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Weaning-induced development of δ-opioid receptors in rat brain: differential effects of guanine nucleotides and sodium upon ligand-receptor recognition

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1 We have previously shown that weaning at day 21 increases δ-opioid receptor binding in the brain at day 25, which might be due to stimulation of the development of a δ-opioid receptor subtype or activation of G-protein coupling processes.

2 We have addressed the possibility that weaning stimulates coupling of the δ-receptor by homogenate binding studies with four agonist and one antagonist radioligand in the presence of a GTP analogue and Na^+ in brain tissue from weaned and non-weaned animals.

3 Saturation studies with three agonist ligands ([^{3}H]-deltorphin I, [^{3}H]-S-Atc-Ile^{5,6}deltorphin I and [^{3}H]-R-Atc-Ile^{5,6}deltorphin II) showed higher levels of maximal binding in brains from 25-day weaned than in brains from non-weaned rats. The magnitude of the effects of GMPPNP and Na^+ in decreasing this binding was ligand dependant and in each case was significantly more marked in brains from weaned animals. GMPPNP and Na^+ were completely without effect on B_{max} for, [^{3}H]-S-Atc-Ile^{5,6}deltorphin I and [^{3}H]-R-Atc-Ile^{5,6}deltorphin II in brains from non-weaned rats.

4 [^{3}H]-Ile^{5,6}deltorphin II and [^{3}H]-naltrindole showed no differences in labelling between weaned and non-weaned groups and both groups responded similarly to the effects of GMPPNP and Na^+ treatment.

5 GMPPNP and Na^+ had small effects on binding affinity (K_D) for some of the agonist radioligands which were similar in both weaned and non-weaned groups.

6 Weaning induced increases in binding of δ-receptors in 25-day rats can be explained in terms of the way δ-agonist radioligands recognize the receptor environment.

Keywords: δ-opioid receptors; development; ontogeny; weaning; G-proteins; guanine nucleotides; receptor coupling

Introduction

Three opioid receptor subtypes (δ-, μ-, and κ) have been cloned (Kieffer, 1995). Although the molecular biology evidence only indicates three genes encoding for three receptor proteins, a substantial amount of pharmacological evidence suggests subdivision of these three receptors and distinct binding profiles and behavioural responses can be ascribed to δ-, κ and μ-subtypes (Clark *et al*., 1989; Jiang *et al*., 1991; Mattia *et al*., 1992; Pasternak & Wood, 1986; Sofuoglu *et al*., 1991; Traynor, 1989; Unterwald *et al*., 1991; Wolleman *et al*., 1993). In developing rats we have provided *in vivo* evidence that there are δ-receptor subtypes (Crook *et al*., 1992; 1993). In addition, we have shown that the process of weaning rat pups from their mother at day 21 is instrumental in activation of some functional δ-receptor responses which may reflect developmental activation of a receptor subtype. For example, δ-mediated stress-induced antinociception (Muhammad & Kitchen, 1993) and antinociceptive responses to some, but not all, δ-receptor agonists (Kitchen *et al*., 1994) are activated by weaning. We have also been able to demonstrate that dependent on the radioligand used to label δ-receptors it is possible to show by homogenate binding studies and autoradiography that weaning activates a subpopulation of δ-receptors (Kitchen *et al*., 1995). These studies demonstrated that when δ-receptors were labelled with [^{3}H]-deltorphin I more sites were recognized in 25-day-old weaned rats compared to non-weaned animals whilst another agonist ligand [^{3}H]-Ile^{5,6} deltorphin II and the antagonist ligand [^{3}H]-naltrindole showed no differences in the level of binding between weaned and non-weaned rats (Kitchen *et al*., 1995). Comparison of mRNA expression by *in situ* hybridization (Kitchen *et al*., 1995) in weaned and non-weaned animals indicated that the differences in receptor binding could not be attributed to activation of new receptor protein at the gene level. An alternative explanation is that weaning activates G-protein coupling processes for the δ-receptor and that the differences in binding observed with agonist and antagonist radioligands reflects the sensitivity of the ligand to coupling states of the receptor. To address this possibility we have now carried out homogenate binding studies with five δ-selective radioligands, under conditions (presence of a GTP analogue and Na^+) which would alter the coupling state of the δ-receptor, in brains from weaned and non-weaned animals. We have used the three ligands employed in our previous studies, [^{3}H]-deltorphin I which recognizes differences in δ-receptor density in brains from weaned and non-weaned rats and [^{3}H]-Ile^{5,6} deltorphin II, [^{3}H]-naltrindole which do not (Kitchen *et al*., 1995). In addition we have also studied two novel deltorphin analogues (Spetea *et al*., 1997; Toth *et al*., 1997) which are amongst the most highly selective δ-receptor agonists developed with >20,000 fold selectivity for this site (Toth *et al*., 1997). The results demonstrate marked differences in sensitivity of δ-agonists to GTP and Na^+ in 25-day- old rat brain and also suggest that weaning produces changes in δ-receptors which represent differences in coupling state.

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Methods

Animals

Wistar Albino rats (University of Surrey strain) were used in all experiments. The day of birth was designated day 1 and litters were cross fostered where possible to give litter sizes of 8–10 pups. The animals were housed in an air conditioned unit maintained at 20°C ± 1°C and 50–60% humidity with light control on a 12 h cycle (lights off 19.00 h–07.00 h) and allowed free access to standard rodent chow and water. Animals were weaned by the removal of the mother from the cage on day 20, whilst non-weaned animals were left with the mother until day 25. Animals were killed by cervical dislocation between 08.00 h and 09.00 h on day 25. Only male pups were used in this study.

Membrane homogenates

The brains were removed (minus cerebella), weighed and placed on ice in 50 nM Tris-HCl (pH 7.4 at 0°C). After homogenizing in 50 volumes of buffer using a Ystral 1500 homogeniser (speed 6, 45 s) the homogenate was centrifuged at $49{,}000 \times g$ for 15 min at 4°C. The resulting pellet was resuspended in 50 volumes of 50 mM Tris-HCl buffer (pH 7.4, 37°C) and incubated for 45 min at 37°C to remove endogenous opioids. After recentrifugation the pellets from three brains were pooled and resuspended at 10 mg (original wet weight)/ml in 50 mM Tris-HCl buffer, pH 7.4 at the subsequent incubation temperature, with or without the addition of 100 mM NaCl.

Receptor binding assays

Saturation binding assays (in triplicate) were carried out to determine K_D and B_{max} for [^{3}H]-deltorphin I, [^{3}H]-Ile^{5,6}deltorphin II, [^{3}H]-S-Atc-Ile^{5,6}deltorphin I, [^{3}H]-R-Atc-Ile^{5,6}deltorphin II and [^{3}H]-naltrindole. Ten to 14 ligand concentrations were used in the ranges 0.1–8.0 nM ([^{3}H]-deltorphin I), 0.2–12 nM ([^{3}H]-Ile^{5,6}deltorphin II), 0.05–3.5 nM ([^{3}H]-S-Atc-Ile^{5,6}deltorphin I), 0.05–2 nM ([^{3}H]-R-Atc-Ile^{5,6}deltorphin II) and 0.01–2.0 nM ([^{3}H]-naltrindole). Assays were carried out at 35°C for 60 min for the deltorphin radioligands or 25°C for 90 min for [^{3}H]-naltrindole. Non-specific binding was determined by using 10 μM naloxone. Each homogenate derived from three pooled brains was used to provide two saturation curves, one in the absence of guanylyl-5′-6′imidodiphosphate (GMPPNP) and the second in the presence of 50 μM GMPPNP. Homogenates prepared with or without Na^+ allowed for the study of the effect of Na^+ alone or Na^+ plus GMPPNP. Assays were terminated by filtration through Whatman GF/B filters, using a Brandel M-24 cell harvester, washing four times with 5 ml of ice cold Tris-HCl buffer (pH 7.4, 0°C) and radioactivity determined by liquid scintillation spectrometry.

Quantitative analysis and statistical procedures

The total protein content of each homogenate was determined by a modification of the Lowry method (Lowry *et al*., 1951) using a bovine serum albumin standard. All saturation binding data was subject to non-linear regression analysis using GraphPad Prism to provide values of K_D and B_{max}.

Analysis of B_{max}, K_D and protein levels was carried out by 3-way ANOVA for the independent variables of ligand, behaviour (weaned or non-weaned) and binding conditions (GMPPNP, Na^+ or GMPPNP and Na^+). For analysis of the behavioural effect of weaning the ligands were separated into those which showed significant difference in absolute binding levels between weaned and non-weaned groups under control conditions and those which did not. Where 3-way ANOVA revealed significant effects or interactions individual comparisons to the control level of binding were made by Duncan's *post hoc* test.

Materials

[^{3}H]-naltrindole, 19.4 Ci mmol^{-1}, [^{3}H]-Ile^{5,6} deltorphin II, 49.5 Ci mmol^{-1}, [^{3}H]-Ile^{5,6}-S-Atc-deltorphin I, 34.5 Ci mmol^{-1} and [^{3}H]-Ile^{5,6}-R-Atc-deltorphin II, 36 Ci mmol^{-1} were provided by Hungarian Academy of Sciences, Szeged, Hungary. The following compounds were purchased: [^{3}H]-deltorphin I, 35 Ci mmol^{-1}, Zeneca, Cambridge Research Biochemicals, UK. Guanylyl-5′-6′-imidodiphosphate, Boehringer Mannheim, U.K. Naloxone hydrochloride, Sigma Chemicals. All other chemicals used were of analytical grade.

Results

Weaned/non-weaned differences

Under control conditions (50 mM Tris HCl buffer) the four agonist ligands ([^{3}H]-deltorphin I, [^{3}H]-Ile^{5,6} deltorphin II, [^{3}H]-S-Atc-Ile^{5,6}deltorphin I and [^{3}H]-R-Atc-Ile^{5,6}deltorphin II) and the antagonist ligand [^{3}H]-naltrindole showed saturable high affinity binding (Figure 1) and their K_D values were the same in weaned and non-weaned groups (Table 1, $P<0.05$). All ligands fitted to a single site model. In common with our previous studies (Kitchen *et al*., 1995) binding with [^{3}H]-deltorphin I revealed a significantly higher B_{max} in brains from 25-day-old weaned rats (Figure 2, $P<0.05$) whilst binding with [^{3}H]-Ile^{5,6}deltorphin II and [^{3}H]-naltrindole was equivalent in brains from both weaned and non-weaned groups. The S-Atc derivative of deltorphin I, [^{3}H]-S-Atc-Ile^{5,6}deltorphin I and the R-Atc derivative of deltorphin II, [^{3}H]-R-Atc-Ile^{5,6}deltorphin II, also revealed a higher number of δ-sites in the brains from weaned rats ($P<0.01$). There were no significant differences in protein levels recorded in any group of animals used in the study (Table 2). All the deltorphin analogues and naltrindole show high levels of specific binding (Table 2) under all conditions.

Effect of GMPPNP and Na^+ on B_{max} values

Representative saturation binding curves for each ligand in the presence of GMPPNP and Na^+ are shown in Figure 1 and the derived B_{max} are given in Figure 2. 3-way ANOVA of all the data showed a significant difference for the ligand factor ($F=68.6$, $df=4$, $P=0.0001$) and for the binding conditions factor ($F=24.2$, $df=3$, $P=0.0001$). For analysis of the influence of weaning on the effects of different binding conditions 3-way ANOVA was applied to those ligands which under control conditions showed differences in B_{max} between weaned and non-weaned treatments ([^{3}H]-deltorphin I, [^{3}H]-S-Atc-Ile^{5,6}deltorphin I and [^{3}H]-R-Atc-Ile^{5,6}deltorphin II). 3-way ANOVA showed a signficant interaction between the factors behaviour (weaned or non-weaned) and binding conditions ($F=5.43$, $df=3$, $P=0.002$). Analysis of those ligands which under control conditions did not show difference in B_{max} between weaned and non-weaned treatments ([^{3}H]-

Ile^{5,6}deltorphin II and [^{5,6}H]-naltrindole) showed no significant interaction between the factors behaviour (weaned or non-weaned) and binding conditions ($F=0.32$, $df=3$, $P=0.81$). *Post hoc* analysis for individual comparison of binding conditions showed that for the antagonist [^{3}H]-naltrindole there were no significant effects of GMPPNP or Na+, either alone or in combination, upon B_{max} in either weaned or non-weaned groups. For the agonist ligand [^{3}H]-Ile^{5,6}deltorphin II, which like naltrindole shows no difference in δ-binding between weaned and non-weaned groups, GMPPNP had no effect, but Na^+ or the combination of GMPPNP and Na^+ decreased binding in brains from both weaned and non-

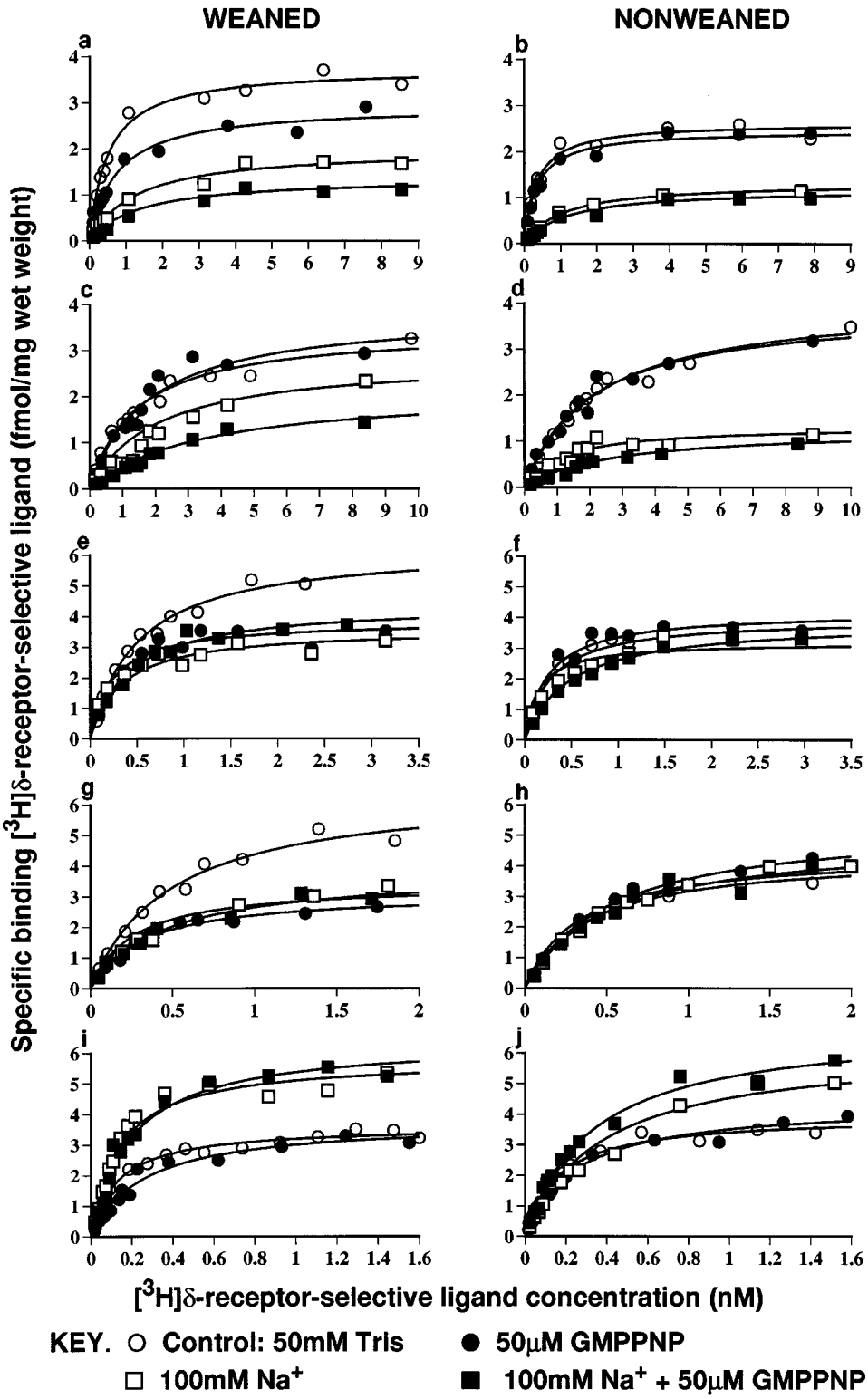

Figure 1 Representative saturation curves for the binding of δ-receptor-selective ligands in homogenate of whole brain (minus cerebellum) from 25-day-old weaned (left panel) or non-weaned (right panel) rats. Binding was carried out in the absence or presence of 50 μM GMPPNP or 100 mM Na^+ or both and is expressed as specific binding in fmol/mg net weight of tissue. (a) and (b) [^{3}H]-deltorphin I, (c) and (d) [^{3}H]-Ille^{5,6}deltorphin II, (e) and (f) [^{3}H]-S-Atc-Ile^{5,6}deltorphin I, (g) and (h) [^{3}H]-R-Atc-Ile^{5,6}deltorphin II, (i) and (j) [^{3}H]-naltrindole.

Table 1 The effect of 50 μM GMPPNP, 100 μM Na^+ or both on receptor binding affinity of δ-receptor selective ligands in 25-day-old rat brain

	K_D (nM)	
	Weaned	*Non-weaned*
[^{3}H]-deltorphin I		
Control Tris	0.50±0.03	0.49±0.05
50 μM GMPPNP	0.82±0.10	1.1±0.6
100 mM Na^+	1.5±0.1**	0.84±0.21
100 mM Na^+ + 50 μM GMPPNP	2.1±0.4**	2.5±0.8*
[^{3}H]-IIe^{5,6}deltorphin II		
Control Tris	2.0±0.3	2.8±0.5
50 μM GMPPNP	2.7±0.7	1.9±0.3
100 mM Na^+	3.6±0.4*	3.1±0.9
100 mM Na^+ + 50 μM GMPPNP	4.3±0.7**	4.2±1.2
[^{3}H]-S-Atc-IIe^{5,6}deltorphin I		
Control Tris	0.39±0.02	0.36±0.06
50 μM GMPPNP	0.28±0.05	0.33±0.04
100 mM Na^+	0.24±0.02*	0.39±0.08
100 mM Na^+ + 50 μM GMPPNP	0.34±0.06	0.46±0.08
[^{3}H]-R-Atc-IIe^{5,6}deltorphin II		
Control Tris	0.73±0.07	0.66±0.06
50 μM GMPPNP	0.40±0.04*	0.47±0.03
100 mM Na^+	0.53±0.02	0.60±0.06
100 mM Na^+ + 50 μM GMPPNP	0.55±0.06	0.70±0.11
[^{3}H]-naltrindole		
Control Tris	0.16±0.03	0.13±0.01
50 μM GMPPNP	0.25±0.04	0.20±0.02
100 mM Na^+	0.17±0.03	0.23±0.11
100 mM Na^+ + 50 μM GMPPNP	0.23±0.02	0.23±0.07

Values are means±s.e.mean for three to eight determinations. Comparison of the effects of GMPPNP and Na^+: significant difference from corresponding control values *$P<0.05$ **$P<0.01$ (1-factor ANOVA and Duncan's *post hoc* test).

weaned groups although the Na^+ effect in the non-weaned animals was not significant.

For the ligands which showed differences in B_{max} between 25-day-old weaned and non-weaned animals differences in sensitivity to GMPPNP and Na^+ were observed (Figures 2 and 3). A decrease in B_{max} for [^{3}H]-deltorphin I was observed in the presence of either GMPPNP or Na^+ and in combination, in brains taken from weaned animals. Although Na^+ or the combination of GMPPNP and Na^+ decreased B_{max} in the non-weaned groups, GMPPNP alone had no effect on maximal binding (Figure 2). For the Atc-Ile^{5,6}derivatives of deltorphin I and II, GMPPNP and Na^+ or Na^+ alone significantly decreased binding in brains taken from weaned groups, but the effect of Na^+ was not as marked as observed for [^{3}H]-deltorphin I. In addition GMPPNP significantly reduced B_{max} for the deltorphin II derviative. Brains, however, from non-weaned animals showed no shifts to either the GTP analogue or Na^+ nor to the combination of GMPPNP and Na^+ for these two deltorphin analogues (Figure 2). The observed changes in B_{max} were similar when expressed per protein or per wet weight (data not shown).

Effect of GMPPNP and Na^+ on K_D values

GMPPNP and Na^+ had no significant effect on K_D values for [^{3}H]-naltrindole alone or in combination in either weaned or non-weaned groups. For [^{3}H]-deltorphin I and [^{3}H]-Ile^{5,6}deltorphin II there were 2–4 fold increases in K_D observed in the presence of the combination GMPPNP and Na^+ which was significant in weaned and non-weaned groups for [^{3}H]-deltorphin I and for [^{3}H]-Ile^{5,6} deltorphin II in weaned groups only. There were also smaller but significant increases in K_D for [^{3}H]-deltorphin I in brains treated with Na^+ from weaned groups only. For the Atc derivatives of the deltorphins there were no indications of decreases in affinity in the presence of GMPPNP or Na^+ or in combination in either weaned on non-weaned groups and indeed two of the weaned groups showed small increases in affinity (Table 1).

Discussion

In agreement with our previous studies (Kitchen *et al*., 1995) the number of δ-receptors was around 30% higher in 25-day-old brains taken from weaned animals than in non-weaned controls when labelled with [^{3}H]-deltorphin I but not when labelled with [^{3}H]-Ile^{5,6}deltorphin II or [^{3}H]-naltrindole. The new deltorphin I and II analogues that we have synthesised (Spetea *et al*., 1997; Toth *et al*., 1997) [^{3}H]-S-Atc-Ile^{5,6}deltorphin I and [^{3}H]-R-Atc-Ile^{5,6}deltorphin II behave similarly to [^{3}H]-deltorphin I and also recognize those receptors which are only apparent after weaning. It might be argued that the differences in B_{max} reflect differences in the number of coupled receptors since for [^{3}H]-deltorphin I and the Atc-deltorphin derivatives there is a clear lack of sensitivity to GMPPNP in the brains taken from non-weaned animals whilst those taken from weaned rats show the characteristic loss of sites in the presence of the GTP analogue and Na^+ ions. In contrast for [^{3}H]-Ile^{5,6}deltorphin II, there is no change in binding in the presence of both in weaned and non-weaned groups suggesting that the receptor recognition by this ligand is unaltered by the weaning process.

The picture however is more complex, because it is quite clear that all four agonist ligands exhibit distinct profiles in their sensitivity to the membrane environment manipulations in brains taken from weaned animals, which might be considered to be control tissue. For example, [^{3}H]-deltorphin I shows a 35% loss of sites in the presence of GMPPNP and a 60% loss of sites in the presence of Na^+ whilst the Atc-deltorphin analogues show a smaller loss of sites with GMPPNP (10–25%) and no greater loss with Na^+ (25–35%, Figure 3). Further, although [^{3}H]-Ile^{5,6}deltorphin II is completely insensitive to GMPPNP it shows a 35% loss of sites in the presence of Na^+(Figure 3). Indeed 3-way ANOVA for all four agonist ligands reveals a highly significant interaction between the factors ligand and binding conditions ($F=5.89$, $df=9$, $P=0.0001$). This complexity is also mirrored in the analysis from non-weaned brain tissue in that the Atc-deltorphin derivatives show complete lack of effect of Na^+ or the combination of GMPPNP and Na^+ whilst [^{3}H]-deltorphin I shows a loss of sites under these conditions. The expression of δ-receptors is thus very much ligand and environment dependent and the process of weaning renders changes in the environment or changes in the receptor which influence its ability to respond in a given environment, manifested as differences in sensitivity to G-protein coupling and cation regulation processes.

Much of the early opioid literature showed that δ-receptors were regulated by guanine nucleotides and Na^+ (Blume, 1978; Pert & Snyder, 1974; Pfeiffer *et al*., 1982; Werling *et al*., 1984; Zajac & Roques, 1985) and that the loss of binding of agonist but not antagonists probably reflected uncoupling of the G-protein from its receptor (Childers, 1991). Although the extent of the reduction of agonist binding for both GTP analogues and Na^+ varies (Chang *et al*., 1983; Puttfarken *et al*., 1986; Szucs *et al*., 1987) these differences have often been suggested to reflect differences in the experimental systems (Cox, 1993).

Nevertheless in this current study, where binding is under identical conditions and the methodological design is in many respects paired, the differences observed are clearly ligand dependent and this accords with many other studies that have demonstrated ligand/receptor dependency for GTP and cation regulation. For example, (Werling *et al*., 1986) showed in guinea-pig membranes that Na^+ reduced δ-receptor density without an effect on affinity but κ- and μ-binding showed loss of affinity without loss of site number. In cell lines expressing opioid receptors [^{3}H]-etorphine binding density is markedly reduced by Na^+ and GMPPNP but [^{3}H]-diprenorphine binding is unaffected (Polastron & Jauzac, 1994). Differences in sensitivity to other cations such as Mg^{2+}, dependent on both opioid receptor subtype and labelling ligand, have also been demonstrated (Kosterlitz *et al*., 1987; Rodriguez *et al*., 1992).

It is apparent that the Atc-substitution within the deltorphin molecule makes substantial differences in how the δ-receptor behaves under uncoupling conditions and these differences are exemplified by a marked difference between Ile^{5,6}deltorphin II and its Atc derivative. It is also worthy of

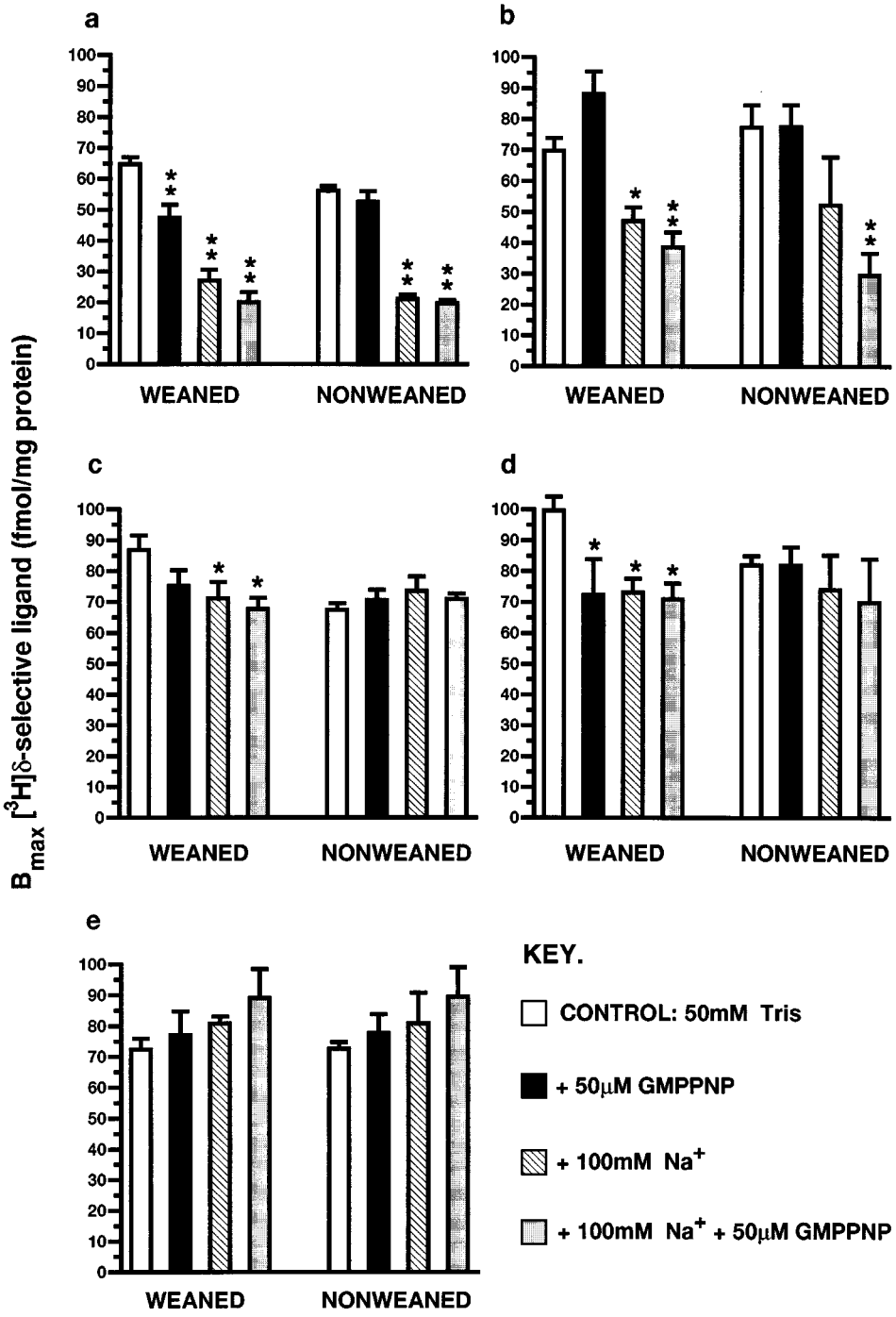

Figure 2 The effects of 50 μM GMPPNP and/or 100 mM Na^+ on δ-receptor density in the brain of 25-day-old weaned and non-weaned rats expressed as specific binding of (a) [^{3}H]-deltorphin I, (b) [^{3}H]-Ille^{5,6}deltorphin II, (c) [^{3}H]-S-Atc-Ile^{5,6}deltorphin I, (d) [^{3}H]-R-Atc-Ile^{5,6}deltorphin II and (e) [^{3}H]-naltrindole. Values represent the means ± s.e.mean of three to eight determinations by saturation binding to homogenates of whole brain minus cerebellum. For 3-way ANOVA showing interaction between ligand, behaviour and binding conditions, refer to results text. Individual comparisons of the effect of binding conditions were made in each group using Duncan's *post hoc* test $*P<0.05$, $**P<0.01$.

Table 2 Homogenate protein levels as a percentage of wet weight of tissue and specific binding ratios at K_d

	Protein (%)		*Specific binding ratio (%)*	
	Weaned	*Non-weaned*	*Weaned*	*Non-weaned*
[^{3}H]-deltorphin I				
Control Tris	5.5±0.2	5.3±0.2	87.1±1.1	80.2±2.8
50 μM GMPPNP	5.3±0.2	4.9±0.3	75.3±4.1	68.5±7.6
100 mM Na^+	5.7±0.3	6.1±0.2	65.3±1.2	52.0±8.9
100 mM Na^+ +50 μM GMPPNP	5.7±0.3	6.1±0.2	56.6±1.0	43.7±5.5
[^{3}H]-IIe^{5,6}deltorphin II				
Control Tris	5.4±0.2	5.2±0.2	43.2±4.0	51.0±4.9
50 μM GMPPNP	4.8±0.7	5.1±0.4	41.3±6.4	39.3±3.5
100 mM Na^+	5.7±0.2	4.9±0.4	21.2±3.0	17.8±0.5
100 mM Na^+ +50 μM GMPPNP	5.7±0.2	5.0±0.4	14.0±1.7	10.8±1.3
[^{3}H]-S-Atc-IIe^{5,6}deltorphin I				
Control Tris	6.4±0.1	6.1±0.01	70.6±1.6	66.5±2.4
50 μM GMPPNP	6.3±0.1	6.1±0.01	71.7±1.8	67.5±2.6
100 mM Na^+	6.1±0.1	5.8±0.01	73.0±1.4	70.0±2.3
100 mM Na^+ +50 μM GMPPNP	6.1±0.1	5.8±0.01	71.4±2.2	70.8±2.2
[^{3}H]-R-Atc-IIe^{5,6}deltorphin II				
Control Tris	6.6±0.2	6.7±0.2	53.0±3.6	58.3±1.8
50 μM GMPPNP	6.2±0.2	6.1±0.2	53.0±4.4	59.0±0.8
100 mM Na^+	6.2±0.2	6.5±0.01	53.3±2.7	54.0±8.2
100 mM Na^+ +50 μM GMPPNP	6.2±0.2	6.5±0.01	54.7±2.1	54.0±3.3
[^{3}H]-naltrindole				
Control Tris	5.3±0.1	4.9±0.2	71.0±3.8	75.5±0.2
50 μM GMPPNP	5.2±0.3	5.4±0.2	65.0±5.8	74.4±1.3
100 mM Na^+	6.1±0.5	6.2±0.4	75.5±2.0	78.0±4.0
100 mM Na^+ +50 μM GMPPNP	6.2±0.5	6.2±0.4	74.5±3.9	78.0±2.5

Values are means±s.e.mean for three to eight determinations. 3-way ANOVA showed no significant effect of behaviour (weaned or non-weaned, $F=0.82$, $P=0.37$) and no significant interaction between ligand, behaviour and binding conditions ($F=0.87$, $P=0.58$).

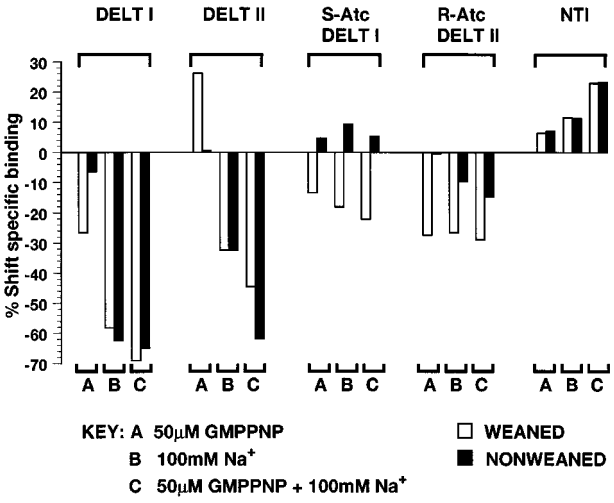

Figure 3 Shifts in specific binding of δ-receptor-selective ligands in the presence of 50 μM GMPPNP, 100 mM Na^+ or both, measured as specific bound in fmol/mg protein and expressed as a percentage of the specific binding under control conditions.

note that the Atc-substitution makes the ligand resistant to affinity changes in contrast to the parent deltorphin molecules which show decreases in agonist affinity consistent with the concept of increased agonist dissociation at the δ-receptor (Childers & Snyder, 1980). Although this might be typically viewed as a difference which may be explained by suggesting that the Atc-derivatives are partial agonists, the functional data on these compounds does not support this assertion (Toth *et al*., 1997). It is becoming increasingly clear that for the δ-receptor the effects of both GTP analogues and Na^+ can be markedly different from ligand to ligand and comparative studies with peptide and novel non-peptide ligands such as BW 373U86 show that this diethylbenzamide ligand is resistant to uncoupling conditions despite it being a full agonist (Childers *et al*., 1993; Wild *et al*., 1993). The evidence that suggests that Na^+ is additive with GTP in the effect δ-binding (Chang *et al*., 1981; Childers, 1991) is clearly not universal for every ligand and probably supports the concept that has been advanced that Na^+ acts at a membrane site distinct from the G-protein/ligand interaction (Costa *et al*., 1989; Ott *et al*., 1988).

It therefore seems likely that the δ-receptor shows multiple ligand recognition states and indeed that probably much of what has been interpreted as δ-subtypes represents different ligand recognition dependent on receptor environment. Such a proposal has been proffered to explain multiplicity of δ-receptor subtypes which might reflect coupled and uncoupled states of a single receptor species (Richardson *et al*., 1992). However, what is also intriguing from the present study is that the behavioural stimulus of weaning appears to alter the receptor or its environment and thus is able to alter ligand recognition. Some ligands show sensitivity to this effect and others do not. Such a modulatory role clearly has potential functional consequences and some of these we have already demonstrated in developing rats (Kitchen *et al*., 1994; Muhammad & Kitchen, 1993).

There is recent evidence in cell systems that the δ-receptor interacts with multiple G-proteins and that the functional effects of agonists are not related to their ability to activate these G-proteins and are independent of receptor density (Prather *et al*., 1994a, b). Nevertheless, our studies now show that a simple behavioural stimulus may produce an alteration in the receptor and/or its membrane environment influencing the recognition of the δ-receptor, which has consequences in the alteration of biological response during development.

In conclusion, we have shown that differences in binding density of δ-receptors in 25-day-old weaned and non-weaned rats can be explained in terms of the way in which various radioligands recognise the receptor environment. It appears that the behavioural separation of the mother causes changes in the δ-opioid receptor and/or its membrane environment. Finally, this study serves to confirm marked differences exist between δ-agonist ligands in their sensitivity to GTP and cations.

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