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G-protein coupling of δ-opioid receptors in brains of μ-opioid receptor knockout mice

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Abstract

μ-opioid receptor knockout mice have been reported to show loss of some δ-opioid receptor function. We hypothesised that this is due to some δ-opioid receptors being uncoupled from G-proteins in the absence of the μ-opioid receptor. To address this possibility, we have used quantitative autoradiography to determine the binding of three δ-opioid receptor agonist ligands ([^{3}H]deltorphin I, [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II, [^{3}H] 4-[(alpaR)-alpha-((2S,5R)-4-propyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC-121)) and the δ-opioid receptor antagonist, [^{3}H]naltrindole in the presence and absence of a GTP analogue, guanylylimidodiphosphate (GMPPNP) in the brains of mice lacking the μ-opioid receptor gene. Guanylylimidodiphosphate caused a decrease in the binding of the agonist ligands (to differing extents) and an increase in binding for the antagonist in wild-type controls. Overall, there were no major differences in the effects of guanylylimidodiphosphate for the agonist ligands in μ-knockout mice although a few structures showed a smaller sensitivity to the effects of this GTP analogue most notably for [^{3}H]naltrindole. These findings suggest that in the majority of brain regions, G-protein coupling is unaltered in μ-opioid receptor knockout mice.

Keywords: Autoradiography; d-opioid receptor; G-protein coupling; Opioid receptor knockout

1. Introduction

The first opioid receptor to be disrupted using transgenic approaches was the μ-opioid receptor (Matthes et al., 1996). There are now five independent groups that have reported the successful generation of mice lacking the μ-opioid receptor using homologous recombination techniques with different exons of the μ-opioid receptor gene targeted (Loh et al., 1998; Matthes et al., 1996; Schuller et al., 1999; Sora et al., 1997; Tian et al., 1997). In all mutant mice, disruption of the μ-opioid receptor gene leads to a complete loss of the main biological actions of morphine, including analgesia, reward, immunosuppression, constipation and physical dependence (see Kieffer and Gaveriaux-Ruff, 2002; Kitchen, 1999).

Overall, homogenate binding studies show that there are no major compensatory changes in either κ- or δ-opioid receptors in μ-knockout animals (Loh et al., 1998; Matthes et al., 1996). However, there have been some indications in the literature that there are some subtle changes in both the expression and function of the δ-opioid receptor in μ-knockout mice. We have shown by quantitative autoradiography small, but significant, decreases in the number of [^{3}H]deltorphin I binding sites in some brain regions of μ-knockout mice. The brain regions demonstrating this down regulation are predominantly non-cortical and areas that are known to have a high expression of μ-opioid receptors in wild-type animals (Kitchen et al., 1997). In agreement with this finding of discrete changes in δ-opioid receptor binding in μ-knockout animals, Matthes et al. (1996) reported that administration of the selective δ-opioid receptor agonist BUBU ([Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)]) did not cause antinociception in the tail flick test in μ-knockout animals whereas it caused significant antinociception in wild-type animals. Furthermore, it has also been reported that other selective δ-opioid receptor ligands, [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and deltorphin II, have partially reduced activity in μ-knockout mice, including complete absence of respiratory depression and reduced analgesia in

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the tail flick model (Matthes et al., 1998; Sora et al., 1997) as well as absence of deltorphin-conditioned place preference and withdrawal (Hutcheson et al., 2001). Such loss of functional responses to DPDPE has also been reported by others (Hosohata et al., 2000). In addition, the action of a non-peptide δ-opioid receptor agonist SNC80 ((+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide) has also been shown to be altered in animals lacking the μ-opioid receptor gene, as acetic acid writhing is reduced by SNC80 in wild type but not μ-knockout mice (Sora et al., 1999).

These findings of altered responses to some δ-opioid receptor agonists in μ-knockout mice could be explained if the agonists used elicited their response through both activation of δ- and μ-opioid receptors. An alternative explanation is the existence of a functional complex between the δ- and μ-opioid receptors, which has been postulated for several years (Lee et al., 1980; Rothman and Westfall, 1982; Vaught et al., 1982). If such a complex exists, it is feasible to assume that the lack of antinociceptive responses to certain δ-opioid agonists in μ-knockout animals could be explained by the removal of the μ-opioid receptor from the complex. The remaining δ-opioid receptor may then have an altered signal transduction pathway, regulation or G-protein-coupled state.

To address this issue, several groups have investigated the G-protein coupling of the δ-opioid receptor in μ-knockout mice but the data are equivocal. Using whole brain homogenates, Matthes et al. (1998) reported that the ability of δ-opioid agonists to stimulate [^{35}S]GTPγS binding in μ-knockout mice was unchanged compared to wild-type controls and this has also shown to be the case for δ- and κ-agonists in spinal cord membranes (Narita et al., 1999). In contrast, a recent study has shown differences in stimulation of [^{35}S]GTPγS binding in the brains of μ-knockout animals by various δ-opioid ligands with maximal responses to pCl-DPDPE being reduced (Hosohata et al., 2000).

Although most of the findings from membrane binding studies suggest that δ-opioid receptors remain functionally coupled in μ-knockout mice (Hosohata et al., 2000; Matthes et al., 1998; Narita et al., 1999), a recent autoradiographic study has reported a lack of DPDPE-induced stimulation of [^{35}S]GTPγS binding in both the caudate and cerebral cortex in μ-knockout mice (Park et al., 2000) and they concluded that the μ-opioid receptor is needed for functional δ-opioid receptor coupling. Only three brain structures were quantified in the autoradiographic study (Park et al., 2000), so it is unclear what the effect of the absence of the μ-opioid receptor has on the coupling of the δ-opioid receptor throughout the brain. In order to resolve the apparent conflict in the literature regarding the coupling of the δ-opioid receptor in μ-knockout animals, we have now carried out a detailed autoradiographic analysis of δ-opioid receptor expression and coupling throughout the brain of μ-knockout animals. We have used both agonist and antagonist ligands to test the hypothesis that δ-receptor G-protein coupling is disrupted in only certain brain regions and that this might be responsible for the some loss of δ-opioid receptor function.

2. Materials and methods

2.1. Animals

μ-Opioid receptor gene knockout animals, disrupted in exon 2, were prepared according to the methods described by Matthes et al. (1996). Mice (of mixed sexes, aged 6–8 weeks) were killed by decapitation and intact whole brains were removed.

2.2. Autoradiography

Autoradiography was performed, as previously described (Kitchen et al., 1997). Adjacent coronal brain sections (20 μm) were used for the determination of total binding, binding in the presence of 50 μM guanylylimidodiphosphate and non-specific binding (determined by naloxone 1 μM) for each of [^{3}H]deltorphin I (7 nM), [^{3}H] [R-Atc^3, Ile^{5,6}]-deltorphin II (8 nM), [^{3}H]SNC-121(10 nM) and [^{3}H]naltrindole (1 nM). Ligand concentrations used were approximately 3–4 times K_d. An incubation time of 60 min was employed for [^{3}H]deltorphin I and [^{3}H]R-Atc^3, Ile^{5,6} deltorphin II, whilst 90 and 120 min were employed for [^{3}H]naltrindole and [^{3}H]4-[(alpaR)-alpha-((2S,5R)-4-propyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC-121), respectively, to achieve equilibrium binding. Sections from individual wild-type and knockout mouse brains were processed in a paired protocol including using identical radioligand stocks, apposition to the same tritium-sensitive film (5 weeks) and parallel image analysis.

2.3. Quantitative analysis and statistical procedures

Quantitative analysis of autoradiographic films was carried out, as detailed previously (Kitchen et al., 1997), using video-based computerised densitometry. Control specific binding and specific binding in the presence of

Fig. 1. Computer enhanced colour autoradiograms of coronal brain sections from wild type (+/+) and μ-opioid receptor knockout (−/−) mice showing binding of [^{3}H]deltorphin I (DELT I) (7 nM), [^{3}H]SNC-121 (10 nM), [^{3}H][R-Atc^3, Ile^{5,6}]deltorphin II (RATGLU) (8 nM) and [^{3}H]naltrindole (NTI) (1 nM) in the presence and absence of guanylylimidodiphosphate (GMPPNP) (50 μM). Panels shown are adjacent sections cut at either the level of the caudate or amygdala. The colour bar shows a pseudo-colour interpretation of relative density of black and white film images calibrated in fmol/mg tissue.

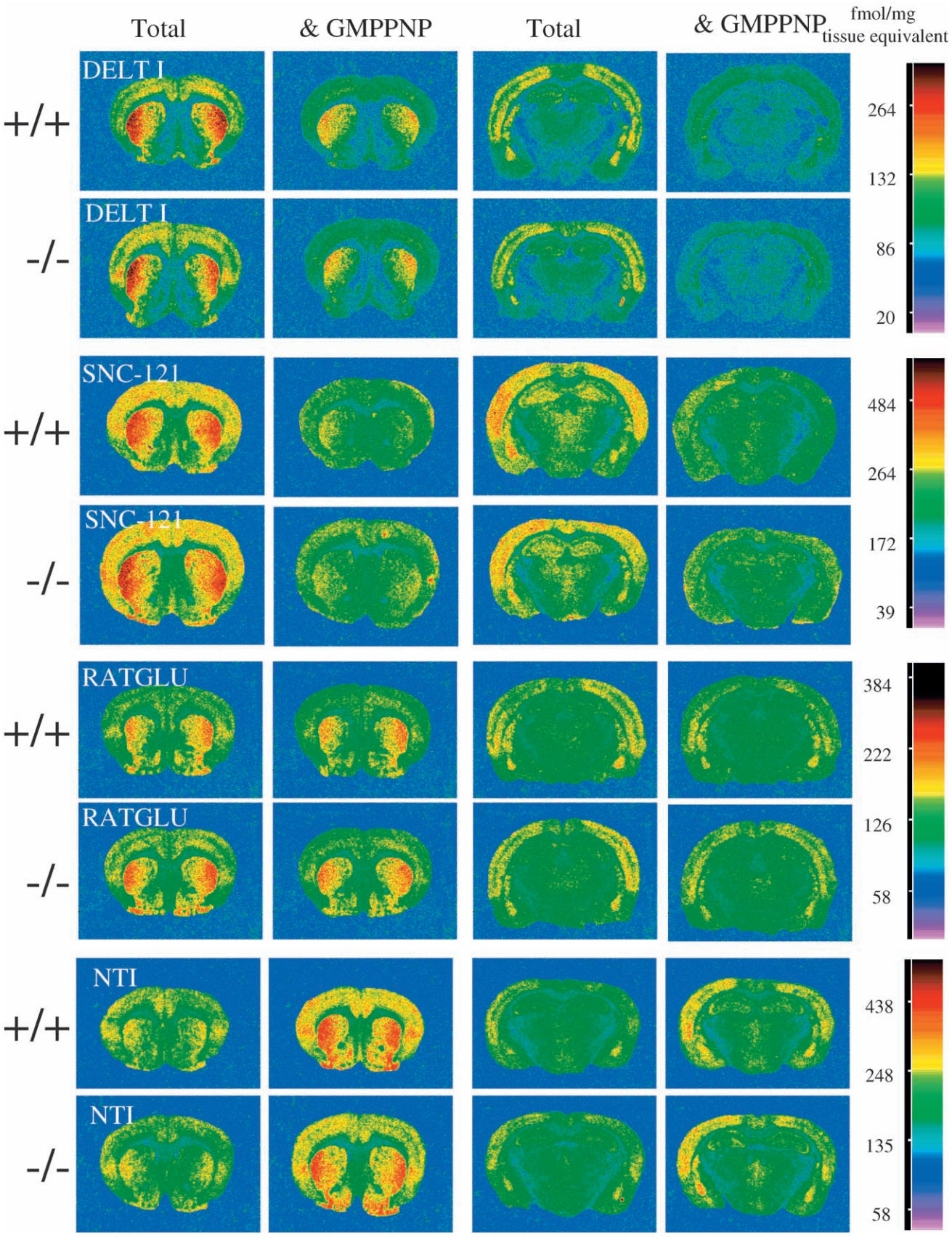
Total
& GMPPNP
Total
& GMPPNP
fmol/mg
tissue equivalent
DELT I
+/+
DELT I
-/-
264
132
86
20
SNC-121
+/+
SNC-121
-/-
484
264
172
39
RATGLU
+/+
RATGLU
-/-
384
222
126
58
NTI
+/+
NTI
-/-
438
248
135
58

Table 1
Mean specific binding (fmol/mg tissue equivalent) ± S.E.M. of [^{3}H]deltorphin I, [^{3}H]SNC-121, [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II (RATGLU) and [^{3}H]naltrindole (NTI) in the presence or absence of guanylylimidodiphosphate (GMPPNP) (50 μM) in cortical brain regions of wild type (+/+) and μ-opioid receptor knockout (−/−) mice (n = 5–6)

Cortical regions		[^{3}H]DELT I		[^{3}H]SNC-121		[^{3}H]RATGLU		[^{3}H]NTI	
		Control	GMPPNP	Control	GMPPNP	Control	GMPPNP	Control	GMPPNP
Motor									
Superficial layer	+/+	71 ± 4.6	30 ± 2.4	37 ± 3.4	6.4 ± 2.5	83 ± 6.3	61.3 ± 7.5	26 ± 7.1	52 ± 7.6
	−/−	70 ± 4.2	28 ± 2.5	44 ± 1.8	8.8 ± 2.2	74 ± 6.9	63.2 ± 7.3	19 ± 3.3	53 ± 5.6
Deep layer	+/+	84 ± 6.4	42 ± 4.1	47 ± 4.2	7.0 ± 2.4	90 ± 5.0	74.1 ± 7.0	24 ± 6.5	46 ± 7.3
	−/−	85 ± 4.3	39 ± 3.1	54 ± 3.7	8.5 ± 3.1	84 ± 7.9	73.9 ± 6.0	28 ± 8.1	52 ± 4.6
Cingulate									
Superficial layer	+/+	66 ± 5.1	30 ± 3.3	35 ± 3.4	5.9 ± 1.4	64 ± 6.2	67.9 ± 7.4	27 ± 9.0	47 ± 4.0
	−/−	76 ± 6.2	30 ± 2.2	40 ± 4.6	10 ± 3.2	77 ± 5.1	69.9 ± 5.2	22 ± 7.0	43 ± 5.8
Deep layer	+/+	74 ± 6.4	35 ± 4.7	43 ± 4.7	7.5 ± 0.6	66 ± 4.3	72.7 ± 6.3	25 ± 7.6	38 ± 5.3
	−/−	79 ± 4.9	38 ± 4.3	46 ± 6.3	9.6 ± 4.0	81 ± 4.6	75.8 ± 5.3	28 ± 8.8	42 ± 4.6
Rostral somatosensory									
Superficial layer	+/+	71 ± 5.1	30 ± 3.2	35 ± 7.3	5.4 ± 2.7	74 ± 8.7	71.2 ± 6.3	37 ± 11.6	49 ± 4.7
	−/−	79 ± 7.6	32 ± 2.5	46 ± 1.9	14 ± 4.7[a]	95 ± 4.7	73.1 ± 6.7	40 ± 8.8	51 ± 6.1
Deep layer	+/+	76 ± 6.7	37 ± 3.6	44 ± 5.8	7.8 ± 2.5	72 ± 6.2	74.7 ± 6.2	23 ± 9.7	36 ± 3.4
	−/−	80 ± 7.3	43 ± 5.0	54 ± 4.0	14 ± 3.6	88 ± 5.5	83.4 ± 5.1	27 ± 6.7	47 ± 9.1
Caudal somatosensory									
Superficial layer	+/+	73 ± 5.6	26 ± 1.8	36 ± 4.4	11 ± 5.8	81 ± 5.9	67.7 ± 8.0	31 ± 5.7	49 ± 5.2
	−/−	70 ± 5.7	35 ± 4.3	42 ± 3.9	7.9 ± 1.8	83 ± 7.0	73.7 ± 8.2	42 ± 10.1	45 ± 1.4
Deep layer	+/+	73 ± 6.2	31 ± 2.0	41 ± 3.9	9.6 ± 3.7	76 ± 5.6	72.0 ± 4.3	27 ± 5.4	39 ± 4.3
	−/−	74 ± 8.3	39 ± 6.3	50 ± 2.9	11 ± 2.5	79 ± 4.5	76.0 ± 7.3	34 ± 8.4	41 ± 6.3
Auditory									
Superficial layer	+/+	71 ± 4.9	27 ± 2.3	41 ± 6.0	5.6 ± 1.4	80 ± 6.5	71.1 ± 5.8	30 ± 8.9	58 ± 6.5
	−/−	76 ± 7.9	35 ± 5.3	41 ± 4.3	8.7 ± 2.7	91 ± 6.5	68.6 ± 4.2	30 ± 6.2	46 ± 12.6
Deep layer	+/+	76 ± 6.2	33 ± 3.1	47 ± 3.0	9.3 ± 2.6	82 ± 3.0	73.9 ± 4.0	32 ± 8.8	39 ± 3.3
	−/−	78 ± 8.4	39 ± 5.8	37 ± 3.3	8.7 ± 2.3	92 ± 5.8	75.9 ± 3.0	37 ± 5.7	37 ± 7.4
Visual									
Superficial layer	+/+	73 ± 2.9	27 ± 1.8	41 ± 6.5	9.1 ± 3.3	84 ± 3.6	65.6 ± 4.9	25 ± 8.3	49 ± 6.9
	−/−	73 ± 9.1	36 ± 4.8	45 ± 3.1	7.2 ± 1.3	88 ± 5.2	76.5 ± 8.2	37 ± 5.5	59 ± 13
Deep layer	+/+	72 ± 4.5	33 ± 2.9	45 ± 1.9	11 ± 3.6	81 ± 5.0	70.5 ± 4.5	23 ± 8.4	41 ± 2.9
	−/−	74 ± 8.4	41 ± 6.7	45 ± 3.1	7.2 ± 1.3	89 ± 3.7	82.3 ± 3.6	32 ± 7.1	39 ± 9.1
Retrosplenial									
Superficial layer	+/+	56 ± 3.8	25 ± 2.1	35 ± 6.1	6.3 ± 3.6	64 ± 1.9	54.8 ± 2.5	15 ± 9.3	34 ± 2.1
	−/−	57 ± 4.1	31 ± 3.6	35 ± 2.5	6.8 ± 3.2	60 ± 2.8	54.9 ± 2.7	20 ± 7.1	30 ± 10
Deep layer	+/+	61 ± 0.6	30 ± 0.7	40 ± 1.2	7.0 ± 2.0	67 ± 1.9	61.8 ± 1.6	26 ± 2.2	33 ± 2.0
	−/−	60 ± 1.5	34 ± 2.3	40 ± 4.2	8.0 ± 1.5	65 ± 2.2	60.8 ± 2.8	26 ± 2.7	30 ± 3.0
Perirhinal	+/+	81 ± 4.8	29 ± 3.4	44 ± 1.3	6.1 ± 0.7	80 ± 3.8	70.3 ± 4.8	35 ± 9.6	57 ± 5.0
	−/−	83 ± 6.9	31 ± 6.8	39 ± 5.1	5.8 ± 3.0	77 ± 4.7	66.1 ± 3.1	39 ± 7.2	47 ± 8.1
Entorhinal	+/+	40 ± 3.7	18 ± 1.3	32 ± 3.5	6.6 ± 2.0	46 ± 5.5	42.0 ± 4.7	24 ± 12.0	48 ± 7.2
	−/−	31 ± 3.1	15 ± 1.8	23 ± 3.8	11 ± 3.7	45 ± 6.2	40.0 ± 3.9	21 ± 7.1	29 ± 7.1

Measures from regions were taken at Bregma coordinates, as previously described (Slowe et al., 1999), taken from the mouse atlas of Franklin and Paxinos (1997).

[a] $P < 0.05$, Fischers LSD post-hoc test.

guanylylimidodiphosphate were determined by subtraction of non-specific binding from either total binding or binding in the presence of guanylylimidodiphosphate, respectively. Structures were identified using the mouse brain atlas of Franklin and Paxinos (1997). For each [^{3}H] δ-opioid receptor ligand, a value for the mean specific binding (± S.E.M.) was determined for all brain structures analysed for both treatment groups (control specific binding and specific binding in the presence of guanylylimidodiphosphate) for wild-type and knockout mice. For statistical purposes, regions were split into cortical and non-cortical subsets before comparison of receptor binding was made. The effect of genotype on binding in cortical or non-cortical regions was determined by Fishers least significant difference (LSD) post hoc test after a one-way analysis of variance (ANOVA) for both control and guanylylimidodiphosphate data sets.

2.4. Materials

[^{3}H]naltrindole (NTI) (44.2 Ci/mmol) (Toth et al., 1993) and [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II (RATGLU) (36 Ci/mmol) were synthesised by the Hungarian Academy of Sciences, Szeged. [^{3}H]deltorphin I (47 Ci/mmol) was cus-

Table 2
Mean specific binding (fmol/mg tissue equivalent) ± S.E.M. of [^{3}H]deltorphin I, [^{3}H]SNC-121, [^{3}H][R-Atc^3, Ile^{5,6}]deltorphin II (RATGLU) and [^{3}H]naltrindole (NTI) in the presence or absence of guanylylimidodiphosphate (GMPPNP) (50 μM) in non-cortical brain regions of wild type (+/+) and μ-opioid receptor knockout (− / −) mice (n = 5–6)

Non-cortical regions		[^{3}H]DELT I		[^{3}H]SNC-121		[^{3}H]RATGLU		[^{3}H]NTI	
		Control	GMPPNP	Control	GMPPNP	Control	GMPPNP	Control	GMPPNP
Olfactory bulb									
External plexifom layer	+/+	247 ± 13	136 ± 13	102 ± 10	24 ± 6.2	237 ± 11	226 ± 26	111 ± 13	215 ± 13
	− / −	230 ± 14[a]	106 ± 5.9[a]	131 ± 5.3[a]	20 ± 3.6	249 ± 12	269 ± 33.2[a]	141 ± 13	212 ± 9.9
Internal plexiform layer	+/+	74 ± 1.2	41 ± 2.2	33 ± 2.7	5.4 ± 1.9	77 ± 5.6	70 ± 4.8	27 ± 4.1	65 ± 13
	− / −	80 ± 11	43 ± 7.0	44 ± 3.0	9.0 ± 2.2	90 ± 5.5	91 ± 7.6	34 ± 7.4	59 ± 10
Nucleus accumbens									
Shell	+/+	68 ± 5.8	28 ± 3.2	33 ± 6.3	3.7 ± 1.8	30 ± 4.3	30 ± 3.2	17 ± 7.3	22 ± 4.4
	− / −	40 ± 3.4[a]	26 ± 2.0	35 ± 1.6	6.4 ± 2.8	28 ± 2.1	33 ± 5.0	11 ± 5.6	12 ± 2.9
Core	+/+	41 ± 3.3	18 ± 1.7	27 ± 3.7	3.9 ± 1.7	52 ± 6.8	58 ± 4.3	23 ± 8.2	54 ± 4.9
	− / −	24 ± 2.1[a]	17 ± 2.0	27 ± 2.9	10 ± 3.4	55 ± 3.9	60 ± 8.1	17 ± 7.4	39 ± 10
Olfactory tubercle	+/+	88 ± 11	42 ± 4.4	51 ± 4.9	6.1 ± 3.5	98 ± 13	100 ± 11	45 ± 3.2	123 ± 6.3
	− / −	78 ± 8.3	41 ± 4.7	58 ± 4.9	16 ± 2.6	117 ± 7.6	100 ± 8.5	59 ± 7.7	80 ± 4.4[a]
Caudate Putamen									
Medial	+/+	84 ± 8.4	53 ± 6.8	52 ± 4.1	9.6 ± 1.6	81 ± 8.0	94 ± 4.6	30 ± 10	64 ± 5.6
	− / −	83 ± 7.7	49 ± 7.3	58 ± 7.8	19 ± 1.9	85 ± 5.2	89 ± 4.5	28 ± 10	49 ± 4.2
Lateral	+/+	147 ± 14	78 ± 8.2	69 ± 10	9.2 ± 3.0	119 ± 12	138 ± 8.5	42 ± 13	106 ± 8.0
	− / −	131 ± 12[a]	73 ± 8.5	92 ± 9.5[a]	7.9 ± 2.3	135 ± 8.3	136 ± 8.8	48 ± 14	89 ± 3.4
Septum									
Medial	+/+	37 ± 2.0	18 ± 2.0	25 ± 4.2	3.8 ± 1.7	34 ± 3.2	34 ± 3.6	12 ± 4.1	36 ± 4.6
	− / −	33 ± 2.6	21 ± 2.4	30 ± 5.5	7.6 ± 2.3	36 ± 3.9	38 ± 1.9	11 ± 5.3	12 ± 4.7
Lateral	+/+	14 ± 0.8	8.4 ± 1.2	12 ± 1.7	2.3 ± 1.8	10 ± 1.6	12 ± 1.4	4.3 ± 1.7	5.7 ± 2.3
	− / −	13 ± 2.1	7.0 ± 0.5	12 ± 4.2	2.2 ± 2.2	13 ± 1.5	13 ± 1.9	5.7 ± 3.0	5.5 ± 3.9
Ventral diagonal band	+/+	38 ± 6.5	21 ± 3.3	26 ± 5.2	7.3 ± 1.3	52 ± 6.3	45 ± 7.2	19 ± 5.3	51 ± 2.8
	− / −	37 ± 4.4	21 ± 3.0	34 ± 6.5	8.4 ± 4.3	52 ± 6.3	49 ± 4.7	18 ± 2.6	27 ± 4.4[a]
Globus pallidus	+/+	17 ± 0.4	15 ± 2.6	15 ± 2.4	7.5 ± 3.3	16 ± 2.2	23 ± 1.2	5.0 ± 1.9	9.1 ± 1.5
	− / −	17 ± 1.9	16 ± 3.3	16 ± 2.4	7.0 ± 2.4	20 ± 2.4	22 ± 3.8	3.6 ± 2.3	7.9 ± 2.2
Stria terminalis	+/+	57 ± 4.7	23 ± 2.5	40 ± 2.1	19 ± 0.9	64 ± 7.0	73 ± 4.7	29 ± 6.1	56 ± 7.4
	− / −	50 ± 5.3	24 ± 4.0	54 ± 13	21 ± 5.0	70 ± 4.7	76 ± 5.2	27 ± 4.5	68 ± 9.8
Bed nucleus of the stria terminalis	+/+	19 ± 0.5	12 ± 2.6	13 ± 2.3	9.8 ± 5.3	21 ± 2.5	22 ± 1.7	9.3 ± 2.3	9.0 ± 1.6
	− / −	18 ± 1.7	12 ± 2.5	16 ± 3.2	6.1 ± 2.8	19 ± 2.1	19 ± 2.4	2.1 ± 1.0	8.6 ± 1.1
Medial pre-optic layer	+/+	10 ± 0.6	6.3 ± 0.7	6.3 ± 1.2	2.7 ± 2.0	13 ± 1.9	17 ± 1.6	5.5 ± 2.2	4.7 ± 2.0
	− / −	9.6 ± 1.5	8.8 ± 2.3	7.9 ± 3.0	6.8 ± 2.1	11 ± 2.2	11 ± 2.8	3.8 ± 2.4	6.5 ± 3.0
Thalamus	+/+	30 ± 1.1	14 ± 0.8	19 ± 8.0	8.0 ± 2.4	27 ± 2.1	24 ± 2.1	5.7 ± 2.6	8.6 ± 2.0
	− / −	26 ± 2.1	17 ± 2.5	24 ± 3.4	7.4 ± 5.4	31 ± 2.8	27 ± 2.1	1.8 ± 4.7	13 ± 2.8
Hypothalamus	+/+	21 ± 1.4	11 ± 1.3	14 ± 2.3	4.5 ± 1.7	21 ± 2.5	20 ± 2.2	3.1 ± 1.7	12 ± 4.3
	− / −	16 ± 2.7	13 ± 2.4	15 ± 5.0	14 ± 12	17 ± 2.7	17 ± 1.2	8.7 ± 4.0	16 ± 0.9
Amygdala									
Basolateral	+/+	99 ± 7.6	30 ± 1.9	42 ± 4.5	8.4 ± 3.1	88 ± 5.1	73 ± 4.4	31 ± 9.6	66 ± 7.7
	− / −	79 ± 7.2[a]	37 ± 4.8	43 ± 5.2	5.3 ± 2.8	95 ± 8.2	83 ± 12	52 ± 6.9[a]	59 ± 6.0
Basomedial	+/+	33 ± 1.5	17 ± 2.2	21 ± 0.7	5.7 ± 3.1	39 ± 2.5	40 ± 3.0	13 ± 5.5	30 ± 8.7
	− / −	31 ± 4.1	23 ± 4.2	21 ± 3.1	3.2 ± 2.0	42 ± 4.4	39 ± 4.5	22 ± 6.6	33 ± 4.9
Medial	+/+	30 ± 0.7	13 ± 2.4	18 ± 1.9	11 ± 6.5	35 ± 2.1	35 ± 3.3	16 ± 7.4	30 ± 7.6
	− / −	28 ± 3.3	19 ± 4.4	16 ± 3.8	4.9 ± 2.5	37 ± 4.6	36 ± 3.7	21 ± 4.7	27 ± 4.3
Hippocampus	+/+	44 ± 3.3	16 ± 1.6	29 ± 2.7	7.6 ± 1.8	44 ± 1.9	36 ± 2.6	12 ± 5.0	22 ± 1.9
	− / −	41 ± 3.9	19 ± 4.6	22 ± 2.9	2.8 ± 1.5	48 ± 3.5	39 ± 4.8	19 ± 6.9	15 ± 1.4
Presubiculum	+/+	71 ± 4.2	36 ± 3.0	60 ± 4.5	10 ± 5.1	93 ± 1.8	83 ± 2.9	38 ± 9.6	64 ± 3.8
	− / −	64 ± 3.5	34 ± 3.5	48 ± 4.5	12 ± 4.0	94 ± 3.7	83 ± 7.4	32 ± 4.9	60 ± 10.4
Interpeduncular nucleus	+/+	10 ± 2.5	5.6 ± 2.2	11 ± 3.6	8.9 ± 4.0	18 ± 4.7	22 ± 7.0	7.2 ± 5.1	5.8 ± 2.3
	− / −	7.7 ± 1.6	3.4 ± 1.5	11 ± 3.3	8.0 ± 3.6	12 ± 2.5	8.4 ± 2.2	5.4 ± 2.1	5.6 ± 3.2
Pontine nucleus	+/+	46 ± 4.0	23 ± 3.6	37 ± 1.5	11 ± 3.4	62 ± 4.0	39 ± 8.5	25 ± 5.5	31 ± 2.1
	− / −	46 ± 4.0	18 ± 0.9	38 ± 4.0	8.2 ± 2.6	67 ± 4.3	53 ± 2.1	25 ± 4.4	35 ± 2.3

Measures from regions were taken at Bregma coordinates, as previously described (Slowe et al., 1999), taken from the mouse atlas of Franklin and Paxinos (1997).

[a] $P < 0.05$, Fischers LSD post-hoc test.

tom synthesised by Amersham Radiochemicals and [^{3}H]SNC-121 (53 Ci/mmol) was purchased from Tocris Cookson. Naloxone hydrochloride and guanylylimidodiphosphate were purchased from Sigma Chemicals and Roche Diagnostics, respectively. All other chemical used were of analytical grade.

3. Results

3.1. Effect of guanylylimidodiphosphate on specific binding

δ-Opioid receptors labelled with [^{3}H]deltorphin I, [^{3}H]R-Atc^3, Ile^{5,6} deltorphin II, [^{3}H]SNC-121 or [^{3}H]naltrindole were observed throughout the brain of wild-type (+/+) animals. The qualitative distribution of the δ-opioid receptors was identical when labelled by the different radioligands and guanylylimidodiphosphate had no effect on the qualitative pattern of expression (Fig. 1). Guanylylimidodiphosphate significantly reduced the level of binding of both [^{3}H]deltorphin I and [^{3}H]SNC-121 in both cortical and non-cortical brain regions quantified (ANOVA $P<0.01$). The effect of guanylylimidodiphosphate on [^{3}H]SNC-121 binding was greater than the effect on [^{3}H]deltorphin I binding (approximately 80% compared to 50% reduction, respectively) (Tables 1 and 2). In contrast, [^{3}H]naltrindole binding was significantly increased by the inclusion of guanylylimidodiphosphate in the incubation buffer in cortical and non-cortical brain regions (ANOVA $P<0.01$; Tables 1 and 2). Guanylylimidodiphosphate did not significantly effect the binding of [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II binding to non-cortical regions, but guanylylimidodiphosphate did cause a significant decrease in binding in cortical regions (ANOVA $P<0.01$; Tables 1 and 2).

3.2. Effect of genotype on specific binding

Some specific non-cortical brain regions showed a significant decrease in [^{3}H]deltorphin I binding in knockout mice (Table 2), and the most marked changes were seen in the nucleus accumbens core and shell (40% decrease) and in the basolateral amygdala (21% decrease). In contrast, post hoc analysis of individual regions for the other agonists ([^{3}H]R-Atc^3, Ile^{5,6} deltorphin II and [^{3}H]SNC-121) did not show any significant decreases in binding in the knockout tissue, with just two regions (olfactory bulb and lateral caudate putamen) showing significant increases in binding. Significant increases in binding were also observed in knockout brains for the antagonist [^{3}H]naltrindole in olfactory tubercle and basolateral amygdala (Tables 1 and 2). Overall, changes in cortical structures between genotypes were minimal.

3.3. Effect of genotype and guanylylimidodiphosphate on specific binding

There was a smaller decrease in [^{3}H]deltorphin I binding induced by guanylylimidodiphosphate in just a few regions in knockout mice compared to wild-type mice. This difference was most marked in non-cortical regions and in regions that also showed a decreased level of binding in knockout animals compared to wild-type controls (Table 2). These regions included the shell and core of the nucleus accumbens, lateral caudate putamen and the basolateral amygdala. Accordingly, after guanylylimidodiphosphate treatment, post hoc analysis showed that there was only one region (olfactory bulb) where significant differences in the levels of binding between the genotypes was observed. After treatment with guanylylimidodiphosphate, the levels of binding of [^{3}H]SNC-121 and [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II levels were very similar in all regions of wild type and knockout mice ($P>0.05$). For [^{3}H]naltrindole, the significant increase in binding after guanylylimidodiphosphate treatment was less marked in the knockout brains in several regions and in addition post hoc analysis showed significant decreases, rather than increases, in binding in the knockout tissue in olfactory tubercle and ventral diagonal band.

4. Discussion

The finding of a decrease in binding of [^{3}H]deltorphin I or [^{3}H]SNC-121 in the presence of guanylylimidodiphosphate in over 90% of regions analysed suggests, as expected from their potent agonist activity, that [^{3}H]deltorphin I and [^{3}H]SNC-121 have greater affinity for the coupled δ-opioid receptor state than the uncoupled receptor state. Despite the reported potent agonist activity of [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II (Toth et al., 1997), guanylylimidodiphosphate did not significantly change binding to non-cortical regions, and this finding of insensitivity of [^{3}H] [R-Atc^3, Ile^{5,6}]deltorphin II binding to guanylylimidodiphosphate is in agreement with our findings in rat brain homogenates (Kelly et al., 1998). In contrast to the finding with the [^{3}H]δ-opioid receptor agonists, the increase in binding of the δ-opioid receptor antagonist [^{3}H]naltrindole in the presence of guanylylimidodiphosphate would indicate that this compound is more selective for the uncoupled receptor state and suggests that [^{3}H]naltrindole may act as an inverse agonist. The cortical/non-cortical difference in the effect of guanylylimidodiphosphate might suggest that the coupling state of the δ-receptor may not be homogeneous throughout the brain and this has been suggested by others for the μ-receptor (Maher et al., 2000). Alternatively, it is known that δ-opioid receptors couple to multiple G-proteins (for a review, see Quock et al., 1999) and that there are regional differences in G-protein subtype expression (Wekesa and Anholt, 1999) and thus the differences we have found could reflect regional variations in G-protein subtype coupling to the δ-opioid receptor.

In agreement with other studies, there were no major compensatory changes in the expression of the δ-opioid receptor in the brains of μ-opioid receptor knockout mice compared to wild type (Chen et al., 2000; Kitchen et al., 1997; Loh et al., 1998; Matthes et al., 1996). However, in support of the findings of our previous study (Kitchen et al., 1997), a small down regulation of [^{3}H]deltorphin I binding was apparent in some regions of the brains of μ-opioid receptor knockout compared to wild-type mice particularly

in non-cortical structures. This effect is most marked in the nucleus accumbens and basolateral amygdala, where the sensitivity to guanylylimidodiphosphate is not so great in the knockout brain. More pronounced differences of the effect of guanylylimidodiphosphate were observed for the binding of the antagonist [^{3}H]naltrindole where several non-cortical structures (e.g. nucleus accumbens, caudate putamen, ventral diagonal band, olfactory tubercle, medial septum and hippocampus) showed less sensitivity to the effects of the GTP analogue. This finding does not directly point to uncoupling of the δ-receptor as it is not widely evident for the agonist ligands. Nonetheless, the alterations in guanylylimidodiphosphate sensitivity for the antagonist naltrindole might suggest altered δ-receptor functioning which might account for the previously reported partial loss of δ-opioid receptor agonist activity in μ-knockout mice (Hosohata et al., 2000; Matthes et al., 1998; Sora et al., 1997) and with the report of Park et al. (2000). Earlier studies have failed to show removal of the μ-opioid receptor influences δ-opioid receptor coupling (Hosohata et al., 2000; Matthes et al., 1998) and it is possible that this is because these studies used homogenate binding approaches which may lack the sensitivity afforded by the autoradiographic approach.

A functional interaction between the μ- and δ-opioid receptor has been postulated for several years (Rothman et al., 1988; Rothman and Westfall, 1982; Vaught et al., 1982). There is also evidence for a close physical interaction and the receptors are not separable on the basis of hydrodynamic parameters (Simon et al., 1986). In addition, receptor dimerisation of opioid receptors has been demonstrated to influence ligand binding and pharmacological properties of ligands (Cvejic and Devi, 1997; Jordan and Devi, 1999). It is therefore feasible that if some δ-opioid receptors do exist as heterodimers with μ-opioid receptors in vivo, the removal of the μ-opioid receptor from the complex could influence the ligand recognition of the δ-opioid receptor. Where there is evidence for altered ligand recognition (most notably for [^{3}H]naltrindole), the altered sensitivity to guanylylimidodiphosphate is predominantly in non-cortical structures pointing to region dependency in alterations of δ-opioid receptor recognition.

In conclusion, these data suggest that for agonist ligands, there is no indication of δ-opioid uncoupling from their G-proteins in the absence of the μ-opioid receptor. However, the data with the antagonist ligand [^{3}H]naltrindole support the possibility of altered ligand recognition of the δ-receptor in μ-knockout mice. In line with this hypothesis, the results provide further support for the existence of functional interactions between the μ- and the δ-opioid receptor.

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