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Postnatal development of δ-opioid receptor subtypes in mice

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1 The density and affinity of binding sites for the δ-selective opioid ligands [^{3}H]-[D-Ala^2, Asp^4]deltorphin (DELT-I), [^{3}H]-[D-Ala^{2}Glu^4]-deltorphin (DELT-II), [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (DPDPE), and [^{3}H]-naltrindole (NTI) were determined in whole brain from 10, 15, 25 and 60 day-old C57BL mice.

2 At all ages, the analyses of the homologous displacement curves, gave best fits to single rather than to multiple site models. The binding capacity (B_{max}) labelled by [^{3}H]-NTI was about one half that labelled by [^{3}H]-DELT-I, [^{3}H]-DELT-II and [^{3}H]-DPDPE. In 25 and 60 day-old mouse brain the DPDPE B_{max} was 25% less than the deltorphin-II B_{max}.

3 In saturation experiments, specific binding of [^{3}H]-DELT-I on adult mouse brain homogenates was best fitted by a two-site model (34% high affinity site, $K_d = 1.08$ nM and 66% low affinity sites, $K_d = 39.9$ nM).

4 DPDPE produced a biphasic inhibition of specific [^{3}H]-DELTI-I binding, from 15 days of age onwards. The relative percentage of high and low affinity sites was 72% and 28% in 15 day-, 65% and 35% in 25 day- and 30% and 70% in 60 day-old mice.

5 In adult mouse brain labelled with [^{3}H]-DELT-I, DELT-II recognized 71% of high-affinity and 29% of low-affinity sites. DELT-I and DPDPE produced monophasic inhibition of specific [^{3}H]-DELT-II binding to brain homogenates of adult mice.

6 These data suggest that a sub-population of δ-sites (probably the δ_2-subtype), recognized by DELT-I, with high affinity for DELT-II and low affinity for DPDPE develops from 25 days onward.

7 In electrically stimulated mouse vas deferens (MVD) the rank order of potency of the three δ-agonists was: DELT-I > DELT-II > DPDPE in 10 day-old mice, and: DELT-I = DELT-II > DPDPE, from 25 days onward. During this time, the potency of DELT-II increased about 15 fold whereas the potency of DELT-I and DPDPE increased only 5 times. The higher efficacy of DELT-II could depend on receptor maturation towards the δ_2-subtype.

Keywords: Postnatal development; δ-receptor subtypes; mouse brain homogenates; mouse vas deferens; deltorphins

Introduction

Although evidence from *in vivo* pharmacological studies (Jiang *et al*., 1991; Mattia *et al*., 1991; Sofuoglu *et al*., 1991), *in vitro* binding experiments (Rothman *et al*., 1984; Negri *et al*., 1991; Fang *et al*., 1994) and the use of antisense oligonucleotides complementary to the cloned δ-opioid receptors (Lai *et al*., 1995; Cha *et al*., 1995) suggests that subtypes of the δ-opioid receptor exist at least in the brain, only a single gene encoding the δ-opioid receptor has been cloned in rats and mice (Evans *et al*., 1992; Kieffer *et al*., 1992; Yasuda *et al*., 1993; Fukuda *et al*., 1993). Yet Northern blots of RNA from most brain regions of these species hybridized with both the coding and 3′ non-coding region of the δ-opioid receptor cDNA have shown multiple transcripts with no evidence for differential expression (Evans *et al*., 1992; Yasuda *et al*., 1993). Hence rather than reflecting multiple encoding genes, the transcript heterogeneity that possibly accounts for receptor subtypes may derive from alternative RNA splicing. In the rat and mouse, the expression of δ-opioid receptors in the brain begins in the first week after birth (Spain *et al*., 1985; McDowell & Kitchell, 1986; Negri *et al*., 1993). Over the next three weeks receptor density increases to reach that in the adult animal. This postnatal development pattern offers an opportunity to ascertain if ontogenetic expression of the various opioid receptor subtypes run in a parallel manner or differentially.

Although inhibition of electrically-induced contraction of mouse vas deferens (MVD) is commonly used to screen compounds for activity at the δ-opioid receptors some evidence indicates that the δ-opioid receptor in MVD may differ from brain δ-opioid receptors (Vaughn *et al*., 1990; Wild *et al*., 1993; Fang *et al*., 1994). No data are available on the postnatal development of MVD opioid receptors.

With the aim of documenting the extent and origin of the receptor heterogenicity, we studied the ontogenesis of the δ-opioid receptor population in mouse brain and vas deferens. In relation to evidence for receptor subtypes based on differences in B_{max} values obtained with different radioligands (Knapp *et al*., 1991; Sofuoglu *et al*., 1992), we conducted a full postnatal ontogenetic study with four highly-selective δ-radioligands. Although the four highly selective δ-opioid ligands, [^{3}H]-[D-Ala^2, Asp^4] deltorphin (DELTI-I), [^{3}H]-[D-Ala^2, Glu^4] deltorphin (DELT-II), [^{3}H]-[D-Pen^2, D-Pen^5]enkephalin (DPDPE) and [^{3}H]-naltrindole (NTI) permitted measurement of the binding parameters of δ-opioid receptor subtypes in mouse brain, the binding assay was not sensitive enough to trace the low receptor density of pup vas deferens. We therefore compared the activity of the three selective δ-opioid agonists DELT-I, DELT-II and DPDPE on electrically-stimulated vas deferens. Unlike radioligand binding studies, bioassay studies measure both the affinity and efficacy of the tested agonists: changes in their relative potencies, during development, may indicate heterogeneity in the receptor-transducer complex.

Methods

Animals and tissue preparations

C57BL mice of either sex were used in all experiments and litters of 6–10 pups were cross fostered at birth. Animals were housed in an air-conditioned unit maintained at 22 ± 2°C and

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50–60% humidity with a natural day/night light cycle until they were killed. Fresh, whole brain homogenates were prepared from 10, 15, 25 and 60 day-old mice. At each age, to obtain sufficient tissue to generate inhibition curves, brain tissue from three to six litters was pooled. Brains were quickly removed on ice, weighed and immediately homogenized in 50 volumes of Tris-HCl buffer (50 mM, pH 7.4, 4°C) with a Kinematica PT 3000 polytron (20 s, speed 16000 r.p.m.). The homogenate was centrifuged at 41000 *g* for 20 min at 4°C, pellets were resuspended in 50 vol of buffer and incubated at 25°C for 30 min to remove endogenous opioids. After centrifugation, pellets were resuspended in buffer, containing 5% glycerol, to give a final w/v of 20 mg ml^{-1}.

Binding assay

For all radioligands K_d and B_{max} were determined by a homologous displacement protocol with 14 cold displacer concentrations against a constant radioligand concentration. The range of concentrations for each displacer was chosen to obtain a defined plateau at the top and the bottom of the displacement curve. For [^{3}H]-DELT-I only, saturation assays were performed with 12 ligand concentrations. All assays were carried out on 20 mg fresh tissue, in a final volume of 2 ml Tris-HCl buffer (50 mM, pH 7.4), at 35°C, for long enough to reach equilibrium (90–120 min) and non specific binding determined with 5 μM naltrindole. Reagents and membranes were distributed with a robotic sample processor (Tecan RSP 5000-series). By use of a Brandel M-24 cell harvester, assays were terminated by filtration through Whatman GF/B filter strips previously treated with 0.5% polyethylenimine for more than one hour. Filters were washed 3 times with 4 ml of ice-cold buffer and radioactivity was counted in a liquid scintillation spectrometer (Betamatic, Kontron). All samples were assayed in triplicate and all experiments were replicated a minimum of three times on different pools of brain membranes.

Homologous displacement of [^{3}H]-DELT-I (0.3 nM) and [^{3}H]-naltrindole (0.1 nM) binding to brain membranes was studied in 10 to 60 day-old mice. Homologous displacement curves of [^{3}H]-DELT-II (0.3 nM) and [^{3}H]-DPDPE (3.0 nM) were obtained in 15 to 60 day-old mice. Saturation of [^{3}H]-DELT-I (0.024–50 nM) was assayed on adult mouse brain membrane. In adult mouse brain membranes, heterologous displacement of 0.3 nM [^{3}H]-DELT-I binding was obtained with DELT-II (0.024–200 nM), DPDPE (0.1–800 nM) and NTI (0.0024–20 nM) and displacement of [^{3}H]-DELT-II (0.3 nM) binding with DELT-I (0.024 200 nM), DPDPE (0.1–800 nM) and NTI (0.0024–20 nM).

Heterologous displacement of 0.3 nM [^{3}H]-DELT-I by DPDPE was also studied in brain membranes from 10 to 60 day-old mice. All binding parameters: K_d, K_i, B_{max} and the Hill coefficient (n_H), were determined, with the computer programme Ligand (LIGAND, Biosoft, Cambridge, U.K.), from displacement and saturation curves. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. A 2-way ANOVA and Tukey's multiple comparison test were used to compare K_d, K_i and B_{max} values in mice at different ages.

Pharmacological assay in mouse vas deferens

One pair (15 to 60 day-old mice) or two pairs (10 day-old mice) of vasa were mounted between platinum strip electrodes in a 10 ml organ bath and bathed in oxygenated (95:5 O_2/CO_2) magnesium-free Krebs buffer (NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $KHPO_4$ 0.93, $NaHCO_3$ 25, glucose 11 mM) at 37°C. Tissues were stimulated with bipolar rectangular pulses of supramaximal voltage as previously described (Melchiorri *et al.*, 1991). Agonists were evaluated for their inhibition of the electrically evoked twitch. The results are expressed as the IC_{50} values obtained from concentration-response curves.

Drugs

DELT-I and DELT-II were synthesized as previously described (Erspamer *et al.*, 1989); DPDPE was bought from Bachem (Bubendorf, Switzerland) and naltrindole from RBI (Research Biochemicals Inc., Natick, MA, U.S.A.); [^{3}H]-DPDPE (41 Ci $mmol^{-1}$), [^{3}H]-DELT-II (49 Ci $mmol^{-1}$), and [^{3}H]-NTI (32 Ci $mmol^{-1}$) were purchased from NEN Products (Du Pont de Nemous Italiana, Milan, Italy); [^{3}H]-DELT-I ([3, 5-^{3}H-Tyr]-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, 35 Ci $mmol^{-1}$) was custom synthesized by CRB (Cambridge, U.K).

Results

Binding assay in mouse brain

In homologous displacement experiments, at all ages, all the four ligands produced linear Hoffstee plots and Hill coefficients around one. Thus the displacement curves fitted best to a single site model. K_d values for the four radioligands remained unchanged from postnatal day 15 to adult age (day 60), except a higher K_d value for [^{3}H]-DELT-I at the earliest age (day 10). At all ages tested, binding affinities of the four radioligands differed significantly, the rank order of affinity being NTI > DELT-I > DELT-II > DPDPE (Table 1). B_{max} values for DELT-I and NTI increased about 1.6 fold between day 10 and day 15; beteween day 15 and day 25, B_{max} values for DELT-I, DPDPE and NTI increased about 1.8 fold, but B_{max} values for DELT-II increased 2.5 fold. At day 25 the receptor density approached that in 60 day-old animals. At all ages, [^{3}H]-NTI labelled about half as many binding sites as [^{3}H]-DELT-I. Although the number of receptors for the three agonists ([^{3}H]-DELT-I, [^{3}H]-DELTI-II and [^{3}H]-DPDPE) did not statistically

Table 1 Dissociation constants (K_d) and δ-receptor maximal binding capacities (B_{max}) in developing mouse brain labelled with [^{3}H]-DELT-I, [^{3}H]-DELT-II, [^{3}H]-DPDPE and [^{3}H]-NTI

Age (days)	K_d (nM)				B_{max} (fmol mg^{-1} protein)			
	DELT-I	*DELT-II*	*DPDPE*	*NTI*	*DELT-I*	*DELT-II*	*DPDPE*	*NTI*
10	1.54±0.11	ND	ND	0.110±0.01	57.2±4.9	ND	ND	26.7±2.2
15	0.87±0.10*	1.92±0.3	4.3±0.5	0.077±0.01	87.9±9.3	76.0±6.1	71.1±8.1	44.8±3.5
25	1.01±0.12*	2.05±0.3	3.7±0.6	0.115±0.01	164.5±12.5†	190.5±12.1†,°	129.7±9.8	78.1±6.6
60	0.84±0.07*	1.99±0.2	4.9±0.5	0.11±0.01	182.2±15.3†	194.1±16.3†	146.3±13.7	81.4±6.0

K_d and B_{max} were derived from homologous displacement analysis of [^{3}H]-DELT-I (0.3 nM), [^{3}H]-DELT-II (0.3 nM), [^{3}H]-DPDPE (3.0 nM), and [^{3}H]-NTI (0.1 nM), by use of the Ligand programme. Values are mean ± s.e.mean of 3–5 determinations.; ND = not detected. For K_d values ANOVA multi-way comparisons across ages for each ligand showed significant differences for [^{3}H]-DELT-I vs day 10 (*$P<0.05$). There were no significant differences in K_d for [^{3}H]-DELT-II, [^{3}H]-DPDPE and [^{3}H]-NTI. For B_{max} values two-way ANOVA shows a significant interaction of age ($P<0.005$) and age x ligand ($P<0.05$). The analysis showed significant differences ($P<0.01$) for NTI vs DELT-I, DELT-II and DPDPE at all ages. †$P<0.05$ vs DPDPE; °$P<0.05$ vs DELT-I and DPDPE.

Table 2 δ-Receptor-specific binding and protein concentrations in mouse brain labelled with [^{3}H]-DELT-I, [^{3}H]-DELT-II, [^{3}H]-DPDPE and [^{3}H]-NTI

Age (days)	*Specific binding ratio* (%) *DELT-I*	*DELT-II*	*DPDPE*	*NTI*	*Protein/wet wt* (%)
10	85	ND	ND	56	3.5±0.1
15	91	86	55	69	4.8±0.2
25	95	90	80	73	5.5±0.3
60	96	94	86	72	6.3±0.4

Specific binding ratio (expressed as % of total binding) was determined at 0.3 nM for [^{3}H]-DELT-I, 0.3 nM for [^{3}H]-DELT-II, 3.0 nM for [^{3}H]-DPDPE and 0.1 nM for [^{3}H]-NTI. Protein values are expressed as a % of brain wet weight. Values are means±s.e.mean of 5–7 determinations. ND = not detected.

Table 3 Inhibition of 0.3 nM [^{3}H]-DELT-I binding to developing mouse brain by DPDPE

Age (days)	*One-site model* K_i (nM)	*Two-site model* K_{i-1} (nM)	%	K_{i-2} (nM)	%	n_H
10	4.9±0.3					0.88±0.02
15		2.03±0.32	72±2	29.8±2.1	28±1	0.72±0.03
25		1.46±0.21	65±5	19.9±1.1	35±2	0.69±0.03
60		1.34±0.19	30±4	15.9±1.8	70±5	0.77±0.03

Values are mean±s.e.mean of 4–6 determinations. One-site binding was observed for DPDPE in brain homogenates from 10 day-old mice. K_i = values derived from monophasic fitting of experimental points; K_{i-1}, K_{i-2} = values for high- and low-affinity sites derived from biphasic fitting of experimental points; % = the percentage of each site recognized by the ligands; n_H = Hill value.

differ on day 15, on day 25 [^{3}H]-DPDPE had a 21% lower binding density than [^{3}H]-DELTI-I and a 32% lower binding density than [^{3}H]-DELT-II.

At the concentrations tested, specific binding of all ligands increased with age. Specific binding of the two tritiated deltorphins increased from about 85 to 96%, whereas that of [^{3}H]-DPDPE and [^{3}H]-NTI increased from about 55% to 80% of total binding. Brain protein concentrations increased 1.8 fold from day 10 to day 60 (Table 2).

In saturation experiments, specific binding of [^{3}H]-DELT-I on adult mouse brain homogenates was best fitted by a two-site model, the equilibrium dissociation constants being 1.08±0.17 and 39.9±10.4 nM and the B_{max} being 58.2±5.8 and 115.2±13.4 fmol mg^{-1} protein (Figure 1).

Whereas DPDPE inhibited [^{3}H]-DELTI-I binding to brain membranes of 10 day-old mice from one site only (K_i = 4.9 nM), from 15 days onward it inhibited [^{3}H]-DELT-I binding from two sites. At 15 days of age, 72% of this displacement occurred at a high-affinity site (K_i = 2.03 nM) and only 28% at a low-affinity site (K_i = 29.8 nM). At 60 days only 30% of the binding was to a high-affinity site (K_i = 1.34 nM) whereas 70% was to a low-affinity site (K_i = 15.9 nM) (Table 3). DELT-II also showed two-site inhibition of [^{3}H]-DELT-I binding to brain homogenates of adult mice: the major part (71%) occurring at a high-affinity (K_i = 0.7 nM) and a minor portion (29%) occurring at a lower affinity site (K_i = 8.3 nM), (Table 4). Heterologous displacement of [^{3}H]-DELT-II, studied only in adult mouse brain membranes, yielded DPDPE (K_i = 2.8 nM) and DELT-I (K_i = 0.45 nM) competition curves best fitted to a single rather than a multiple site model. In adult mouse brain, NTI displaced both [^{3}H]-DELTI-I (K_i = 0.14 nM) and [^{3}H]-DELT-II (K_i = 0.09 nM) from a single binding site (Table 4).

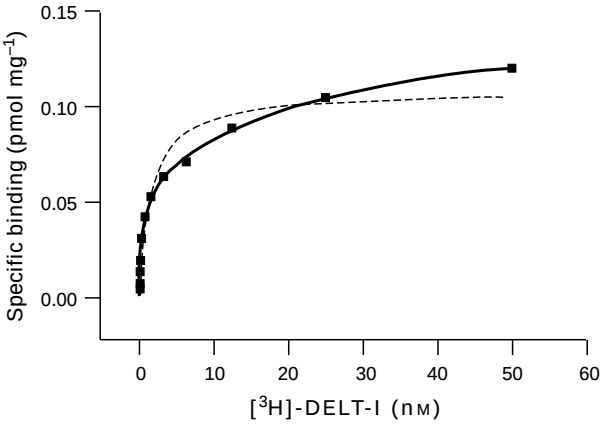

Figure 1 Saturation plot of [^{3}H]-DELT-I binding to adult mouse brain membranes. The brain homogenates (0.8 mg protein) were incubated with 0.024 to 200 nM [^{3}H]-DELT-I for 90 min at 35°C. The nonspecific binding was defined by 10 μM naltrindole. The data were best fitted by a two-site model (solid line) rather than a one-site model (dotted line). The K_D values obtained by nonlinear regression analysis were 1.08 and 39.9 nM, respectively, and the B_{max} values 58.2 and 115.2 fmol mg^{-1} protein, respectively. The curve is representative of three experiments.

Bioassay on mouse vas deferens

None of the tested agonists yielded valid dose-response curves on postnatal day 10. Nonetheless in 50% (4/8) of the experiments in which DELT-I showed some activity, its IC_{50} exceeded 100 nM. On postnatal day 15, DELT-I produced potent inhibition of the electrically stimulated twitch of MVD (IC_{50} = 1.74±0.19 nM (Table 5). On day 25 its potency (IC_{50} = 0.31±0.025 nM) increased 5 fold, reaching the value obtained in adult mice (IC_{50} = 0.49±0.048 nM). On postnatal day 15, DELT-II (IC_{50} = 6.8±1.26 nM) was four times and DPDPE (IC_{50} = 65.8±2.4) 38 times less potent than DELT-I. DELT-II potency rapidly increased with age: on postnatal day 25 (IC_{50} = 0.45±0.04 nM) it equalled that of DELT-I, whereas DPDPE (IC_{50} = 11.5±1.1) remained 37 times less potent.

Discussion

In this study we have shown that, in mice, between the postnatal day 15 and day 25, both in the brain and in the MVD a new δ-opioid receptor develops. This receptor has the characteristics of the δ_2-subtype. Strong pharmacological evidence suggests the existence of δ-opioid receptor subtypes. On the basis of antagonist data (Jiang *et al.*, 1991; Sofuoglu *et al.*,

Table 4 Inhibition of [^{3}H]-DELT-I and [^{3}H]-DELT-II binding to adult mouse brain by selected δ-opioid ligands

Displacing ligand		*[^{3}H]-DELT-I* K_i (nM)	n_H	*[^{3}H]-DELT-II* K_i (nM)	n_H
DELT I		0.84±0.07	0.96±0.09	0.45±0.03	0.98±0.09
DELT II	High affinity	0.72±0.11 (71%)	0.78±0.05	1.99±0.21	0.89±0.09
	Low affinity	8.33±0.72			
DPDPE	High affinity	1.34±0.19 (30%)	0.79±0.03	2.80±0.18	0.89±0.10
	Low affinity	15.9±1.8			
NTI		0.14±0.01	0.84±0.05	0.09±0.01	0.88±0.05

Values are mean±s.e.mean of 3–5 experiments. Heterologous displacement of [^{3}H]-DELT-I (0.3 nM) with the two agonists, DELT-II and DPDPE, yielded two-site binding curves. The percentage of high-affinity sites recognized by the ligands is given in parentheses.

Table 5 Inhibition of the electrically stimulated twitch in the developing mouse vas deferens

Age (days)	*IC$_{50}$* (nM) *DELT-I*	*DELT-II*	*DPDPE*
10	334±147	ND	>1000
15	1.74±0.19	6.8±1.26	65.8±10.1
25	0.31±0.025*	0.45±0.043‡	11.5±1.1†
60	0.49±0.048*	0.54±0.054‡	16.2±1.5†

Values are means±s.e.mean of 8–12 determinations. IC_{50} = the peptide concentration that produces 50% inhibition of contraction. *$P<0.001$, vs day 15; ‡ and †$P<0.01$, vs day 15.

1991; Mattia *et al*., 1992; Portoghese *et al*., 1992; Buzas *et al*., 1994), subtypes have been tentatively classified as δ_1 (i.e. activated by DPDPE and sensitive to antagonism by [D-Ala^2, Leu^5,Cys^6]enkephelin (DALCE) and 7-benzylidenenaltrexone) and δ_2 (i.e., activated by DELT-II and sensitive to antagonism by 5′-naltrindole isothiocyanate and naltriben). Both *in vivo* and *in vitro* data suggest that the cloned mouse δ-opioid receptor corresponds to that pharmacologically classified as δ_2. The finding that the treatment with an antisense oligodeoxynucleotide directed towards the N-terminal portion of the cloned δ-receptor selectively inhibits the supraspinal antinociceptive actions of DELT-II (putative δ_2 agonist), but not those of DPDPE (putative δ_1 agonist) (Bilsky *et al*., 1994; Lai *et al*., 1994), whereas treatment with an antisense oligo, directed to a conserved region of the μ, δ and κ cloned opioid receptors inhibits the antinociceptive actions of DPDPE (Lai *et al*., 1995), suggests that DPDPE produces its actions via δ-opioid receptor, which shares a region common to all presently known cloned opioid receptors, but which differs, from the cloned δ-receptor, at least in the N-terminus sequence. The presence of two introns in the coding region of the mouse δ-opioid receptor gene suggests the possible existence of alternatively spliced forms (Simonin *et al*., 1994). Because of their high selectivity in binding studies for δ-receptors, the four radioligands studied here, should be suitable tools to reveal the eventual appearance of δ-receptor isoforms by alternative splicing during ontogeny.

In a previous paper, while studying the pattern of appearance and development of [^{3}H]-DELT-I binding sites in Swiss Albino mice, we found a significant increase in receptor affinity from the 25th day onwards, owing to a high-affinity binding site that appeared one month after birth (Negri *et al*., 1991). In this study, in the C57BL strain, DELT-I receptor affinity in 10 day old mice was significantly lower than in adult mice. Displacement of [^{3}H]-DELT-I binding with DPDPE gave monophasic curves in 10 day-old mouse membrane preparations but biphasic curves when we used membrane preparations from 15 day-old mice onward: the percentage of high affinity sites decreased from 72% in 15 day-old to 30% in 60 day-old mice. DELT-II inhibition of [^{3}H]-DELT-I binding in the same 60 day-old mice marked out 71% high affinity sites. DELT-I appears non-selective because it has high affinity at the preferred site labelled by [^{3}H]-DELT-II (K_i = 0.45 nM) and [^{3}H]-DPDPE (K_i = 0.3 nM). We conclude that the agonists detected only one form of receptors in 10 day-old mice, but at later ages, a sub-population of δ-sites that has high affinity for DELT-II and low affinity for DPDPE develops. Support for this hypothesis came from the relative density in δ-receptors marked out by the three agonist ligands at different ages. In 15-day-old mice, DELTI-I, DELT-II and DPDPE labelled an equal number of receptors, but in adult mice DPDPE binding sites were only 80% of those of DELT-I and less than 75% of those of DELT-II suggesting the presence of a δ-receptor subtype preferentially labelled by the deltorphins. The possibility that DELT-I and DELT-II labelled a non δ-site is remote because these ligands are > 3000 fold selective for δ-receptors (Erspamer *et al*., 1989). The most likely explanation is that a new δ-receptor with the characteristics of the δ_2-subtype develops after day 15, being fully expressed at day 25. Although the differing B_{max} might depend on receptor affinity states (Richardson *et al*., 1992; Standifer *et al*., 1993; Xu *et al*., 1993), this possibility is excluded by evidence that naltrindole, a neutral antagonist that has no preference for G-protein-coupled or uncoupled receptor states (Fang *et al*., 1994), labelled 50% fewer binding sites than the opioid agonists deltorphi-I and -II. If the binding site of the heterocyclic compound NTI on the δ-receptor molecule differs from that of the peptide agonists (Kong *et al*., 1993) presumably the δ-receptor exists in two isoforms: one binds the peptides and NTI and the other binds the peptides alone.

In the electrically stimulated MVD we found that the potency rates of the three δ-agonists tested differed between day 10 (DELT-I > DELT-II > DPDPE) and day 25 (DELT-I = DELT-II > DPDPE): during this period DELT-I and DPDPE potency increased only 5 times whereas DELT-II potency increased 15 fold. Even though in the MVD we measured the efficiency of a system rather than the number of receptors, the situation resembles that in the brain, where between day 15 and day 25, DELT-II binding sites increased 2.5 fold whereas DELT-I and DPDPE binding sites increased 1.8 fold. Most published data provide support for only one functional subtype of opioid δ-receptor in the MVD but other data do not exclude the possible presence of subtypes (Fang *et al*., 1994): binding experiments suggest that the δ-receptors in MVD differ from the brain δ-receptors of the rat (Vaughn *et al*., 1990), and of the mouse (Wild *et al*., 1993; Fang *et al*., 1994) bioassay experiments suggest they resemble the δ_2 site in the mouse brain (Wild *et al*., 1993). Our data do not allow us to demonstrate the actual presence of two distinct δ-receptor subtypes in adult MVD but they do indicate that the δ-system matures between days 15 and 25, clearly assuming the features of a δ_2-subtype. Possible explanations could be structural changes in the δ-opioid receptor, that is, diverse developmental mRNA splicing, or changes in G-protein content. The latter hypothesis presumes that developmental changes in the transduction system increase the efficacy of DELT-II more than the efficacy of DELT-I or DPDPE. Whereas this could hold true for the structurally diverse compounds DELT-II and

DPDPE, it seems far less likely for DELT-II and DELT-I, which differ only in the substitution of the Glu4 residue with an Asp4 residue. A more reasonable hypothesis would be that developmental changes in mRNA transcription lead to the appearance of a δ-opioid receptor subtype on which DELT-II displays higher efficacy than DELT-I and DPDPE. The prominent development of the DELT-II-sensitive system could result from tissue-specific δ-opioid receptor mRNA splicing regulated by factors linked to the change from pup to adult (weaning, or sexual maturation?). The onset of puberty occurs as early as 24 days of age in C57BL mice (Whittingham & Wood, 1983).

In rats, weaning stimulates the development in the CNS of δ-opioid receptors that are recognized by [^{3}H]-DELT-I but not by [^{3}H]-[Ile5,6]DELT-II (Kitchen *et al*., 1995). Quantitative autoradiography has shown that this effect is localized to the deep layers of the frontal-parietal cortex and pontine nuclei. Autoradiographic competition studies indicate that δ-receptors activated by weaning during development can be differentiated by DPDPE suggesting that these sites fit into the class of subtype designated as δ_1 (Kitchen *et al*., 1996). Classification of compounds as putative δ_1- and δ_2-receptor agonists has been based on *in vivo* studies in the mouse, which point to deltorphin analogues being selective for δ_2-receptors, whereas, both *in vivo* (Kitchen *et al*., 1994) and autoradiographic (Renda *et al*., 1993) studies support the hypothesis that DELT-I acts on the same receptors as DPDPE (δ_1-receptors) in the rat. Hence, both in mice and in rats a δ-receptor subtype becomes evident around the adult age: it resembles the δ_2-subtype in the mouse and the δ_1-subtype in the rat. It has recently become apparent that equivalent receptors from different species can exhibit distinct pharmacological profiles with respect to some synthetic ligands (Fong *et al*., 1992) or that small differences in primary structure can relate to changes in pharmacology (Oksenberg *et al*., 1992). Sequence differences in human, rat and mouse δ-opioid receptors occur at regions potentially involved in ligand binding (Simonin *et al*., 1994), hence various opioid ligands may display species selectivity. A detailed comparison of non-conserved amino acid residues in the mouse and rat δ-opioid receptor may provide the information needed for molecular identification of the other δ-opioid receptor subtype.

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