlin, 1983). Since it has been reported that both agonists and antagonists also alter levels of opioid receptors in discrete regions of neonatal rat brain under similar conditions, it is possible that these trophic effects are receptor mediated (Bardo *et al.*, 1982; Handelmann, 1983; Handelmann and Quirion, 1983).

Levels of enkephalins, β-endorphin, and dynorphins have been monitored during development by radioimmunochemical methods (Bayon et al., 1979; Patey et al., 1980; Tsang et al., 1982; Khatchaturian et al., 1983) with inconsistent results. All three types of opioid peptides are present during embryonic development and increase after birth. The major discrepancy appears to be whether they increase, decrease, or remain the same in the second week postnatal. More than likely, these variations reflect differences in regions examined (Bayon et al., 1979). Until this issue is resolved, however, no definitive conclusions can be drawn concerning a correlation between postnatal changes of opioid peptides and their receptors. Nevertheless, the fact that opioid peptide levels generally increase prior to the elevation of μ and δ sites in the second week postnatal is consistent with the attractive hypothesis that these neuropeptides play a role in the development of their receptors.

The results on the ontogeny of δ sites complement and corroborate earlier work (Kent et al., 1982; Wohltmann et al., 1982; Tsang et al., 1982), whereas the data on the μ and κ receptors represent new and interesting findings. In general, the site selectivity afforded by the assay conditions of the present study provided data that represent an improvement over the results previously obtained in developmental binding experiments. The more specific κ assay, for example, is clearly superior to the use of EKC or bremazocine alone (Gillan and Kosterlitz, 1982). As a result, the developmental profile shown here (Fig. 2) is quite different from that obtained using ^{3}H-EKC alone (Spain et al., 1983). Understandably, our earlier findings reflected the binding of EKC to sites other than κ. Recently, using less site-specific assay conditions, Leslie et al., (1982) reported that a μ-specific ligand was less efficient in displacing ^{3}H-EKC binding to 6-day-old rat brain membranes than in displacing binding to those of adults. However, they could not conclude whether μ sites increased or κ sites decreased in this time period. The results obtained in this study prove the former is the case.

Similarly, the use of ligands that exhibit cross-reactivity can lead to ambiguities regarding the absolute concentrations of μ and δ sites. Previous investigations on the developmental profile of specific binding of tritiated [D-ala^2-met^5]enkephalinamide (Patey et al., 1980; Zhang and Pasternak, 1981), met-enkephalin (Tsang and Ng, 1980; Tsang et al., 1982), DADL (Kent et al., 1982), and naloxone (Coyle and Pert, 1976; Kent et al., 1982) in neonatal rat brain yielded patterns similar to that observed in the present study for δ and μ sites. However, since the affinity constants were not measured in those studies, when low levels of specific binding were determined it was not certain whether the desired sites were actually being

measured (Wohltmann et al., 1982). In fact, with the exception of an early study with ^{3}H-naltrexone (Clendeninn et al., 1976), this is the only investigation in which binding affinities and individual concentrations of μ, δ, and κ sites were measured over an extended time course in the neonatal period.

In summary, it is clear that the three types of opioid receptors examined display differential postnatal development providing independent evidence for multiplicity previously demonstrated by pharmacologic (Martin et al., 1976), anatomical (Pert et al., 1976; Chang et al., 1979; Goodman et al., 1980), and biochemical (Chang and Cuatrecasas, 1979; Kosterlitz et al., 1981; Pfeiffer and Herz, 1982) experimentation.

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