**Knockout Subtraction Autoradiography: a novel *ex vivo* method to detect heteromers
finds sparse KOPr/DOPr heterodimerization in the brain**

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Abstract

Several methodological approaches suggest receptor heteromers exist in cell systems, but their presence in physiological tissue is widely contentious. We describe a novel method to determine if heterodimers exist in brain tissue sections using autoradiographic binding comparisons from single and double gene knockout mice, where tissues either have a full receptor complement and can form heterodimers, or are incapable of making heterodimers. We have tested this model, which we have named Knockout Subtraction Autoradiography, to determine if heterodimerisation of the kappa (KOPr) and delta (DOPr) opioid receptors occurs, as evidence from binding studies in cell systems suggest they are present in the brain. Using labeling of putative KOPr/DOPr heterodimers with either [^3H] bremazocine or with [^3H] naltrindole, two ligands which were used to provide evidence suggesting that these opioid receptor subtypes heterodimerize, we have applied a subtraction equation model based on the principle that receptor gene double knockout of either MOPr/KOPr (DOPr expression only) or MOPr/DOPr (KOPr expression only) produces tissue incapable of making the KOPr/DOPr heterodimer. Should heterodimers exist, binding profiles from these should differ from tissues with a full receptor complement. We have shown in most brain regions the labeling fits a simple additive model of monomer labeling, but that in a few brain regions opioid receptor heterodimerization does occur. The data does not support the conclusion that KOPr/DOPr heterodimerisation is widespread in the central nervous system, but does indicate this novel methodology can detect heterodimerisation, when ligands with distinct binding affinities for monomer and heterodimer forms exist.

Keywords: autoradiography, opioid, receptor, subtraction, mouse

Abbreviations:

AcbC, Nucleus accumbens core; AcbSh, Nucleus accumbens shell; Amy, Amygdala; BRET, bioluminescence resonance energy transfer; Cg, Cingulate cortex; Cl, Claustrum; CPu, Caudate putamen; DEn, Dorsal endopiriform nucleus; DOPr, delta opioid receptor; FRET, fluorescence resonance energy transfer; GPCRs, G-protein coupled receptors; Hip, Hippocampus; Hyp, Hypothalamus; IP, Interpeduncular nucleus; KO, knockout; KOPr, kappa opioid receptor; KSA, Knockout Subtraction Autoradiography; MCx, Motor cortex; MHb, Medial habenular nucleus; MOPr, mu opioid receptor; OB, Olfactory bulb; PAG, Periaqueductal gray; PCR, polymerase chain reaction; PrL, Prelimbic cortex; SN, Substantia nigra; SuG, Superficial gray layer of the superior colliculus; Th, Thalamus; Tu, Olfactory tubercle; VTA, Ventral tegmental area; WT, wild-type

1. Introduction

Opioid receptors belong to the superfamily of G-protein coupled receptors (GPCRs) and were originally believed to be present as monomeric entities, but like many other GPCR families current thinking suggests they form heteromers that modulate the physiological and pharmacological function of the receptor (Gurevich and Gurevich, 2008). Cell studies over more than a decade have suggested that mu opioid receptors (MOPr) and delta opioid receptors (DOPr), as well as kappa opioid receptors (KOPr) and DOPr may exist as heteromers (Gomes et al., 2004; Gomes et al., 2000; Jordan and Devi, 1999). In cell studies, co-expression of KOPr/DOPr heteromers (Jordan and Devi, 1999) or MOPr/DOPr heteromers (Gomes et al., 2004; Gomes et al., 2000) exhibited distinct ligand binding and signaling characteristics compared to cells individually expressing the monomeric form of these receptors, suggesting dimerisation between the different subtypes of opioid receptors could modulate receptor function. More recent studies have focused on conditions that might drive the process of opioid heterodimerisation and evidence has been provided that chronic morphine treatment increases the abundance of heteromers (Gupta et al., 2010), which might be of relevance to the treatment of addiction disorders (Stockton and Devi, 2012). Moreover for other G-protein coupled receptors (e.g dopamine and adenosine) there is evidence that implicates heteromer dysfunction in diseases such as depression (Pei et al., 2010) and schizophrenia (Faron-Górecka et al., 2008).

Whilst several methods have been developed to investigate oligomerisation of GPCRs *in vitro* (Harrison and van der Graaf, 2006), including biochemical (co-immunoprecipitation and western blotting), biophysical (fluorescence resonance energy transfer, FRET and bioluminescence resonance energy transfer, BRET) and pharmacological (competitive ligand binding) approaches, only one method in intact physiological tissue has to date been

advanced (Gupta et al., 2010). This has led most recently to the development of a cell based system using fusion proteins that may be used as a novel screening assay for detecting heteromer signaling (van Rijn et al., 2013), demonstrating potency differences of some ligands for the monomer and heteromer forms of the DOPr and MOPr receptors

In the original paper demonstrating opioid heterodimerisation, Jordan and Devi (1999) provided pharmacological evidence for the existence of heteromer forms of the KOPr and the DOPr *in vitro*. In a cell culture study they showed co-immunoprecipitation and distinct ligand binding profiles of co-expressed KOPr and DOPr in HEK cells proposing that these receptors heterodimerise. In the cell studies, (5 α ,7 α ,8 β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide (U-69,593), a selective ligand for the KOPr and [D-Ala²,D-Leu⁵]enkephalin (DPDPE), a selective ligand for the DOPr, were shown to be inactive at the KOPr/DOPr heteromer, whereas [³H]bremazocine (a putative ligand that is thought to bind to KOPr/DOPr heteromer) had a 3-fold lower affinity for the KOPr/DOPr heteromer than for the KOPr monomer, but a 6-fold higher for the DOPr monomer compared to the KOPr/DOPr heteromer (Jordan and Devi, 1999). They also showed that the selective DOPr-selective antagonist, naltrindole, had a 40 fold lower affinity for the KOPr/DOPr heteromer than for the DOPr, but was equipotent at the KOPr monomer.

To determine if such heterodimerisation occurs in physiological tissue as well as in cell systems we have hypothesised that quantitative receptor autoradiography with [³H]bremazocine and [³H]naltrindole could be used to show binding to a monomer and heteromer form of the KOPr and the DOPr if binding was carried out in single and combinatorial opioid receptor knockout (KO) mice. The approach, which we have named Knockout Subtraction Autoradiography (KSA) (Fig. 1A) is based on the principle that

receptor gene double knockout of either MOPr/KOPr (DOPr expression only) or MOPr/DOPr (KOPr expression only) produces tissue incapable of making the KOPr/DOPr heterodimer. Should heterodimers exist, binding profiles from these should differ from tissues with a full receptor complement. We used [³H]bremazocine, and [³H]naltrindole to label the receptor forms of the KOPr and DOPr, respectively, two ligands which provided key *in vitro* evidence suggesting that these opioid receptor subtypes heterodimerize (Jordan and Devi, 1999). The data shows that heterodimerisation of KOPr and DOPr can be observed in brain slice sections, but that across a wide range of anatomical structures throughout the mouse brain the KOPr and the DOPr exist predominantly in monomeric forms.

2. Materials and Methods

2.1 Animals

2.1.1. Single MOPr, double MOPr/DOPr and MOPr/KOPr knockout mice

Mice lacking MOPr (Matthes et al., 1996), DOPr (Filliol et al., 2000) and KOPr (Simonin et al., 1998) were generated in Prof Kieffer's laboratory (IGBMC-CERBM, France). Breeding pairs of single MOPr and double homozygous (MOPr/DOPr and MOPr/KOPr) KO mice, maintained on a pure C57BL/6 genetic background, were obtained from hybrid mutant mice by interbreeding of single knockout strains, originally created on a mixed of C57BL/6J and 129Sv background. Congenic C57BL/6 MOPr, MOPr/DOPr and MOPr/KOPr KO mice were derived following at least 10 generations of successive backcrossing of 129Sv × C57BL/6 heterozygotes to C57BL/6 mice. Animals used in this study were between 32 and 45 weeks old adult male (WT: n=3, MOPr KO: n=4, MOPr/KOPr KO: n= 3 and MOPr/DOPr KO: n=4)

and female (WT: n=3, MOPr KO: n=2, MOPr/KOPr KO: n= 3 and MOPr/DOPr KO: n=2) mice and were genotyped by PCR at weaning.

2.1.2. Triple opioid receptor knockout mice

As described previously by Simonin et al. (2001), triple mutant mice lacking MOPr, DOPr and KOPr genes were generated by interbreeding of single MOPr (Matthes et al., 1996), DOPr (Filliol et al., 2000), and KOPr (Simonin et al., 1998) KO mice. In brief, double MOPr/DOPr KO mice (50% C57BL/6 and 50% 129S2) were mated with double MOPr/KOPr KO mice (50% C57BL/6 and 50% 129S2) to generate triple opioid receptor KO mice maintained on a mixed background (50% C57BL/6J and 50% 129S2). Both the triple opioid receptor KO and wild-type (WT) mice are on a mixed C57BL/6J and 129Sv background. Animals used in this study were between 21 and 23 weeks old adult male and female mice and were genotyped by PCR at weaning. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986.

2.2. Materials

[³H]Bremazocine (26.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). [³H]naltrindole (40.6 Ci/mmol) was synthesized by Dr. Géza Tóth, Dr. Anna Borsodi and Dr. Sandor Benyhe at the Hungarian Academy of Sciences, Szeged. Naloxone was purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK).

2.3. Autoradiography

Animals were killed and the brains were immediately removed and snap frozen at -20 °C in isopentane for 30 sec. Brains were subsequently stored at -80 °C for a maximum period of one month before sectioning. Coronal sections (20 µm) were cut at 300 µm intervals from fore to hindbrain onto precleaned and subbed microscope slides (Kitchen et al., 1997) from single MOPr, double MOPr/DOPr and MOPr/KOPr. Sagittal sections (20 µm) were cut from triple opioid receptor KO and WT at an interval of 300 µm to assess the levels of binding throughout forebrain to hindbrain structures.

All slides were preincubated in 50 mM Tris-HCl, pH 7.4, plus 0.9 % NaCl for 30 min, to remove endogenous opioids (Kitchen et al., 1997). The concentration of [³H]bremazocine (2 nM) and [³H]naltrindole (1 nM) was chosen from previous autoradiographic binding studies in wild-type mice to represent a level approaching a receptor saturating concentration (Kitchen et al., 1997; Oakley et al., 2003; Simonin et al., 2001). Quantitative autoradiographic binding of the ligands was carried out in 50 mM Tris-HCl, pH 7.4, at room temperature for 60 min in brains of WT and mice lacking either MOPr, MOPr/KOPr or MOPr/DOPr as well as in mice lacking all three classical opioid receptors. Non-specific binding was determined by adding 10 µM naloxone. Slides were washed 3 times with ice-cold Tris-HCl buffer, pH 7.4, for 5 min and rapidly cool-air dried.

Autoradiograms were generated by apposing the labelled tissue sections to Kodak BioMax MR films (Eastman Kodak Co., Rochester, NY, USA). Cassettes were sealed using opaque tape to prevent light entry and stored in a dark place for a period of 6 to 9 weeks in X-ray film cassettes along with Amersham tritiated polymer standards at room temperature. Kodak BioMax MR films were developed in 50 % Kodak D19 developer solution. Sections from WT and KO animals were processed together at all stages of experimental procedures to ensure a paired protocol for binding, film apposition and image analysis.

All films were analysed by video-based computerized densitometry using an MCID image analyser (Imaging Research, Canada) as previously described (Kitchen et al., 1997). Films were placed onto a light box (Kaiser Pro-lite, Germany) and calibration took place using the exposed images of the tritium microscale standards. Brain regions were analyzed in duplicate for each brain using left and right hemispheres. The cortical structures and the olfactory tubercle were analyzed by sampling 5-20 times with a box tool (2×2 mm). All other regions were analyzed by freehand drawing tools. Structures were identified by reference to the mouse atlas of Franklin & Paxinos (2001) (Franklin and Paxinos, 2001). Specific binding was calculated by subtracting the level of non-specific binding from the total binding level.

Confirmation of the PCR genotyping was carried out by autoradiographic labeling of MOPr, DOPr and KOPr with receptor selective ligands as described in our previous studies (Kitchen et al., 1997), and was also used to determine any compensatory changes in each remaining opioid receptor in combinatorial knockouts.

2.4. Data analysis

Comparison of specific binding of either [3 H]bremazocine and [3 H]naltrindole in MOPr, double MOPr/DOPr and MOPr/KOPr KO mice was made using the equations described in Figure 1A. A model was developed to test statistically whether, within experimental error, across the set of the component values for the KSA equation, the value for the MOPr KO was distinguishable from the sum of the remaining two. For each triplet of values, the difference of the MOPr KO value from the sum of the remaining two was formed. The resultant set of differences was subjected to a null ANOVA model using PROC MIXED of the SAS suite of

programs (SAS Institute, Cary, NC, USA), both with intercept included and, separately, with intercept excluded. A 1 degree of freedom chi-squared test applied to the difference of the resultant two deviance model-goodness-of-fit measures, for the statistical significance of including the intercept, yielded the desired test of additivity within the triples. P values <0.05 demonstrated significant deviation from a model of additivity, indicative of heterodimer labeling.

3. Results

Coronal brain sections were cut from single MOPr (DOPr and KOPr present, both monomers and potential heterodimers), or double MOPr/KOPr (DOPr only, monomers) and MOPr/DOPr (KOPr only, monomers) KO mice. [³H]bremazocine and [³H]naltrindole binding were measured at a single ligand concentration which in our previous autoradiographic binding experiments represents a near saturation concentration (Oakley et al., 2003; Simonin et al., 2001). If binding of the ligands used in this study is to monomer forms of the KOPr and DOPr, then binding from the equation $\text{MOPr KO} - \text{MOPr/KOPr KO} = \text{MOPr/DOPr KO}$ should apply (Fig. 1A, upper panel). However if binding of these ligands is to heterodimer forms then $\text{MOPr KO} - \text{MOPr/KOPr KO} \neq \text{MOPr/DOPr KO}$ should apply (Fig. 1B lower panel). We have used a statistical analysis of additivity to determine in each brain region studied if binding is, or is not, additive.

3.1. [³H]Bremazocine binding in wild-type, MOPr, MOPr/DOPr and MOPr/KOPr knockout mice

[³H]Bremazocine binding was higher in all brain regions of WT mice compared to single MOPr KO, double MOPr/DOPr and MOPr/KOPr KO mice demonstrating some labeling to all three opioid receptors (Fig. 1B). In MOPr KO, high levels of binding were detected in several regions showing substantial binding of this ligand to KOPr and DOPr (Fig. 1B). When quantitative subtraction (Fig. 1C and Table 1) was applied to the [³H]bremazocine binding results obtained from single MOPr KO and double MOPr/KOPr and MOPr/DOPr KO mice the subtraction binding fitted best to the monomer model (MOPr KO – MOPr/KOPr KO = MOPr/DOPr KO) in twenty brain regions. Only two brain regions (olfactory bulb and periaqueductal gray) approached statistical significance for lack of additivity (P<0.09, Table 1).

3.2. [³H]naltrindole binding in wild-type, single and double opioid receptor knockout mice

[³H]Naltrindole binding was similar in many brain regions of WT and MOPr KO mice showing only a small amount of labeling of the MOPr by this ligand (Fig. 2A). Binding in double MOPr/KOPr KO mice was markedly higher than in MOPr/DOPr KO mice in several forebrain structures signifying high DOPr monomeric labeling (Fig. 2A). When quantitative subtraction (Fig. 2B and Table 1) was applied to the [³H]naltrindole binding results obtained from single MOPr KO and double MOPr/KOPr and MOPr/DOPr KO mice, the subtraction binding fitted best to the monomer/homo-oligomer model (MOPr KO – MOPr/KOPr KO = MOPr/DOPr KO) in twelve out of fifteen regions examined (Fig. 2B and Table 1). Three regions however (thalamus, amygdala and interpeduncular nucleus) showed a best fit to the heterodimer/hetero-oligomer model with highly significant differences from simple additivity (Fig. 2B and Table 1). The hippocampus also approached statistical significance (P<0.07).

3.3. [³H]bremazocine and [³H]naltrindole binding and compensatory changes in single and combinatorial knockout brains

Deletion of both single and double receptor knockout produces minor changes in the remaining opioid receptor complements and we have historical (Kitchen et al., 1997) and unpublished data for all of the brain regions studied using the KSA approach. We therefore made corrected adjustments to the raw binding data for each brain region for both [³H]bremazocine and [³H]naltrindole binding. The data corrected for compensatory changes is shown in Table 2 and shows a very similar pattern to the binding differences presented in the raw data histograms (Fig. 1C and Fig. 2B). The corrected values (Table 2) did not alter the monomer/heteromer fit analysis confirming that where significant deviation from additivity had been observed this was also robust if compensatory changes in the gene knockout models were accounted for.

3.4. [³H]bremazocine and [³H]naltrindole binding in triple opioid receptor knockout mice

Triple opioid receptor KO mice were used to confirm whether both ligands studied bind to other receptors in addition to all three classical opioid receptors. No specific [³H]bremazocine or [³H]naltrindole binding was detected in the sagittal brain sections of mice lacking the three classical opioid receptor genes confirming the ligands can only bind to the three classical opioid receptors (Fig. 3).

4. Discussion

Although several methods such as FRET, BRET, ligand binding, co-immunoprecipitation and Western blotting have been developed to investigate the existence of heterodimer/heteroligomers *in vitro* (Gomes et al., 2004; Gomes et al., 2000; Harrison and van der Graaf, 2006; Jordan and Devi, 1999), proving the existence of heteromers in physiological systems has been a more challenging task. Gupta et al. (2010) using a subtractive immunization strategy to generate antibodies to selectively recognize MOPr/DOPr heteromers have been able to show that chronic morphine treatment leads to increased heterodimer formation in endogenous brainstem neurons. However, concern over the limitations of immunization techniques, particularly in relation to lack of reliability of antibody selectivity, makes alternative approaches attractive. The model described in our study has aimed, using a subtractive receptor binding model, to determine if KOPr/DOPr heterodimers are widespread in brain tissue. The model is based on the principle that receptor KO of either KOPr or DOPr produces tissue incapable of making heteromers, and when compared to tissues with a full receptor complement should show different binding profiles if heterodimerisation exists.

In the KSA model it is prudent to consider whether compensatory changes of other opioid receptors, which may occur due to the deletion of single or double opioid receptors, might influence the additivity analysis. As deletion of both single and double receptor knockout produces only minor changes in the remaining opioid receptor complements the corrected adjustments to the raw binding data made little material alteration to the monomer/heteromer fit analysis. Thus, the observed [³H]bremazocine and [³H]naltrindole binding in single MOPr,

double MOPr/DOPr and MOPr/KOPr KO mice are not influenced to any great extent by compensatory changes occurring due to the deletion of genes for the receptors. This lack of compensatory changes in single and double opioid receptor knockout mice makes analysis by KSA robust for opioid receptor systems, and this would be an important validation criteria for using the model for other G-protein coupled receptor systems. We also confirmed using triple opioid receptor KO mice that both ligands studied bind only to all three classical opioid receptors. Again selectivity of binding to only the receptors involved in the heterodimerisation activity would be a key criteria for use of KSA for other receptor families.

One limitation of the autoradiographic method to detect heterodimers is the achievable resolution, and the lack of ability to take into account the level of individual receptor and study at the single cell level.

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Although the KSA method cannot provide molecular mechanisms underpinning DOPr/KOPr heterodimer/hetero-oligomer formation it can infer the presence or absence of the heterodimer in a physiological system, which is a useful starting point to address specific regional experiments that could be carried out to confirm molecular co-localisation. As binding properties (K_d , B_{max}) of bremazocine and naltrindole at the MOPr, DOPr, KOPr and their heterodimers differ considerably (Jordan and Devi, 1999), we are confident that these concentrations of radioligand would be adequate to detect heterodimers, should they exist. Although we failed to find evidence for widespread heterodimerisation in normal tissue the potential role for heteromers in mouse models of disease states (Pei et al., 2010), or after chronic drug treatment (Gupta et al., 2010) should be recognized. Indeed using KSA after treatment conditions might be another approach to address the importance of heterodimerisation after disease or chronic treatment intervention.

In conclusion, using the KSA approach our data shows only sparse heterodimerization of KOPr and DOPr's across several brain regions. In line with our findings, a role for opioid heterodimers in modulating the actions of opioid drug actions requires their co-expression in a single cell, a concept that is controversial and a previous study has failed to find DOPr's, fused with enhance green fluorescent protein in MOPr containing small dorsal root ganglion neurons (Scherrer et al., 2009). Despite the lack of evidence in our study for widespread heterodimerization throughout the brain, the importance of heterodimer formation in a few structures should not be underestimated, and the potential functional role for heterodimerisation in thalamus and amygdala may point to their relevance in pain and emotional behaviours. Pharmaceutical interest in heterodimers is driven by the realization that agonist drugs could differentially target these structures and indeed if this occurs in a few target brain regions then this could be extremely advantageous. The KSA technique does have the potential for studying heterodimerization of all GPCRs where appropriate single and double knockout tissue is available and could be used to screen for heterodimer interactions of existing and new chemical entities.

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Table 1. Probability values for [^3H]bremazocine and [^3H]naltrindole binding

Region	P value	
	[^3H]Bremazocine	[^3H]Naltrindole
Olfactory bulb	0.0882	0.3619
Prelimbic cortex	0.7480	-
Caudate putamen	0.9990	0.2701
Nucleus accumbens core	0.7635	0.5237
Nucleus accumbens shell	0.5729	0.4662
Olfactory tubercle	0.6001	0.9450
Clastrum	0.1921	0.3285
Dorsal endopiriform nucleus	0.8875	0.9574
Cingulate cortex	0.7985	0.3776
Motor cortex	0.2736	-
Hippocampus	0.9410	0.0692
Thalamus	0.8501	0.0192
Hypothalamus	0.6542	0.5716
Amygdala	0.8009	0.0142
Medial habenular nucleus	0.5805	-
Substantia nigra	0.1066	0.1208
Ventral tegmental area	0.7067	0.2217
Interpeduncular nucleus	0.5674	0.0265
Periaqueductal gray	0.0859	-
Superficial gray layer of the superior colliculus	0.3575	-

Legend to table 1

Probability values for [^3H]bremazocine and [^3H]naltrindole binding tested for fit to a model of additivity in several brain regions as described in the methods. For [^3H]bremazocine binding, no region showed significant ($P < 0.05$) deviation from an additivity model showing binding best fits monomer labeling throughout the brain (see Figure 1C). For [^3H]naltrindole binding three regions (Thalamus, Amygdala and Interpeduncular nucleus) showed significant ($P < 0.05$) deviation from an additivity model showing best fit to heterodimer labeling in these regions and monomer labeling in all other regions (see Figure 2B)

Table 2. Compensated values for [³H]bremazocine and [³H]naltrindole binding

Region	[³ H]Bremazocine			[³ H]Naltrindole		
	MOPr -/-	MOPr/KOPr -/-	MOPr/DOPr -/-	MOPr -/-	MOPr/KOPr -/-	MOPr/DOPr -/-
Olfactory bulb	124.4	129.8	29.6	151.0	137.8	12.2
Caudate putamen	142.4	106.8	43.2	79.0	67.2	8.3
Nucleus accumbens core	104.7	34.3	51.5	42.3	12.7	13.5
Nucleus accumbens shell	107.0	25.2	51.9	36.7	6.6	9.8
Olfactory tubercle	122.8	70.8	60.6	69.9	53.4	14.1
Clastrum	207.1	70.3	174.1	50.6	30.6	17.7
Cingulate cortex	89.3	75.3	23.1	59.6	63.1	9.6
Hippocampus	34.1	26.3	7.8	30.8	33.1	5.9
Thalamus	45.5	22.9	21.5	13.0	18.8	9.8
Hypothalamus	77.9	14.7	55.4	11.2	7.1	7.5
Amygdala	76.7	39.6	34.4	29.4	36.1	11.0

Legend to table 2

Table 2 shows the binding levels (fmol/mg tissue) for [³H]bremazocine and [³H]naltrindole binding in MOPr KO, MOPr/DOPr KO and MOPr/KOPr KO mice after correction for compensatory changes in gene knockout mice have been applied. The compensatory changes in monomeric DOPr and KOPr in brain regions of single and double opioid receptor KO mice were applied to absolute values (Figures 1C and 2B) in order to obtain the corrected values (Table 2). The observed [³H]bremazocine and [³H]naltrindole binding in single MOPr (n=6), double MOPr/DOPr (n=6) and MOPr/KOPr (n=6) KO mice were not significantly influenced by compensatory changes occurring due to the deletion of genes for the receptors and the model of additivity revealed the equivalent analysis as for the uncorrected values.

Figure legends

Figure 1. (A) Model showing quantitative subtraction equation to identify monomer or heterodimer binding in single and double opioid receptor knockout (KO) mice. If binding of [³H]bremazocine or [³H]naltrindole is to monomer/homo-oligomer forms of the KOPr and DOPr then binding from the equation $\text{MOPr KO} - \text{MOPr/KOPr KO} = \text{MOPr/DOPr KO}$ should apply (A; upper row). However if binding of [³H]bremazocine or [³H]nor-BNI is to heterodimer/ hetero-oligomer forms then $\text{MOPr KO} - \text{MOPr/KOPr KO} \neq \text{MOPr/DOPr KO}$ should apply (A; lower row). (B) Representative pseudo-colour images showing [³H]bremazocine (2nM) binding in brains of wild-type (WT), single MOPr and double MOPr/KOPr and MOPr/DOPr KO mice at the level of the caudate and hippocampus. Non-specific binding (NSB) was determined in the presence of 10 μM of naloxone. The colour bar represents pseudo-colour interpretation of black and white film images in fmol/mg tissue. (C) Quantitative autoradiography of [³H]bremazocine (2 nM) binding in the brains of WT (n=6), single MOPr KO (n=6), double MOPr/KOPr (n=6) and MOPr/DOPr (n=6) KO mice. Non-specific binding was determined in the presence of 10 μM of naloxone. Values are expressed as mean $\pm$ SEM of 6 mice.

Abbreviations: AcbC, Nucleus accumbens core; AcbSh, Nucleus accumbens shell; Amy, Amygdala; Cg, Cingulate cortex; Cl, Claustrum; CPu, Caudate putamen; DEn, Dorsal endopiriform nucleus; DOPr, delta-opioid receptor; Hip, Hippocampus; Hyp, Hypothalamus; IP, Interpeduncular nucleus; KOPr, kappa-opioid receptor; MCx, Motor cortex; MHb, Medial habenular nucleus; MOPr, mu-opioid receptor; OB, Olfactory bulb; PAG, Periaqueductal gray; PrL, Prelimbic cortex; SN, Substantia nigra; SuG, Superficial gray layer of the superior colliculus; Th, Thalamus; Tu, Olfactory tubercle; VTA, Ventral tegmental area

Figure 2. (A) Representative pseudo-colour images showing [³H]naltrindole (1 nM) binding in brains of wild-type (WT), single MOPr and double MOPr/KOPr and MOPr/DOPr knockout (KO) mice. Non-specific binding (NSB) was determined in the presence of 10 µM of naloxone. The colour bar represents pseudo-colour interpretation of black and white film images in fmol/mg tissue. (B) Quantitative autoradiography of [³H]naltrindole (1 nM) binding in the brains of WT (n=6), single MOPr KO (n=6), double MOPr/KOPr (n=6) and MOPr/DOPr (n=6) KO mice. Non-specific binding was determined in the presence of 10 µM of naloxone. Values are expressed as mean ± SEM of 6 mice.

Abbreviations: AcbC, Nucleus accumbens core; AcbSh, Nucleus accumbens shell; Amy, Amygdala; Cg, Cingulate cortex; Cl, Claustrum; CPu, Caudate putamen; DEn, Dorsal endopiriform nucleus; DOPr, delta-opioid receptor; Hip, Hippocampus; Hyp, Hypothalamus; IP, Interpeduncular nucleus; KOPr, kappa-opioid receptor; MOPr, mu-opioid receptor; OB, Olfactory bulb; SN, Substantia nigra; Th, Thalamus; Tu, Olfactory tubercle; VTA, Ventral tegmental area

Figure 3. Representative pseudo-colour images showing [³H]bremazocine (2 nM) and [³H]naltrindole (1 nM) binding in sagittal brain sections of WT and triple opioid receptor (MOPr/DOPr/KOPr) KO mice. Non-specific binding (NSB) was determined in the presence of 10 µM of naloxone. The colour bar represents pseudo-colour interpretation of black and white film images in fmol/mg tissue.

A **B**