Characterization of [^{3}H] oxymorphone binding sites in mouse brain: quantitative autoradiography in opioid receptor knockout mice

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ABSTRACT

Oxymorphone, one of oxycodone's metabolic products, is a potent opioid receptor agonist which is thought to contribute to the analgesic effect of its parent compound and may have high potential abuse liability. Nonetheless, the *in vivo* pharmacological binding profile of this drug is still unclear. This study uses mice lacking mu (MOP), kappa (KOP) or delta (DOP) opioid receptors as well as mice lacking all three opioid receptors to provide full characterisation of oxymorphone binding sites in the brain. Saturation binding studies using [^{3}H]oxymorphone revealed high affinity binding sites in mouse brain displaying K*d* of 1.7 nM and B*max* of 147 fmol/mg. Furthermore, we performed quantitative autoradiography binding studies using [^{3}H]oxymorphone in mouse brain. The distribution of [^{3}H]oxymorphone binding sites was found to be similar to the selective MOP agonist [^{3}H]DAMGO in the mouse brain. [^{3}H]Oxymorphone binding was completely abolished across the majority of the brain regions in mice lacking MOP as well as in mice lacking all three opioid receptors. DOP and KOP knockout mice retained [^{3}H]oxymorphone binding sites suggesting oxymorphone may not target DOP or KOP. These results confirm that the MOP, and not the DOP or the KOP is the main high affinity binding target for oxymorphone.

Key words: autoradiography; knockout; mouse; [^{3}H]oxymorphone; opioid

1. Introduction

Oxycodone is widely used opioid medication in the US [16]. Oxymorphone, an active metabolite of oxycodone through *O*-demethylation, is a potent opioid agonist [18]. Oxycodone's pharmacodynamic effects in humans and other mammalian species were shown to be produced by the parent drug, rather than its metabolite oxymorphone [13, 14]. Despite its low metabolism to oxymorphone, oxymorphone may still contribute towards the pharmacological effects of its parent compound depending on its pharmacological potency.

There is evidence suggesting that oxycodone and oxymorphone activates distinct molecular pathways [1, 29]. For instance, oxycodone is 10- to 100-fold less potent than oxymorphone in activating intracellular G-proteins (agonist-stimulated GTPγ[^{35}S] binding assay) in several CNS regions of rats [20]. In addition, oxymorphone-induced antinociceptive effects were blocked by naloxone in rats [20] and abolished in mice lacking mu opioid (MOP) receptors [32] whereas oxycodone-mediated antinociception could be attenuated by blocking kappa (KOP) or delta opioid (DOP) receptors in rats [24] and mice [32], respectively. Furthermore, intrathecal administration of oxymorphone produced longer and significantly greater antinociception compared to oxycodone in rats [20]. Altogether, these studies indicate that although oxymorphone is not the main metabolite of oxycodone, it may contribute to its analgesic effect due to its higher potency to cause MOP desensitisation and G-protein activation in the CNS.

Oxymorphone is effective for the relief of moderate and severe pain [12, 15]. Oxymorphone displayed 320- to 410-fold higher affinity for human MOP expressing CHO-K1 cells (K_i = 0.36 nM) than for the human KOP (K_i = 148 nM) or mouse DOP (K_i = 118 nM) expressing cells [18]. However, there is some evidence that oxymorphone can also interact with the DOP. For instance oxymorphone was able to competitively displace the DOP selective ligand [^{3}H]DADLE binding at its high affinity binding site

but had a non-competitive effect at a lower affinity binding site in the rat brain [25], thus proposing binding activity of oxymorphone with putative DOP subtypes.

To further provide the full characterization of receptor binding profile of oxymorphone *in vivo*, we synthesised [^{3}H]oxymorphone and carried out saturation binding studies in mouse brain homogenates. We performed detailed quantitative [^{3}H]oxymorphone autoradiographic receptor mapping in brains of mice lacking either MOP, KOP or the DOP as well as in mice lacking all three classical opioid receptors (MOP/KOP/DOP) in order to fully characterise the oxymorphone opioid receptor binding sites *in vivo*. This is the first complete autoradiographic mapping study using [^{3}H]oxymorphone to directly and fully characterise oxymorphone binding sites. The results suggest that the MOP and not DOPs or KOPs is the main binding target of oxymorphone throughout the brain tissue.

2. Material and methods

2.1. Animals. Mice lacking MOP, DOP or KOP (congenic C57BL/6 mice) and appropriate wild-type (WT) controls were derived following at least 10 generations of successive backcrossing of 129/SV × C57BL/6 heterozygotes to C57BL/6 [9, 21, 27]. Triple mutant mice lacking MOP, DOP and KOP genes and wild-type (WT) mice were generated by interbreeding of congenic C57BL/6 MOP [21], DOP [9], and KOP [27] knockout (KO) mice [26], maintained on a pure C57BL/6 genetic background. Animals used in this study were > 8 weeks old adult male and female mice and were genotyped by PCR at weaning. Membrane fractions were prepared from brains of C57BL/6 mice (2 months old). All housing and experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 and the Hungarian legislation for the Protection of Animals in Research (XXVIII.tv. Section 32.).

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2.2. Materials. [^{3}H]Oxymorphone (34 Ci/mmol) was synthesised at the Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences. Naloxone was purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK).

2.3. Homogenate binding studies. Freshly dissected whole brains excluding cerebellum of mice were homogenized in ice-cold 50 mM Tris-HCl buffer solution (pH 7.4, 1 g wet weight/10 mL) with an electrically driven Braun teflon-glass rota-machine. The crude homogenate was centrifuged at 40,000*g*, 4°C, for 20 min. Pellets were resuspended in the same volume of fresh 50 mM Tris-HCl pH 7.4 buffer and centrifuged again as above. The final pellet was taken up and homogenized with buffer and used immediately in the binding assays. Radioligand binding studies were conducted in 50 mM Tris-HCl buffer in a final volume of 1 mL. Mouse brain

homogenates were incubated in the presence of the [^{3}H]oxymorphone at a range of different concentrations (0.16 nM – 32 nM) for 30 min at 24°C. The incubation time was chosen based on time course binding studies (**Fig. 1A**). Association kinetics was performed by incubating the samples for different times as indicated in the **Fig. 1A**. Reaction mixtures were filtered together to determine bound radioactivity. The reaction was terminated and bound and free radioligands were separated by rapid filtration under vacuum through Whatman GF/C glass fiber filters using a Brandel M24R Cell Harvester. Filters were dried, then immersed into UltimaGold™ (Packard) solution. Bound radioactivity was determined by a Packard TriCarb 2300TR Liquid Scintillation Analyzer. Saturation binding studies were carried out by incubating the samples in the presence of increasing concentrations of the radioligand (**Fig. 1B**). Non-specific binding (NSB) was measured in the presence of 10 μM naloxone. NSB was subtracted from total [^{3}H]oxymorphone binding to determine equilibrium specific binding for calculation of K_d and B_{max}. Equilibrium competition studies were performed by incubating the samples in the presence of 2 nM radioligand and increasing concentration of unlabelled naloxone.

2.4. Preparation of knockout tissue for sectioning. Single MOP, DOP and KOP KO mice and triple opioid receptor KO mice and their respective WT controls were killed and the brains were immediately removed and snap frozen at -20 °C in isopentane. Frozen brains were removed from storage in a -80 °C freezer and were placed into a Cryostat (Zeiss Microm HM505E, Jena, Germany), and equilibrated at -20 °C. 20 μm coronal (300 μm apart) or sagittal sections from the brains of opioid receptor KO and WT mice, were then cut and thaw-mounted onto gelatine coated ice-cold slides to assess levels of binding from fore- to hind-brain regions. Consecutive sections were taken for determination of total and non-specific binding.

2.5. Quantitative [^{3}H]oxymorphone Autoradiographic binding. Autoradiographic binding was carried out according to the general principles described by our group [17, 33]. All sections were preincubated in 50 mM Tris-HCl, pH 7.4, plus 0.9 % NaCl for 30 min at room temperature, to remove endogenous opioids. Sections were then incubated with 5 nM (3x K_d) [^{3}H]oxymorphone in 50 mM Tris-HCl, pH 7.4, for 60 min at room temperature for total binding. Adjacent section slides were incubated with [^{3}H]oxymorphone (5nM) in the presence of 10 µM naloxone to determine non-specific binding. 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 tissues 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 7 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 (Eastman Kodak Co., Rochester, NY, USA) and fixed with Kodak Rapid fixer (Eastman Kodak Co., Rochester, NY, USA). Sections from WT and KO animals were processed together 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, St. Catharines, ON, Canada) as previously described [17, 33]. Structures were identified by reference to the mouse atlas of Franklin & Paxinos [10]. Specific binding was calculated by subtracting the level of non-specific binding from the total binding level.

2.6. Data analysis. For autoradiographic studies, statistical analysis was performed by one-way ANOVA for each brain region, followed by Dunnett's post hoc test to compare all the other group to the WT. The data from competition and saturation

binding studies were analyzed and graphically processed by GraphPad Prism (San Diego, CA, USA).

3. Results

3.1. Binding of [^{3}H]oxymorphone to brain homogenates. Radioligand binding studies revealed the presence of specific, high affinity binding sites for [^{3}H]oxymorphone in mouse brain homogenates (**Fig. 1, Table 1**). Binding of [^{3}H]oxymorphone to mouse brain membranes at 24°C was saturable and of high affinity (**Fig. 1A**). [^{3}H]Oxymorphone displayed binding to mouse brain membranes with a K_d of 1.7 nM and B_{max} of 147 fmol/mg (**Fig. 1B, Table 1**). The universal opioid antagonist, naloxone displaced [^{3}H]oxymorphone with a K_i value of 1.8 nM (**Fig. 1C, Table 1**).

3.2. [^{3}H]oxymorphone binding in wild-type, MOP, KOP and DOP receptor knockout mice. At a near saturating concentration (5nM, 3-4 x K*d*) (**Table 1**), high levels (>28 fmol/mg) of [^{3}H]oxymorphone binding were detected in the nucleus accumbens, thalamus, hypothalamus, medial habenular nucleus, superficial gray layer of the superior colliculus and periaqueductal gray of WT mice (**Fig. 2 and Table 2**). The qualitative pattern of [^{3}H]oxymorphone binding in WT mice was similar to that of DOP and KOP KO mice (**Fig. 2 and Table 2**). One-way ANOVA revealed a significant genotype effect in the nucleus accumbens core ($P < 0.01$) and shell ($P < 0.01$), caudate putamen ($P < 0.01$), cingulate cortex ($P < 0.05$), secondary motor cortex ($P < 0.05$), primary motor cortex ($P < 0.01$), hippocampus ($P < 0.001$), thalamus ($P < 0.001$), hypothalamus ($P < 0.001$), amygdala ($P < 0.001$), medial habenular nucleus ($P < 0.001$), superficial gray layer of the superior colliculus ($P < 0.001$), periaqueductal gray ($P < 0.001$), substantia nigra ($P < 0.001$) and ventral tegmental area ($P < 0.001$). [^{3}H]Oxymorphone binding was almost completely abolished in the majority of the brain

regions analysed in MOP KO mice vs WT (83% mean decrease in binding vs WT brain across all regions) (**Table 2**). Unlike the case for MOP receptor KO mice, [^{3}H]oxymorphone binding was completely retained at WT levels in DOP and KOP KO mice across all regions. No significant differences in [^{3}H]oxymorphone binding was detected in WT vs KOP KO and DOP KO groups in any regions with the exception of the hippocampus where higher levels of binding was observed in KOP KO (78% increase vs WT, $P<0.05$) (**Table 2**).

3.3. [^{3}H]oxymorphone binding in triple opioid receptor knockout mice. Triple opioid receptor KO mice were used to determine whether the ligand bind to non-opioid receptors. No [^{3}H]oxymorphone binding was detected in brains of mice lacking all three classical opioid receptors (triple KO mice) (**Fig. 3**).

4. Discussion

In the present study, we showed that the anatomical distribution of oxymorphone in mouse brain was similar to the MOP agonist [^{3}H]DAMGO. To further investigate whether oxymorphone interacts to any other opioid receptor and/or non-opioid binding sites, we characterise the pharmacological binding profile of [^{3}H]oxymorphone in mice lacking MOP, KOP and DOP receptor as well as in the mice lacking all three classical opioid receptor genes. No specific [^{3}H]oxymorphone binding was detected in the mice lacking MOP and three classical opioid receptor genes, whereas binding remained mostly unchanged in brains of KOP and DOP knockout mice, indicating a lack of interaction of the ligand with any other sites apart from the classical opioid receptor binding sites. Thus oxymorphone binds specifically to high affinity MOP sites but not to the KOP or the DOP.

The distribution of [^{3}H]oxymorphone binding in the brains of WT mice was found to be similar to that of MOP receptor binding as labelled with the selective MOP receptor agonist [^{3}H]DAMGO in the mouse brain [17]. High levels of [^{3}H]oxymorphone binding was detected in the nucleus accumbens, thalamus, hypothalamus, medial habenular nucleus, superficial gray layer of the superior colliculus and the periaqueductal gray which are all regions of high MOP receptor expression [17]. Moderate binding levels of [^{3}H]oxymorphone were detected in the olfactory tubercle, caudate putamen, amygdala and substantia nigra which is in complete accordance with the presence of moderate MOP receptor expression as observed by Kitchen *et al*. [17] with the use of [^{3}H]DAMGO. Finally, low levels of [^{3}H]oxymorphone binding were found in the prelimbic cortex, frontal association cortex, cingulate cortex, secondary motor cortex, primary motor cortex and hippocampus which are regions of low MOP receptor binding [3, 4, 17].

To further demonstrate whether MOP receptor is the prime target for oxymorphone, [^{3}H]oxymorphone autoradiographic binding was carried out in the brains of mice lacking the MOP receptor. There was almost complete abolition of [^{3}H]oxymorphone binding in the majority of the brain regions of MOP receptor KO mice, demonstrating that MOP

receptor is the main binding target for oxymorphone in mouse brain tissue. In support of this evidence, a competition binding study in a cell membrane preparation expressing recombinant human MOP receptor showed oxymorphone has a high affinity to the MOP receptor (K*i* = 0.4055 nM) [30]. Moreover, in agreement with the present study, oxymorphone had relatively high affinity for human MOP receptor expressing CHO-K1 cells (K_i = 0.36 nM), but 410-fold lower for the human KOP (K_i = 148 nM) and 320-fold lower for mouse DOP (K_i = 118 nM) expressing cells than the human MOP receptor expressing CHO cells [18].

Despite the substantial loss of [^{3}H]oxymorphone binding sites in MOP receptor KO mice, [^{3}H]oxymorphone binding was retained in DOP and KOP KO mice demonstrating a lack of binding interaction of oxymorphone to DOP or KOP. In support of this evidence, oxymorphone-induced antinociception in both thermal and mechanical tests in rats was not reversed by the KOP selective antagonist norbinaltorphimine suggesting that oxymorphone-induced antinociception is not mediated by the KOP [20]. The present study, however, with the use of MOP, KOP and DOP receptor KO mice provides definitive evidence that the prime binding site of oxymorphone in mouse brain is the MOP receptor and not the DOP or KOP receptor. Results from our study show that oxymorphone binds almost exclusively to MOP in brain regions involved in pain modulation (thalamus, periaqueductal grey), reward (VTA, nucleus accumbens, caudate putamen) and emotionality (amygdala, hippocampus, cingulated cortex, hypothalamus). Oxycodone is partly metabolized to oxymorphone *via* *O*-methylation and is mainly metabolized to noroxycodone via *N*-demethylation in humans [19, 31]. It is still an open question whether oxymorphone's MOP activity contributes greatly to the action of oxycodone. Recently, Babalonis et al. [2] accessed the abuse potential of oxymorphone and oxycodone in healthy non-physically dependent opioid abusers and showed that the high dose of oxymorphone (40 mg/kg) increased the ratings of drug linking as being similar to oxycodone. These certainly explain the potent analgesic effect and the potential abuse liability of oxymorphone itself [6] but may be also of its parent compound oxycodone.

Indeed the abuse of oxycodone containing analgesics has increased among drug abusers [7].

Interestingly, a significantly higher level of [^{3}H]oxymorphone binding was observed in the hippocampus of KOP KO mice compared to WT animals. As we established that [^{3}H]oxymorphone binds to MOP and not the KOP or DOP with high affinity, one possible explanation is that this increase may reflect increase of the MOP in the hippocampus of KOP KO. This is unlikely to be the case as autoradiographic binding of MOP with [^{3}H]DAMGO in the brains of KOP KO mice did not show any compensatory alterations of MOP sites in the brain [28]. Another possibility explaining higher levels of [^{3}H]oxymorphone binding in the hippocampus of KOP KO mice is the absence of low affinity KOP-MOP dimers which would be present in WT mice. As a result, the proportion of MOP/MOP-KOP+MOP would be higher in the KOP KO mice vs WT. As oxymorphone binds to high affinity MOP, this may explain the higher oxymorphone binding in the hippocampus of KOP KO mice. Although it is not yet known whether opioid receptors exist as dimers, which may be present as homo- or heterodimers/oligomers in physiological systems [5, 8, 11, 22, 23], various studies have suggested that some opioid receptor agonists or antagonists might be able to distinguish monomer and heterodimers in physiological systems, *in vivo* and *in vitro*.

In conclusion, these results indicate that the MOP, and not the DOP or the KOP is the main high affinity binding target for oxymorphone. This suggests that the pharmacological effect of oxymorphone is most likely to be mediated via a MOP dependent mechanism.

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Figure legends

Fig. 1. Binding properties of [^{3}H]oxymorphone in mouse brain homogenates time course association kinetics (**A**), saturation binding curve (**B**), equilibrium competition (**C**). Time-dependence of binding interaction was examined by incubating the mouse brain membrane preparations with 2 nM [^{3}H]oxymorphone at various time points (**A**). For saturation binding, mouse brain membranes (≈0.2 mg protein, 1 mL volume) were incubated with increasing concentration of 0.16 - 32 nM [^{3}H]oxymorphone in the presence and absence of 10 µM naloxone for 30 min at 24 °C (**B**). For competition binding studies, mouse brain membranes were incubated with 2 nM [^{3}H]oxymorphone in the presence of increasing concentration of naloxone 10^{-12} – 10^{-5} M (**C**). The symbols in the graph represent mean ± SEM of three independent determinations performed in duplicates.

Fig. 2. Representative pseudo-colour images showing [^{3}H]oxymorphone (5 nM) binding in the brains of wild-type, MOP, KOP and DOP opioid receptor knockout (KO) mice. Images were taken at bregma level +2.8mm (prelimbic cortex and frontal association cortex), +1.1mm (nucleus accumbens core and shell, olfactory tubercle, caudate putamen, cingulate cortex, secondary motor cortex and primary motor cortex), -1.9mm (hippocampus, thalamus, hypothalamus, amygdala and medial habenular nucleus) and -3.6mm (superficial gray layer of the superior colliculus, periaqueductal gray, substantia nigra and ventral tegmental area). 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.

Fig. 3. Representative pseudo-colour images showing [^{3}H]oxymorphone (5 nM) in sagittal brain sections of wild-type and all three classical opioid receptor (MOP/KOP/DOP) 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.

Statement of conflicts of interest

The authors report no conflicts of interest.

Table 1. Binding parameters for oxymorphone in mouse brain homogenates

Parameter	**[^{3}H]oxymorphone**
Equilibrium dissociation constant[1] $\mathbf{K_d}$ (nM)	1.7 ± 0.4 (3)
Binding capacity[1] $\mathbf{B_{max}}$ (fmol/mg protein)	147 ± 16 (3)
$\mathbf{pIC_{50}}$ [2]	8.3 ± 0.09 (3)
Equilibrium inhibition constant[2,3] $\mathbf{K_i}$ (nM)	1.8

Legend to Table 1.

Data are mean values ± SEM, number of independent experiments are given in parenthesis.

1) from equilibrium saturation binding studies
2) from homologous competition studies
3) calculated by the Cheng-Prusoff equation

Table 2. 5 nM [^{3}H]oxymorphone binding in MOP, KOP and DOP knockout mice.

Region	[^{3}H]oxymorphone-specific binding (fmol/mg tissue)				% Change in binding (vs. wild-type)		
	Wild-type	MOP KO	KOP KO	DOP KO	MOP KO	KOP KO	DOP KO
Prelimbic cortex	17.9 ± 5.2	8.8 ± 1.8	20.5 ± 3.9	16.8 ± 4.0	-51	15	-6
Frontal association cortex	17.2 ± 3.9	8.4 ± 1.6	17.3 ± 4.0	11.4 ± 2.4	-51	1	-34
Nucleus accumbens core	42.0 ± 12.3	6.1 ± 2.8 **	44.6 ± 4.9	40.1 ± 6.9	-85	6	-5
Nucleus accumbens Shell	38.2 ± 11.6	5.0 ± 2.5 *	47.3 ± 5.9	38.5 ± 7.8	-87	24	1
Olfactory tubercle	24.5 ± 7.7	10.1 ± 2.9	22.8 ± 5.4	21.7 ± 3.8	-59	-7	-11
Caudate putamen	25.3 ± 5.9	3.6 ± 2.3 *	23.6 ± 5.2	24.5 ± 4.1	-86	-7	-3
Cingulate cortex	16.7 ± 4.0	2.4 ± 1.0 *	18.1 ± 4.7	15.1 ± 3.8	-86	9	-9
Secondary motor cortex	16.2 ± 4.3	1.8 ± 1.1 *	14.1 ± 3.6	12.0 ± 2.7	-89	-13	-26
Primary motor cortex	13.9 ± 3.1	1.0 ± 0.6 **	12.1 ± 3.2	12.8 ± 2.3	-93	-12	-8
Hippocampus	5.6 ± 0.7	1.8 ± 0.9	10.0 ± 1.0 *	4.7 ± 1.3	-68	78	-16
Thalamus	28.2 ± 3.9	1.0 ± 0.5 ***	30.7 ± 2.7	25.8 ± 3.6	-97	9	-9
Hypothalamus	29.7 ± 3.5	0.7 ± 0.3 ***	33.9 ± 1.6	28.7 ± 3.6	-98	14	-3
Amygdala	24.1 ± 2.4	1.9 ± 0.6 ***	30.4 ± 2.7	28.7 ± 2.5	-92	26	19
Medial habenular nucleus	43.7 ± 9.0	2.4 ± 2.0 ***	55.3 ± 5.2	55.0 ± 4.6	-95	27	26
Superficial gray layer of the superior colliculus	41.4 ± 4.8	4.8 ± 2.8 ***	46.2 ± 2.2	38.4 ± 2.5	-88	12	-7
Periaqueductal gray	31.5 ± 4.1	2.9 ± 0.8 ***	28.3 ± 3.5	28.8 ± 2.8	-91	-10	-9
Substantia nigra	20.3 ± 5.2	0.6 ± 0.2 **	22.1 ± 4.2	22.1 ± 2.6	-97	9	9
Ventral tegmental area	22.4 ± 5.0	2.9 ± 0.7 **	27.5 ± 4.7	28.5 ± 1.5	-87	23	27

Legend to Table 2.

Quantitative autoradiography of 5 nM [^{3}H]oxymorphone binding in the brains of wild-type (WT), MOP, KOP and DOP knockout (KO) mice. Values are expressed as mean ± SEM of 4 - 5 mice. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to WT mice (Dunnett's post hoc test). The mean decreases in binding across all regions were 83 % and 4 % in MOP and DOP KO mice vs WT, respectively. The mean increase in binding across all regions was 11 % in KOP KO mice vs WT.

Figure 1

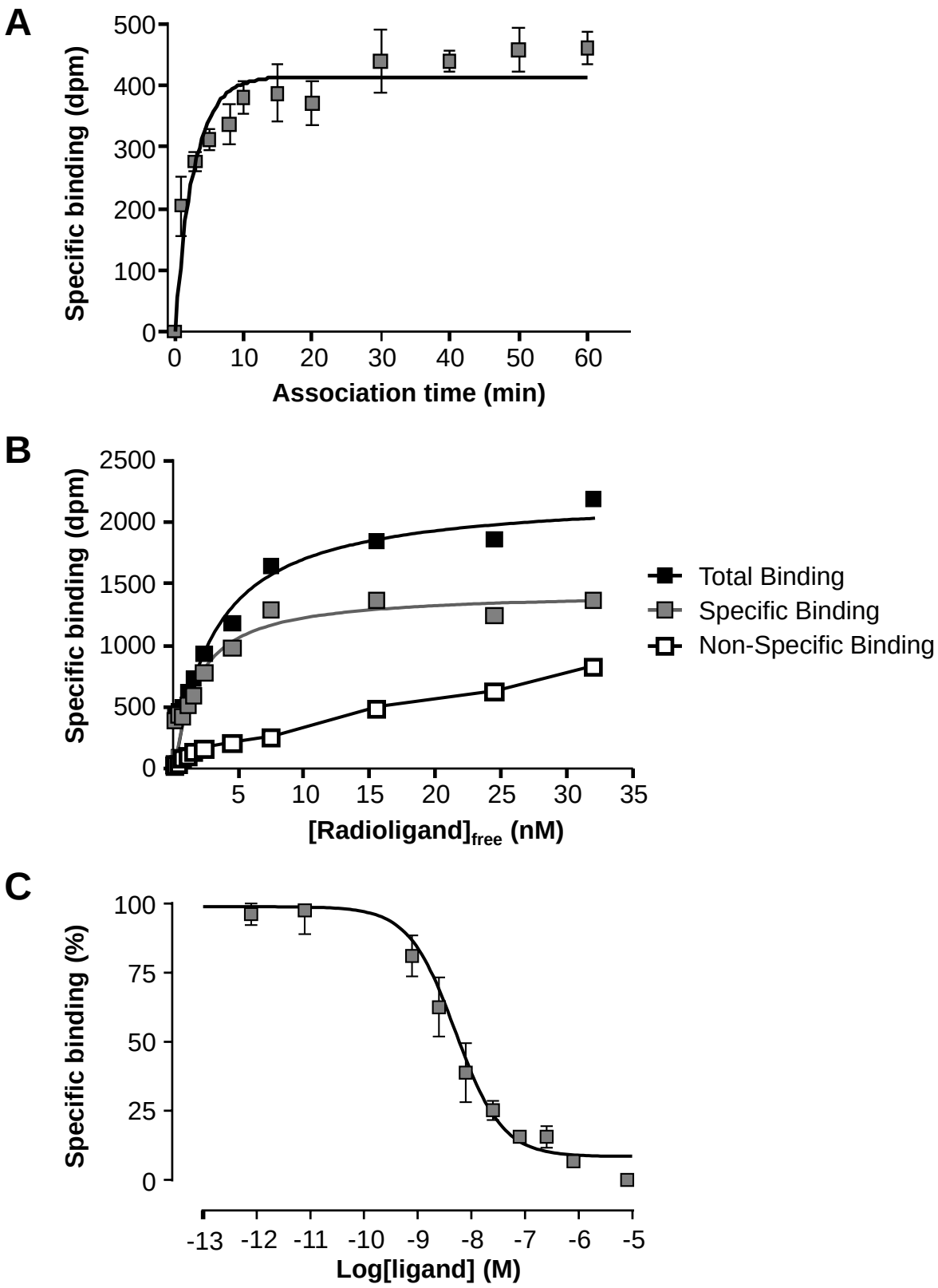
A
Specific binding (dpm)
500
400
300
200
100
0
0
10
20
30
40
50
60
Association time (min)
B
Specific binding (dpm)
2500
2000
1500
1000
500
0
5
10
15
20
25
30
35
[Radioligand]free (nM)
Total Binding
Specific Binding
Non-Specific Binding
C
Specific binding (%)
100
75
50
25
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log[ligand] (M)

Figure 2

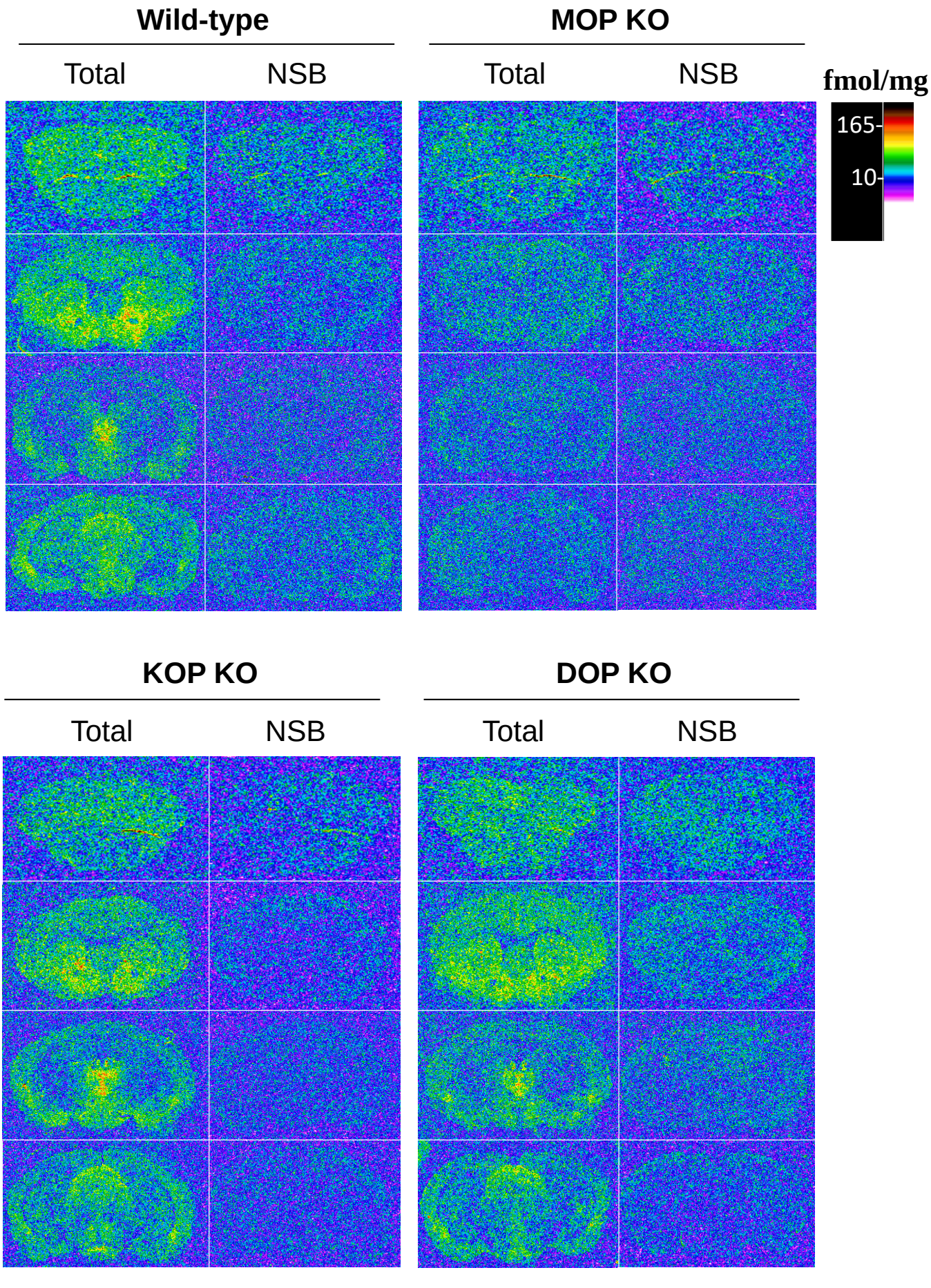
Wild-type
Total
NSB
MOP KO
Total
NSB
fmol/mg
165-
10-
KOP KO
Total
NSB
DOP KO
Total
NSB

Figure 3