A mu–delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks

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Abstract Opioid receptors are G protein-coupled receptors (GPCRs) that modulate brain function at all levels of neural integration, including autonomic, sensory, emotional and cognitive processing. Mu (MOR) and delta (DOR) opioid receptors functionally interact in vivo, but whether interactions occur at circuitry, cellular or molecular levels remains unsolved. To challenge the hypothesis of MOR/DOR heteromerization in the brain, we generated redMOR/greenDOR double knock-in mice and report dual receptor mapping throughout the nervous system. Data are organized as an interactive database offering an opioid receptor atlas with concomitant MOR/DOR visualization at subcellular resolution, accessible online. We also provide co-immunoprecipitation-based evidence for receptor heteromerization in these mice. In the forebrain, MOR and DOR are mainly detected in separate neurons, suggesting system-level interactions in high-order processing. In contrast, neuronal co-localization is detected in subcortical networks essential for survival involved in eating and sexual behaviors or perception and response to aversive stimuli. In addition, potential MOR/DOR intracellular interactions within the nociceptive pathway offer novel therapeutic perspectives.

Keywords Mouse mu opioid receptor · Mouse delta opioid receptor · Receptor brain atlas · Heteromer · Aversive stimuli

Abbreviations
3N Oculomotor nucleus
4N Trochlear nucleus

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| 6N | Abducens nucleus |
|----|----------------|
| 7N | Facial nucleus  |
| 10N| Dorsal motor nucleus of vagus |
| 12N| Hypoglossal nucleus |
| 3V | Third ventricle  |
| A5 | A5 noradrenaline cells |
| AAV| Anterior amygdaloid area, ventral part |
| Acbc| Accumbens nucleus, core |
| AcbS| Accumbens nucleus, shell |
| ACo| Anterior cortical amygdaloid nucleus |
| AD | Anterodorsal thalamic nucleus |
| AHA| Anterior hypothalamic area, anterior part |
| AHC| Anterior hypothalamic area, central part |
| AHP| Anterior hypothalamic area, posterior part |
| AHi| Amygdalohippocampal area |
| Al | Agranular insular cortex |
| AM | Anteromedial thalamic nucleus |
| Amb| Ambiguous nucleus |
| AOE| Anterior olfactory nucleus, external part |
| AVpe| Anteroventral periventricular nucleus |
| APiR| Amygdalopiriform transition area |
| APT| Anterior pretectal nucleus |
| Aq | Aqueduct (Sylvius) |
| Arc| Arcuate hypothalamic nucleus |
| AStr| Amygdastratiatal transition area |
| Atg| Anterior tegmental nucleus |
| aud| Auditory cortex |
| AVDM| Anteroventral thalamic nucleus, dorsomedial part |
| AVVL| Anteroventral thalamic nucleus, ventrolateral part |
| B | Basal nucleus |
| BAOT| Bed nucleus of the accessory olfactory tract |
| Bar| Barrington’s nucleus |
| BIC| Nucleus of the brachium of the inferior colliculus |
| BLA| Basolateral amygdaloid nucleus, anterior part |
| BLP| Basolateral amygdaloid nucleus, posterior part |
| BMA| Basomedial amygdaloid nucleus, anterior part |
| BMP| Basomedial amygdaloid nucleus, posterior part |
| BSTIA| Bed nucleus of the stria terminalis, intraamygdaloid division |
| BSTLD| Bed nucleus of the stria terminalis, lateral division, dorsal part |
| BSTLI| Bed nucleus of the stria terminalis, lateral division, intermediate part |
| BSTLP| Bed nucleus of the stria terminalis, lateral division, posterior part |
| BSTLV| Bed nucleus of the stria terminalis, lateral division, ventral part |
| BSTMA| Bed nucleus of the stria terminalis, medial division, anterior part |
| BSTMP| Bed nucleus of the stria terminalis, medial division, posterior part |
| BSTMV| Bed nucleus of the stria terminalis, medial division, ventral part |
| CA1| Field CA1 of hippocampus |
| CA3| Field CA3 of hippocampus |
| CbCx| Cerebellar cortex |
| cCM| Central medial thalamic nucleus, caudal part |
| CeC| Central amygdaloid nucleus, capsular part |
| CeL| Central amygdaloid nucleus, lateral division |
| CeM| Central amygdaloid nucleus, medial division |
| Cg | Cingulate cortex |
| CGA| Central gray, alpha part |
| CGPn| Central gray of the pons |
| CIC| Central nucleus of the inferior colliculus |
| CI | Claustrum |
| CL | Centrolateral thalamic nucleus |
| CN | Cochlear nuclei |
| CNF| Cuneiform nucleus |
| CPO| Caudal periolivary nucleus |
| CPu| Caudate putamen |
| Cu | Cuneate nucleus |
| CVL| Caudoventrolateral reticular nucleus |
| CxA| Cortex-amygdala transition zone |
| DC| Dorsal cochlear nucleus |
| DCP| Dorsal periolivary region |
| DCT| Dorsal tegmentum |
| DPO| Dorsal periolivary region |
| DRC| Dorsal raphe nucleus, caudal part |
| DRD| Dorsal raphe nucleus, dorsal part |
| DRI| Dorsal raphe nucleus, interfascicular part |
| DRV| Dorsal raphe nucleus, ventral part |
| DTg| Dorsal tegmental nucleus |
| DTgC| Dorsal tegmental nucleus, central part |
| DTgP| Dorsal tegmental nucleus, pericentral part |
| DTT| Dorsal tenia tecta |
| ECIC| External cortex of the inferior colliculus |
| Abbreviation | Structure Description |
|--------------|-----------------------|
| Ect          | Ectorhinal cortex      |
| Ecu          | External cuneate nucleus |
| Epl          | External plexiform layer of the olfactory bulb |
| EPIA         | External plexiform layer of the accessory olfactory bulb |
| Eth          | Ethmoid thalamic nucleus |
| EVe          | Nucleus of origin of efferents of the vestibular nerve |
| EW           | Edinger-Westphal nucleus |
| fi           | Fimbria of the hippocampus |
| fr           | Fasciculus retroflexus |
| FrA          | Frontal association cortex |
| GI           | Granular insular cortex |
| GiA          | Gigantocellular reticular nucleus, alpha part |
| GiV          | Gigantocellular reticular nucleus, ventral part |
| Gl           | Glomerular layer of the olfactory bulb |
| GrA          | Granule cell layer of the accessory olfactory bulb |
| GrC          | Granular layer of the cochlear nuclei |
| GrO          | Granule cell layer of the olfactory bulb |
| HDB          | Nucleus of the horizontal limb of the diagonal band |
| I            | Intercalated nuclei of the amygdala |
| I5           | Intertrigeminal nucleus |
| IAD          | Interanterodorsal thalamic nucleus |
| IAM          | Interanteromedial thalamic nucleus |
| ICjM         | Islands of Caleja, major island |
| IF           | Interfascicular nucleus |
| IGL          | Intergenicate leaflets |
| IL           | Infralimbic cortex |
| ILL          | Intermediate nucleus of the lateral lemniscus |
| IMD          | Intermediodorsal thalamic nucleus |
| InC          | Intertitial nucleus of Cajal |
| InG          | Intermediate gray layer of the superior colliculus |
| IntP         | Interposed cerebellar nucleus, posterior part |
| InWh         | Intermediate white layer of the superior colliculus |
| IO           | Inferior olive |
| IPAC         | Intertstitial nucleus of the posterior limb of the anterior commissure |
| IPC          | Interpeduncular nucleus, caudal subnucleus |
| IPDL         | Interpeduncular nucleus, dorsolateral subnucleus |
| IPDM         | Interpeduncular nucleus, dorsomedial subnucleus |
| IPI          | Interpeduncular nucleus, intermediate subnucleus |
| IPI          | Internal plexiform layer of the olfactory bulb |
| IPL          | Interpeduncular nucleus, lateral subnucleus |
| IPR          | Interpeduncular nucleus, rostral subnucleus |
| IPRl         | Interpeduncular nucleus, rostral subnucleus, lateral part |
| IRt          | Intermediate reticular nucleus |
| LA           | Lateroanterior hypothalamic nucleus |
| Lat          | Lateral (dentate) cerebellar nucleus |
| LC           | Locus coeruleus |
| LDDM         | Laterodorsal thalamic nucleus, dorsomedial part |
| LDMM         | Laterodorsal thalamic nucleus, dorsomedial part |
| LDTg         | Laterodorsal tegmental nucleus |
| LDVL         | Laterodorsal thalamic nucleus, ventrolateral part |
| LEnt         | Lateral entorhinal cortex |
| LGP          | Lateral globus pallidus |
| LH           | Lateral hypothalamic area |
| LHb          | Lateral habenular nucleus |
| LM           | Lateral mammillary nucleus |
| LOT          | Nucleus of the lateral olfactory tract |
| LPAG         | Lateral periaqueductal gray |
| LPBC         | Lateral parabrachial nucleus, central part |
| LPBD         | Lateral parabrachial nucleus, dorsal part |
| LPBE         | Lateral parabrachial nucleus, external part |
| LPAI         | Lateral parabrachial nucleus, internal part |
| LPBS         | Lateral parabrachial nucleus, superior part |
| LPBV         | Lateral parabrachial nucleus, ventral part |
| LPGi         | Lateral paragigantocellular nucleus |
| LPO          | Lateral preoptic area |
| LRt          | Lateral reticular nucleus |
| LSD          | Lateral septal nucleus, dorsal part |
| LSI          | Lateral septal nucleus, intermediate part |
| LSO          | Lateral superior olive |
| LSV          | Lateral septal nucleus, ventral part |
| LVe          | Lateral vestibular nucleus |
| LVPO         | Lateroventral periolivary nucleus |
| M1           | Primary motor cortex |
| M2           | Secondary motor cortex |
| MA3          | Medial accessory oculomotor nucleus |
| mcp          | Middle cerebellar peduncle |
| MCPO         | Magnocellular preoptic nucleus |
| MD           | Mediodorsal thalamic nucleus |
| MDC          | Mediodorsal thalamic nucleus, central part |
| MdD          | Medullary reticular nucleus, dorsal part |
| MDL          | Mediodorsal thalamic nucleus, lateral part |
| MDM          | Mediodorsal thalamic nucleus, medial part |
| ME           | Median eminence |
| Me5          | Mesencephalic trigeminal nucleus |
| MeAD         | Medial amygdaloid nucleus, anterior dorsal |
| MeAV         | Medial amygdaloid nucleus, anteroventral part |
| Med          | Medial (fastigial) cerebellar nucleus |
| MEEnt        | Medial entorhinal cortex |
| MePD         | Medial amygdaloid nucleus, posterodorsal part |
Opioid receptors and endogenous opioid peptides are largely expressed throughout the nervous system (Le Merrer et al. 2009). The opioid system plays a key role in reward and motivation, and regulates emotional responses and cognition. The system also modulates nociception, neuroendocrine physiology and autonomic functions (Feng et al. 2012). The three opioid receptors mu (MOR), delta (DOR) and kappa (KOR) are homologous G protein-coupled receptors (GPCRs) (Filizola and Devi 2013), and their respective implication in pain control, drug abuse and mood disorders has been extensively studied (Pradhan et al. 2011; Lutz and Kieffer 2012).

Several decades of opioid pharmacology have uncovered the complexity of opioid system physiology. In particular, the analysis of opioid drug effects in vivo has revealed functional interactions across receptors, particularly documented for MORs and DORs. The best-known example is the implication of DORs in the development of analgesic tolerance to morphine, a prototypical MOR agonist (Cahill et al. 2007). Whether in vivo receptor interactions occur at systems level across neural circuits, within neurons via signaling pathways, or at molecular level by direct receptor–receptor contact, however, is highly debated, and is extremely difficult to tackle with existing tools.

Within a cell, functional interactions between two receptors may arise from a competition for downstream effectors or a shared association with intracellular partners within protein complexes. Intracellularly, the two receptors may also interact physically, and operate as homo- or heteromers with signaling and trafficking properties distinct from monomeric receptors (Rozenfeld and Devi 2011). The latter hypothesis stems from initial reports using recombinant cell systems in which receptor heteromerization has been demonstrated for many GPCRs.
including MOR and DOR (Jordan and Devi 1999; George et al. 2000). Whether these mechanisms indeed operate in vivo remains a central question in GPCR research. So far, little evidence supports in vivo MOR/DOR co-expression. In vivo co-localization has only been reported in a limited number of brain areas using antibodies specifically raised against MOR–DOR heteromers (Gupta et al. 2010). As for most GPCRs, however, in-depth anatomical mapping of opioid receptors in the brain with subcellular resolution is still lacking.

The present study provides a proof-of-principle brain atlas for GPCR co-expression in vivo, using MOR and DOR as model interacting receptors. We previously targeted the enhanced Green Fluorescent Protein into the DOR gene locus to produce DOR-eGFP knock-in mice (Scherrer et al. 2006). Mutant animals express a fully functional receptor with a fused C-terminal eGFP (DOR-eGFP) in place of the native receptor and show no detectable alteration of behavior and responses to drugs. DOR-eGFP mice allowed visualizing DOR in vivo, with subcellular resolution, in DRGs (Scherrer et al. 2009), spinal cord (Gomes et al. 2004) and rostroventral medulla (Pedersen et al. 2011; Kivell et al. 2004). MOR/DOR co-expression was also reported in a limited number of brain areas using antibodies from brain networks related to water and food consumption, sexual behavior or perception and responses to aversive stimuli that may endanger the animal. Localization in

**Fig. 1** Expression of functional receptors in MOR-mcherry knock-in mice. a Targeting strategy: Oprm1 exons, mcherry cDNA, and the FRT (triangle) flanked neomycin cassette are, respectively, displayed as exon number, mcherry, and neo. Homologous recombination (HR) was followed by FLP recombinase treatment (FLP) in ES cells. Positions of the oligonucleotides (BAZ 43, BAZ 44) used for genotyping are indicated. b Western blot: detection of MOR-mcherry fusion by immunoblotting with antibodies directed against mcherry on membranes from striatum and periaqueductal gray (PAG) from wild-type (Oprm1+/+), heterozygous (Oprm1+/mch) and homozygous (Oprm1−/−mch) mice (MOR-mcherry fusion indicated by arrow). Cos cells transfected with a plasmid encoding mcherry (cos) were added as a control for unbound mcherry protein detection with the antimcherry antibody (arrow head). c G protein activation: similar [%35S]GTPγS incorporation was measured on brain membranes from wild-type (filled square) (n = 8), heterozygous (n = 7) (filled diamond) and homozygous (n = 11) (filled circle) mice following stimulation with the mu-selective agonist DAMGO. Data are the mean ± sem from independent experiments performed in triplicate (n = 3 animals per genotype). d Tail immersion test: similar tail withdrawal latencies were measured at 52 °C in wild-type (Oprm1+/+) and MOR-mcherry (Oprm1mch/mch) mice after saline or morphine injection (5 or 10 mg/kg, i.p.). Data are presented as mean ± sem (n = 16 animals/group). *p < 0.05, ***p < 0.001 morphine effect compared to baseline. e Hot plate test: similar jump latencies from a hot plate at 52 °C were measured in wild-type (Oprm1+/+) and MOR-mcherry (Oprm1mch/mch) mice after saline or morphine injection (5 or 10 mg/kg, i.p.). Data are presented as mean ± sem (n = 16 animals/group). *p < 0.05, ***p < 0.001 morphine effect compared to baseline. f Locomotor sensitization: wild-type (Oprm1+/+), MOR-mcherry (Oprm1mch/mch) mice received daily morphine (25 mg/kg, i.p.) or saline injections for 5 days. Similar locomotor activities were recorded for 1 h. Data are expressed as total traveled distance (mean ± sem) (n = 8–10 animals/group). g Conditioned place preference: wild-type (Oprm1+/+) or MOR-mcherry (Oprm1mch/mch) mice showed similar preference for the compartment associated with morphine (10 mg/kg, s.c.) following three conditioning sessions. Place preference corresponds to the time spent in the drug-paired compartment expressed as a percentage of time spent in the two compartments during the 20 min pre- and post-stimulating sessions (n = 8 animals/group). Data are presented as mean ± sem. Treatment effect ***p < 0.001. h Physical dependence: global scores of pharmacological withdrawal precipitated by naloxone (1 mg/kg, s.c.) were similar in wild-type (Oprm1+/+) or MOR-mcherry (Oprm1mch/mch) mice treated with escalating doses of morphine (20, 40, 60, 80, 100 mg/kg) or in saline-treated controls (n = 8/group). Data are presented as mean ± sem. Drug effect ***p < 0.001
stop codon (Scherrer et al. 2006). MOR-mcherry knock-in mice expressing the mu opioid receptor fused its C-terminus to the red protein mcherry were generated by homologous recombination following a procedure similar to the one used for DOR-eGFP knock-in mice. A targeting construct in which the \textit{Oprm1} stop codon has been replaced by a Gly-Ser-Ile-Ala-Thr-mcherry encoding cDNA followed by a neomycin resistance gene flanked by FRT sites was transfected into ES cells (Fig. 1). Two independent homologous recombinants were electroporated with a FLP recombinase expressing plasmid to excise the neomycin gene and microinjected into C57Bl6/J blastocysts. Chimeric mice were crossed with C57Bl6/J mice to obtain F1 heterozygous progenies. Heterozygous animals were

![Diagram A](image1)

![Diagram B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

![Graph F](image6)

![Graph G](image7)

![Graph H](image8)
intercrossed to generate mice homozygous for Oprm1-mcherry that are fertile and develop normally. DOR-eGFP mice were crossed with MOR-mcherry mice to obtain mice homozygous for both constructs. Wild-type mice were used as control in behavioral experiments. The genetic back-ground of all mice was C57/Bl6/J: 129svPas (50:50 %). Mice genotyping was performed by standard PCR tech-nique using a 5′ oligonucleotide located on the fourth exon of the oprm1 gene (BAZ 43 tgacctcagctcagttgagattt) and a 3′ oligonucleotide located in the 3′ UTR untranslated region (BAZ 44 tccccaaacccctgacagcaac). Introduction of the coding sequence for mcherry increased the size of the amplified fragment by about 800 bp enabling identification of wild type oprm1+/+, heterozygous oprm1+/mch and homozygous oprm1/mch/mch animals by PCR.

Mice were housed in a temperature- and humidity-controlled animal facility (21 ± 2 °C, 45 ± 5 % humidity) on a 12-h dark–light cycle with food and water ad libitum. Male and female mice aged 8–14 weeks were used in all protocols. All the experiments were conducted during the light period. All experiments were performed in accordance with the European Communities Council Directive of 26 May 2010 and approved by the local ethical com-mittee (Com’Eth 2012-006).

Drugs

Morphine chloride (Francopia, Lyon, France) was administered s.c. or i.p. at doses of 5, 10, 25 or 30 mg/ kg. Naloxone hydrochloride (Sigma) was used at 1 mg/kg (s.c.) for the pharmacological induction of morphine withdrawal. SNC 80 (Tocris) was used in vivo at 10 mg/kg. Naloxone hydrochloride (Sigma) was used at 1 mg/kg administered s.c. or i.p. at doses of 5, 10, 25 or 30 mg/kg. Naloxone hydrochloride (Sigma) was used at 1 mg/kg (s.c.) for the pharmacological induction of morphine withdrawal. SNC 80 (Tocris) was used in vivo at 10 mg/kg and 5 % humidity) on a 12-h dark–light cycle with food and water ad libitum. Male and female mice aged 8–14 weeks were used in all protocols. All the experiments were conducted during the light period. All experiments were performed in accordance with the European Communities Council Directive of 26 May 2010 and approved by the local ethical com-mittee (Com’Eth 2012-006).

Real-time quantitative PCR analysis

RNA extraction, cDNA synthesis and qPCR assays were carried out as described previously (Befort et al. 2008). Total brain tissue was collected from three animals for each genotype to isolate RNA using Trizol reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer’s instructions. Briefly, 2.5 μg of total RNA was reverse transcribed using Superscript II (200 or 400 U, Invitrogen, Cergy Pontoise, France) with anchored-oligoDT primer (8 mmol/L), random Hexamer (16 mmol/L), and deoxy-nucleotide triphosphates (500 μmol/L each). Real-time PCR was performed in triplicate on a MyIq BioRad instrument using iQSYBRGreen supermix (Bio-Rad, Marnes-la-Coffete, France), cDNA (0.5 μL), and gene-specific primers (200 nmol/L) in a 25 μL reaction as recom-mended by the manufacturer. Gene-specific primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). Sequences of primers are provided below. Thermal cycling parameters were 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 30 s at 72 °C. Relative expression ratios were normalized to the level of HPRT reference gene, and the 2-ΔΔCt method was used to evaluate the differential expression level. Two reference genes (β-actin, Rplp0) were tested in each run as an internal control.
Ex vivo tissue analysis of MOR-mcherry mice

Membrane preparations were carried out as described previously (Befort et al. 2001). Briefly, whole brain were removed, immediately frozen on dry ice, and stored at −80 °C prior to use. Whole brain were prepared by homogenizing the brain in ice-cold 0.25 M sucrose solution 10 vol (ml/g wet weight of tissue). Samples were then centrifuged at 1,100 g for 10 min. Supernatants were collected and diluted five times in buffer containing 50 mM Tris–HCl (pH 7.4) and 1 mM EDTA, following which they were centrifuged at 35,000 g for 30 min. The pellets were homogenized in 2 ml ice-cold sucrose solution (0.32 M) and aliquots kept at −80 °C until further use.

Scatchard analysis

50 μg of membrane proteins was incubated in the presence of variable concentrations (3 10^{-9} to 2 10^{-10} M) of [3H] DAMGO for 1 h at 25 °C. Membranes were washed and filtered, and radioactivity was quantified using a liquid scintillation counter. Assays were performed in triplicates in eight experiments using six different membrane preparations.

[^35]S GTPγS binding assay

5 μg of membrane proteins was used per well. Samples were incubated with the mu agonist DAMGO, the delta agonist AR-M1000390 or the kappa agonist U50-488H (10^{-4} to 10^{-11} M) for 1 h at 25 °C in assay buffer 50 mM Tris–HCl (pH 7.4), 3 mM MgCl₂, 100 mM NaCl, 0.2 mM EGTA containing 30 μM GDP and 0.1 nM [35S] GTPγS. Incubation was terminated by rapid filtration and washing in ice-cold buffer (50 mM Tris–HCl, 5 mM MgCl₂, 50 mM NaCl, pH 7.4). Bound radioactivity was quantified using a liquid scintillation counter. Non-specific binding was defined as binding in the presence of 10 μM GTPγS, and basal binding was assessed in the absence of agonist. Assays were performed in triplicates in nine experiments using six different membrane preparations.

Co-immunoprecipitation

Membrane preparations (500 μg) were solubilized in Tris–HCl 50 mM pH 7.4, 100 mM NaCl, 10 % CHAPS, complete protease inhibitor cocktail (Roche applied Bioscience, Mannheim, Germany) for 1 h at 4 °C, immunoprecipitated with either 1 μg anti-eGFP or 1 μg anti-mcherry antibodies for 1 h at 4 °C and isolated by incubation with G protein Sepharose for 1 h at 4 °C. Samples were washed three times with Tris–HCl 50 mM pH 7.4 and resuspended in SDS-PAGE sample buffer.

Western blot analysis

Total protein content of brain membranes was determined by Bradford assay. Samples were heated in loading buffer (62.5 mM Tris–HCl, pH 6.8, 5 % (wt/vol) β-mercaptoethanol, 2 % (wt/vol) SDS, 10 % (vol/vol) glycerol, 0.1 % (wt/vol) Bromophenol blue) for 5 min at 95 °C. 50 μg proteins were loaded onto an 8 % SDS-PAGE gel. Proteins were transferred onto Immobilon P polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Following blocking in 5 % (wt/vol) non-fat dry milk in 50 mM Tris–HCl pH 8, 150 mM NaCl, 0.2 % (vol/vol) Tween 20 (TBST) for 1 h, PVDF membranes were incubated overnight at 4 °C with a 1:1,000 dilution of the anti mu opioid receptor or a 1:1,000 dilution of the anti mcherry antibody. PVDF membranes were washed three times for 10 min with 5 % (wt/vol) non-fat dry milk in TBST, incubated for 2 h with a 1: 10 000 dilution of HRP-conjugated anti-mouse (Fab’₂) fragment antibody in 5 % (wt/vol) non-fat dry milk in TBST. PVDF membranes were washed three times for 10 min in TBST. Chemiluminescence was detected using ECL+ according to the manufacturer’s instructions.

Behavioral testing

Experiments were performed in stable conditions: 21 ± 2 °C, 45 ± 5 % humidity, 40 ± 2 lux. All experiments were preceded by 2 days of animal handling. Tail

| Gene   | Forward             | Reverse          | Target sequence |
|--------|---------------------|------------------|-----------------|
| mMOR   | GAGCCACAGCCTGTGCCCT | CGTGCTAGTGCTGAAGCATC | Exon1/exon2     |
| mDOR   | GCTCGTATGTGGTGATC   | AAGTACCTGGGGCTGCTGGAA | Exon1/exon2     |
| mKOR   | CCTGGCAGTCTGTTGAT   | GGAATCTGCGAGACATTTC | Exon2/exon3     |
| HPRT   | TGACACTGTGAAACATTGCA| GGTCCCTTTACACGCAAGCT   |
| Rplp0  | TGAGATTCCG GATATGCTGTTG | TTCAATGGTGCTCTGGAGAT | |
| β-Actin| GACGGCCAGGTCACTCATAT | CCACCGATCCACACAGAGTA  |
immersion and hot plate tests were used to evaluate antinociceptive responses.

Tail immersion test

The mouse was maintained in a cylinder and the tail immersed into a heated water bath set at 52 °C. Morphine (5 or 10 mg/kg) or a saline solution were injected i.p. Tail withdrawal latencies were measured 45 min later with a 10 s cutoff time. Baseline responses were measured 1 h prior drug injection.

Hot plate test

Morphine (5 or 10 mg/kg) or a saline solution was injected i.p. The mouse was placed on a 52 °C hot plate 45 min later and latencies to jump were recorded with a 300 s cutoff time.

Conditioned place preference test

Apparatus Place conditioning experiments were performed in unbiased computerized boxes (Imetronic, Pessac, France) formed by two Plexiglas chambers (15.5 × 16.5 × 20 cm) separated by a central alley (6 × 16.5 × 20 cm). Two sliding doors (3 × 20 cm) connected the alley with the chambers. Two triangular prisms of transparent polycarbonate were arranged in one chamber, and one rectangular prism in the other to form different shape patterns (covering the same surface). Distinct-textured removable floors made of translucent polycarbonate provided additional contextual cues. The activity and location of mice were recorded using five photocells located throughout the apparatus. Behavioral data were collected by an interface connected to a PC. Light intensity in the chambers was set at 30 Lux.

Experimental protocol Animals were naive when conditioning started. Morphine conditioning consisted of 3 phases. On day 1, naive mice were placed in the central alley and allowed to freely explore the apparatus for 20 min for a pretest session. Based on the individuals' spontaneous preference during this pretest phase, the drug-paired chamber was assigned in such a way that saline and morphine groups were counterbalanced and unbiased toward contextual cues. Statistical analysis on pre-test data indicated no bias between the two chambers (p = 0.99). Conditioning phase lasted 3 days. Mice underwent two daily conditioning sessions, vehicle and drug paired, 7 h apart. Drug pairings were performed in the morning (10:00 AM). The animals were injected with either morphine (10 mg/kg, s.c.) or saline (controls) immediately before being confined in the “drug-paired” chamber. Vehicle pairings were performed in the afternoon (4:00 PM). All the animals received an injection of saline and were confined in the vehicle-paired compartment. Testing phase was conducted on day 5. The animals, in a drug-free state, were placed in the neutral central alley and allowed to explore the apparatus for 20 min with the two sliding doors opened. The time spent in each chamber was recorded.

Naloxone-precipitated withdrawal

Mice daily received escalating doses (20, 40, 60, 80, 100 mg/kg) morphine i.p. or a saline solution for 6 days. Physical dependence to morphine was verified by measuring withdrawal syndrome precipitated by a naloxone (1 mg/kg, s.c.) injection 2 h after the last morphine injection. A global withdrawal score was calculated as previously described (Berrendero et al. 2003).

Locomotor sensitization

Locomotor activity was assessed in clear Plexiglas boxes (21 × 11 × 17 cm) placed over a white Plexiglas infrared platform. Light intensity of the room was set at 15 lux. The trajectories of the mice were analyzed and recorded via an automated tracking system equipped with an infrared-sensitive camera (Videotrack; View Point, Lyon, France). Behavioral testing started when the animals were placed in the activity boxes for a 60-min habituation period. They were then injected with saline and locomotor activity was measured for another 1 h. Animals were then injected with morphine (25 mg/kg) or saline and activity was measured for 2 h. Locomotor activity was assessed during five consecutive days (Contet et al. 2008).

Tissue preparation and immunohistochemistry

Mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and perfused intracardiacally with 50 ml of 4 % paraformaldehyde (PFA) (at 2–4 °C) in PB 0.1 M or PBS 1× (Dulbecco’s Phosphate Buffer Saline, Sigma Aldrich), pH 7.4. Brains were post-fixed for 24 h at 4 °C in 4 % PFA solution, cryoprotected at 4 °C in a 30 % sucrose, PB 0.1 M pH 7.4 solution, embedded in OCT (Optimal Cutting Temperature medium, Thermo Scientific), frozen and kept at −80 °C. 30-μm thick brain sections were cut with a cryostat (CM3050, Leica) and kept floating in PB 0.1 M pH 7.4.

Immunohistochemistry was performed according to standard protocols (Erbs et al. 2012). Briefly, 30-μm thick sections were incubated in blocking solution (PB 0.1 M pH 7.4, 0.5 % Triton X100 (Sigma, St. Louis, MO, USA), 5 % normal goat or donkey serum (Invitrogen, Paisley, UK) depending on the secondary antibody) for 1 h at room temperature (RT). Sections were incubated overnight at
4 °C in the blocking solution with appropriate primary antibodies. Sections were washed three times with PB 0.1 M pH 7.4, 0.5 % Triton X100, incubated for 2 h at RT with appropriate AlexaFluor-conjugated secondary antibodies. Sections were washed three times and mounted on Superfrost™ glass (Menzel-Glaser) with Mowiol (Calbiochem, Darmstadt, Germany) and 4′, 6-diamidino-2-phenylindole (DAPI) (Roche Diagnostic, Mannheim, Germany) (0.5 μg/ml).

DOR-eGFP fluorescence was enhanced by detection with an anti-GFP antibody and a secondary antibody coupled to the AlexaFluor 488. MOR-cherry fluorescence was enhanced by detection with an anti-mcherry antibody and a secondary antibody coupled to AlexaFluor 594. Double labeling was performed to co-localize DOR-eGFP or MOR-mcherry with the chosen neuronal marker. Antibodies specific for the neuronal markers were detected with a secondary antibody coupled to the AlexaFluor 594 or 488 depending on amplification of the DOR-eGFP or MOR-mcherry signal, respectively, or with secondary antibody coupled to the AlexaFluor 350 for triple labeling.

Immunocytochemistry on MOR-mcherry primary neuronal cultures

Primary neuronal cultures were performed as previously described (Pradhan et al. 2009). Briefly, P0 mice pups were decapitated, and hippocampi were dissected and digested with papain (15 U/ml, Worthington). Cells were plated on glass coverslips coated with poly-l-lysine (PLL, Sigma) in B27/NeurobasalA medium (Invitrogen) completed with 0.5 mM glutamine and antibiotics. Cells were plated at a density of 8 × 10^4 cells/cm^2. Medium was replaced 60 min after plating, and half the medium changed every 5–7 days. Cultures were maintained for 15 days in vitro (DIV). Fully matured primary neurons (DIV 10–14) were used for DAMGO-induced receptor internalization studies. Cells were fixed with 4 % PFA in PBS before or at various time points after 1 μM DAMGO addition. Immunological detection with an anti-mcherry antibody was then performed as described previously (Massotte 2006). Briefly, cells were incubated in blocking solution (PB 0.1 M pH 7.4, 0.2 % Tween 20 (Sigma, St. Louis, MO, USA), 5 % normal goat serum (Invitrogen, Paisley, UK) for 1 h at room temperature (RT). Coverslips were incubated overnight at 4 °C in the blocking solution with anti-mcherry antibodies (1:1,000), washed three times with PB 0.1 M pH 7.4, 0.2 % Tween 20 and incubated for 2 h at RT with goat anti rabbit AlexaFluor 594-conjugated secondary antibodies. Coverslips were washed three times and mounted with Mowiol (Calbiochem, Darmstadt, Germany) and 4′, 6-diamidino-2-phenylindole (DAPI) (Roche Diagnostic, Mannheim, Germany) (0.5 μg/ml).

Statistical analysis

Statistical analysis was performed with Graph-Pad Prism v4 (GraphPad, San Diego, CA) and Statistica v9 (StatSoft, Maisons-Alfort, France). In vitro pharmacology experiments were analyzed using a one-way ANOVA. Behavioral experiments were analyzed using a two-way ANOVA. Multiple comparisons were made using Newman-Keuls or Tukey tests for post hoc analysis. A paired t test was performed to verify that the apparatus used in the conditioned place preference test was unbiased. Place conditioning data were expressed as percentage of time spent in the drug-paired compartment. Four-way ANOVA was performed with gender, genotype and treatment as between-group factors and conditioning (pretest versus test session) as a within-group factor.
Results

Generation and characterization of MOR-mcherry knock-in mice

We generated MOR-mcherry knock-in mice expressing MOR in fusion with the red fluorescent protein mcherry at the C terminus (Fig. 1a), as previously done for DOR and the green fluorescent protein eGFP (Scherrer et al. 2006). DNA sequencing showed accurate insertion of the mcherry cDNA at genomic level in homozygous mutant mice (Oprm1\textsuperscript{mch/mch} or MOR-mcherry mice). Quantitative mRNA analysis revealed that the genomic modification does not disrupt Oprm1 transcription, which was slightly increased in knock-in animals similarly to DOR-eGFP knock-in mice (Scherrer et al. 2006) (online resource Fig. 1). Western blot analysis of brain tissue using antibodies recognizing mcherry showed expression of a protein with expected molecular mass for the fusion construct, and no free mcherry protein could be detected (Fig. 1b). Scatchard analysis of [\textsuperscript{3}H] DAMGO binding to brain membranes from Oprm1\textsuperscript{+/+}, Oprm1\textsuperscript{mch/mch} and Oprm1\textsuperscript{mch/mch} animals showed similar ligand affinity (Kd 0.65 ± 0.12, 0.51 ± 0.16 and 0.51 ± 0.07 nM, respectively) and receptor density (207 ± 39, 230 ± 41 and 293 ± 18 fmol/ mg, respectively, p = 0.223). Further, the MOR-selective agonist DAMGO activated G proteins in brain membranes from Oprm1\textsuperscript{+/+}, Oprm1\textsuperscript{mch/mch} and Oprm1\textsuperscript{mch/mch} mice with similar potency (184 ± 33, 120 ± 27 and 184 ± 27 nM, respectively) and maximal efficacy (210 ± 11, 196 ± 16 and 199 ± 12 %, respectively) (Fig. 1c). Binding and signaling properties of DOR (AR-M1000390) and KOR (U50-488H) agonists were otherwise unchanged in mutant mice (online resource Fig. 1).

Next, we compared well-described behavioral effects of morphine in Oprm1\textsuperscript{mch/mch} mice and their wild-type Oprm1\textsuperscript{+/+} controls. Thermal antinociception following acute morphine administration was identical in animals from the two genotypes using both tail immersion and hot plate tests at two doses (5 and 10 mg/kg, s.c.) (Fig. 1d). A single injection of morphine (25 mg/kg, i.p.) also produced comparable locomotor activation in the two genotypes, and maximal efficacy (210 ± 0.51) was measured in the two genotypes upon naloxone injection (1 mg/kg i.p.) (Fig. 1g). Altogether, data demonstrate that functional properties of MOR are maintained in MOR-mcherry mice both in vitro and in vivo, as previously observed for DOR-eGFP knock-in mice (Scherrer et al. 2006; Pradhan et al. 2009).

Receptor subcellular localization in MOR-mcherry knock-in mice

Unlike DOR-eGFP that predominantly localizes at the plasma membrane under basal conditions (Scherrer et al. 2006; Pradhan et al. 2009), the MOR-mcherry fluorescent signal was strong inside neurons (Fig. 2a) and rather weak at the plasma membrane. Because fusion to mcherry may alter receptor distribution, notably the inside/outside receptor ratio, we compared the cellular distribution of MOR-mcherry to that of the native receptor using heterozygous Oprm1\textsuperscript{+/mch} animals co-expressing the two receptor forms. Double labeling using antibodies raised against mcherry or MOR demonstrated overlapping patterns with a similar weak signal at the neuronal surface (Fig. 2b). This result indicates that the low amount of cell surface MOR-mcherry does not result from deficient receptor trafficking, but rather reflects the genuine distribution of the native receptor. This is consistent with the previous reports describing substantial intracellular localization of endogenous untagged receptors (Poole et al. 2011). In addition, the intensity of the intracellular fluorescent signal varied across brain regions. Combined with the observation of intact in vivo morphine responses in MOR-mcherry mice (Fig. 1), our data strongly suggest that, under physiological conditions, only a small proportion of MOR is present at the cell surface, and that this distribution is compatible with full morphine effects. Electron microscopy showed that intracellular fluorescence is present in large multivesicular bodies suggesting that intracellular proteins are at least in part involved in the degradative pathway (not shown).

We finally examined whether MOR-mcherry internalizes upon agonist treatment, as largely described for MOR expressed in transfected cells (Borgland et al. 2003) or neurons (Arttamangkul et al. 2008; Haberstock-Debic et al. 2005). Although strong intracellular expression of MOR-mcherry hampers easy detection of receptor trafficking, internalization was detectable in primary hippocampal neurons from MOR-mcherry mice upon DAMGO exposure (Fig. 2c). A typical internalization punctate pattern was visible after 10 min, a time when surface staining had entirely disappeared, and the distribution returned to the basal pattern after 30 min. MOR-mcherry therefore shows normal trafficking response with kinetics similar to previous reports for the untagged receptor expressed in neurons (Rodriguez-Munoz et al. 2007; Trafton et al. 2000).
Neuroanatomy of MOR-mcherry and DOR-eGFP in double mutant mice

MOR-mcherry and DOR-eGFP mice were crossed and the two fluorescent signals mapped throughout the brain, spinal cord and DRGs. Data are presented as an interactive virtual atlas accessible at http://mordor.ics-mci.fr/ and summarized in Fig. 3 and Table 1. MOR-mcherry was more readily visualized in cell bodies than neural processes, because of the high intracellular/extracellular protein ratio. In contrast, DOR-eGFP was predominantly seen at the plasma membrane, therefore receptor expression in neurites or passing fibers was better detected for DOR-eGFP than for MOR-mcherry.

Both MOR-mcherry and DOR-eGFP distributions in the brain are in full agreement with the previous reports in mice and rats based on ligand binding (Slowe et al. 1999; Lesscher et al. 2003; Kitchen et al. 1997; Goody et al. 2002), GTP\(_7\)S incorporation (Tempel and Zukin 1987; Pradhan and Clarke 2005) or mRNA detection (Mansour et al. 1995; George et al. 1994; Cahill et al. 2001) (for a review see (Le Merrer et al. 2009)). In addition, we detected MOR-mcherry expression in discrete groups of neurons (Fig. 4; online atlas) that could not be previously resolved using autoradiography or in situ hybridization. Therefore, the approach further refines our current knowledge of MOR distribution.

In the spinal cord, localization of the fluorescent signals associated with MOR-mcherry and (Mansour et al. 1987) DOR-eGFP is also in agreement with mRNA distribution, radioligand binding and immunohistochemical data collected in rat (Wang et al. 2010; Trafton et al. 2000; Gray et al. 2006) and mice (Scherrer et al. 2009). The fluorescence associated with MOR-mcherry was predominantly present in the superficial layers of the dorsal horn, mainly lamina II, but somas could be detected in all layers (online atlas).

In DRGs, DOR-eGFP was present in neurons with small-, medium-, and large-diameter somata with predominance in the latter, consistent with enrichment in myelinated afferents as previously reported (Scherrer et al. 2009). MOR-mcherry was also expressed in the three types of neurons but was most abundant in DRG neurons with small diameter cell in agreement with previous immunohistochemical detection in rats and mice (Scherrer et al. 2009; Wang et al. 2010; Rau et al. 2005).

Altogether, both MOR-mcherry and DOR-eGFP fluorescent signals are consistent with currently available data from the literature. This designates the double fluorescent knock-in mouse as a unique tool to fine map receptor distribution.
expression at subcellular level, and addresses brain sites of receptor co-expression.

Neuroanatomy of MOR-mcherry/DOR-eGFP neurons in double mutant mice

Under basal conditions, we identified a limited number of regions in which both MOR-mcherry and DOR-eGFP fluorescent proteins could be detected in the same neuron distribution of the DOR-eGFP construct. The size of the green circle is indicative of the abundance of the receptor in the given area. A pale green circle indicates low expression level. See list for abbreviations (Fig. 5a, b; online atlas). Regions with most significant co-expression were the hippocampus, the hypothalamus, the lateral parabrachial nucleus and vestibular nuclei. Additional regions included the piriform cortex, the auditory pathway, as well as regions involved in the control of movement and posture or relaying somatosensory or motor information to the autonomic nervous system. Cellular co-expression of MOR-mcherry and DOR-eGFP in the lateral hypothalamus and the rostral ventral medulla is consistent with the function of mu and delta opioid receptors in the nervous system.
Table 1  Relative distribution of MOR-mcherry and DOR-eGFP signals in the brain

| Mu | Delta | Mu | Delta | Brainstem |
|---|---|---|---|---|
| **Olfactory structures** | **Dorsal thalamus** | **Brainstem** |
| Accessory olfactory bulb | Anterior group | Interpeduncular nucleus |
| EPIA | ++ | + | AD | +/- | IPC | ++ | +/- |
| GrA | - | + | IAD | ++ | + | IPL | ++ | +/- |
| MiA | ++ | + | AVDM | +/+ | + | |
| Olfactory bulb | AVVL | - | + | IPC | ++ | +/- |
| Epl | - | +++ | AM | + | + | Oculomotor |
| Gl | + | - | IAM | + | + | 3N | - | + |
| GrO | - | ++ | Lateral group | EW | - | + |
| IPI | - | + | LDVL | - | +/- | Dk | - | + |
| Mi | ++ | + | LDDM | - | + | InC | - | + |
| Anterior olfactory nucleus | LDMM | - | + | MA3 | - | + |
| AOE | +++ | + | Ventrual group | RI | + | + |
| DTT | ++ | ++ | VA | + | + | Su3/Su3C | + | +/- |
| VTT | ++ | - | VL | - | + | 4N | - | + |
| LOT | + | - | VM | ++ | +/- | Pa4 | +++ | + |
| Tu | - | ++ | VPM/VPL | - | + | Pr | - | + |
| **Cerebral cortex** | **VPPC(Gus)** | **Parabraunal group** |
| Orbital | +++ | +/- | Medial group | DMPAG | - | + |
| FrA | + | ++ | MD | + | +/- | DLPAG | + | +/- |
| DP | - | ++ | MDC | +/- | +/- | LPAG | +/+ | + |
| PrL | + | ++ | MDL | ++ | +/- | VLPAG | + | + |
| IL | + | ++ | MDM | + | +/- | |
| Cg | + | ++ | Sub | - | +/- | LPBS | + | +/- |
| M1/M2 | + | + | Lateral geniculate | LPBD | +/- | +/- |
| AI | + | ++ | DLG | - | - | LPBC | +/- | + |
| Di/Gl | + | ++ | VLGMC | + | - | LPBV | + | + |
| Pir | + | + | VLGPC | - | - | LPBI | + | + |
| S1/S2 | +++ | ++ | IGL | +/- | - | LPBE | ++++ | - |
| Ect | + | ++ | Medial geniculate | MPB | + | + |
| LEnt | + | + | MGD | - | - | MPBE | + | + |
| MEnt | + | + | MGV | - | - | Raphe |
| RSA | + | - | MGM | + | + | RLi | + | +/- |
| Te + aud | + | - | SG | + | + | MnR | +/+ | + |
| V1 + V2 | - | + | Posterior group | PMnR | ++ | +/- |
| **Basal forebrain** | **Midline group** | **Red nucleus** |
| Cl | - | + | PoT | ++ | +/- | DRV/DRI/DRD | +/- | +/- |
| IC/M | - | - | Eth | - | + | RMg/RPa/Rob | ++ | +/- |
| MS(Ld) | ++ | ++ | Reth | +/- | +/- | Red nucleus |
| VDB | ++ | ++ | PVA | +++ | - | RMC | - | + |
| HDB | ++ | ++ | PV | ++ | - | RPC | ++ | + |
| LSD | - | + | PVP | +/- | - | RPF | ++ | +/- |
| LSI | ++ | + | PT | +++ | + | RR | + | + |
| LSV | + | - | IMD | ++ | +/- | Reticular formation |
| TS | - | + | Re | + | +/- | CnF | - | + |
| Shi | ++ | + | VRe | ++ | +/- | DpMe | + | + |
| SFI | ++ | + | Rh | +++ | - | PhC | + | + |
| B (Meynert) | + | + | | | | | | |
Table 1 continued

| Mu | Delta | Mu | Delta | Mu | Delta |
|----|-------|----|-------|----|-------|
| SI | ++ | + | Xi | +++ | – | PnO | +/– | + |
| VP | ++ | + | Intralaminar group | +/– | + | Gi | + | + |
| DEn | ++ | +/– | rCM | +++ | +/– | DPGi | +/– | – |
| SFO | ++ | + | cCM | ++ | ++ | LPGi | + | +/– |
| **Basal ganglia** | | | | | | | | |
| AcbC | ++ | + | PC | +/– | + | GiV | + | + |
| AcbS | ++ | + | OPC | – | + | PCRtA | +/– | + |
| CPu | +++ | +++ | PIL | + | + | PCRt | +/– | + |
| LGP | ++ | + | PF | – | + | Rl | ++ | + |
| MGP | + | + | SPFP | +/- | +/- | Lrl | +/- | ++ |
| **Amygdala** | | | | | | | | |
| AAV | + | + | | | | | | |
| ACo | ++ | + | MnPO | – | +/- | MdD | + | + |
| PLCo | + | + | VMPO/AVPe/Pc | – | + | Tectum | | |
| PMCo | ++ | + | PaLM | +/- | + | APT | +/- | + |
| APir | + | ++ | PaV/PaAP | +/- | – | PPG/MPT/OPT | ++ | +/- |
| AHi | + | + | SCh | – | – | SuG | +/- | – |
| CxA | + | + | SO | +/- | + | Op | +/- | – |
| ASTr | +/- | + | Arc | + | +/++ | InG | + | + |
| BAOT | +++ | – | ME | – | + | InWh | + | + |
| LA | +/- | + | Medial | | | | | |
| BLA | +/- | +++ | MPA | ++ | +/- | PBG | + | – |
| BLP | +/- | ++ | MPOM/MMPOL | ++ | ++ | CIC | ++ | + |
| BMA | ++ | + | AHA/AHC/AHP | + | + | ECIC | + | ++ |
| BMP | + | + | DM | ++ | + | DCIC | +/- | – |
| I | ++ | – | VMH | ++ | + | BIC | + | + |
| **Extended amygdala** | | | | | | | | |
| BSTLD | +/- | +/- | LM | – | +/- | VTA | ++ | + |
| BSTLP/I/V | + | +/- | MM | ++/+/- | – | SNC | +/++ | +/++ |
| CeC | + | – | MMn | – | – | SNL | + | + |
| CcL | +/- | – | ML | +/- | – | SNR | + | + |
| Cel | +++ | + | PMV/PMD | ++ | + | ATg/VTg | – | + |
| CeM | +/++ | + | SuML | ++ | – | Mtg | + | ++ |
| IPAC | +/- | + | SuMM | +++ | + | PPTg | ++ | +/- |
| **Medial extended amygdala** | | | | | | | | |
| BSTMA | ++ | + | Lateral | | | SPTg | + | + |
| BSTMP | ++ | + | MCPO | +/- | + | RlTg | ++ | +/++ |
| BSTMV | ++ | +/- | LPO | + | +/- | DTgC | + | + |
| MeAD | +++ | + | LH | ++/+/- | + | DTgP | – | – |
| MeAV | ++ | + | PeF | + | + | DMTg | + | + |
| MePD | ++ | + | PSTh | + | + | DPTg | – | + |
| MePV | + | + | **Cerebellum** | | | | | |
| BSTIA | + | + | ChCx | – | – | Me5 | – | + |
| **Hippocampal formation** | | | | | | | | |
| DG | + | + | Lat | + | + | PC5 | + | +/- |
| CA1/CA3 | + | + | Med | + | + | Su5/I5 | +/- | + |
| Py | + | + | mcp | + | + | Pr5VL | +/- | – |
with previous identification, respectively, by a combination of electrophysiology and immunohistochemistry (Pedersen et al. 2011). Also, cellular co-expression of MOR-mcherry and DOR-eGFP in the main nucleus of the trapezoid body, the rostroventrolateral medulla, the hippocampus, the pons and the hypothalamus is in agreement with MOR–DOR detection using heteromer-specific antibodies (Gupta et al. 2010).

In the literature, DRGs represent one of the few sites where MOR/DOR co-localization was studied (Scherrer et al. 2009; Wang et al. 2010; Rau et al. 2005). Here, we found that MOR-mcherry and DOR-eGFP co-expression was restricted to discrete populations of small-, medium-, and large-size DRG neurons (Fig. 6a). We estimate that about 40 % of DOR-eGFP-positive (43 ± 8 %, n = 20) and one-third of MOR-mcherry-positive (35 ± 5 %, n = 20) neurons express the two receptors. Large neurons represent about 37 ± 8 % (n = 20) of the total number of neurons co-expressing the two receptors. The extent of receptor co-expression in this study is larger than previously reported using native MOR immunodetection in DOR-eGFP knock-in mice (Scherrer et al. 2009) but remains consistent with MOR and DOR being predominantly expressed on distinct populations of somatosensory neurons.

Using DOR-eGFP mice, we previously identified neuronal populations expressing DOR in the hippocampus (Erbs et al. 2012). Here, co-localization of MOR-mcherry and DOR-eGFP fluorescent signals was observed in GABAergic interneurons that control the firing rate of glutamatergic neurons (Fig. 7a). Co-expression in parvalbumin-positive neurons from the pyramidal layer suggests that these neurons are basket or chandelier cells (Erbs et al. 2012). Co-expression is also observed in horizontal somatostatin-positive cells close to the alveus which points to oriens-lacunosum moleculare or hippocampo-septal neurons (Erbs et al. 2012).

We then investigated whether MOR/DOR heteromers are detectable in the hippocampus, where extensive co-localization was observed. Co-immunoprecipitation experiments using antibodies directed against the fluorescent proteins indeed revealed close physical proximity between the two receptors supporting the hypothesis that MOR/DOR heteromers can exist in this structure (Fig. 7b).
Fig. 4 Brain mapping of MOR-mcherry expression at cellular resolution MOR-mcherry expression is observed in discrete neuronal populations at the level of the a cortex, b striatum, c habenula, d lateral hypothalamus, e periaqueductal gray matter, f paratrophic nucleus, g interpeduncular nucleus, h locus coeruleus area, i nucleus ambiguus, j detail of the lateral hypothalamus. Scale bars 200 and 20 μm (j)
Neuroanatomy of MOR-mcherry/DOR-eGFP neurons in double mutant mice following treatment with SNC80

To increase detection of DOR-eGFP expressing cell bodies, mice were treated with the DOR-selective agonist SNC80 (10 mg/kg, s.c. for 2 h) before perfusion. This drug induces DOR internalization in vivo, and subsequent degradation in lysosomal compartments (Pradhan et al. 2009), leading to concentrate DOR-eGFP fluorescence in the soma while depleting DOR-eGFP staining from neuron terminals. Upon treatment, the number of detected DOR-eGFP cell bodies increased substantially, revealing about twice as many regions with MOR-mcherry/DOR-eGFP co-expressing neurons compared to basal conditions (Fig. 5a). Most of these new areas concentrate in the brainstem and midbrain and are associated with distinct functional sensorimotor pathways (see “Discussion”). SNC80 treatment also revealed co-expression of the two receptors in neurons distributed across all layers of the spinal cord (Fig. 6b). This latter observation is concordant with a previous study reporting physical MOR/DOR interaction in the spinal cord using co-immunoprecipitation experiments (Gomes et al. 2004).

Notably, SNC80 treatment did not reveal any co-localization in the telencephalon other than the piriform cortex.
already identified under basal conditions. In essence, whether or not DOR agonist treatment was used, we could not detect MOR/DOR co-expression in brain areas where opioid receptors are most extensively studied. This includes the cortex, as well as ventral and dorsal striatum, where receptor cross talk may not be a major operating mechanism. A previous study using ELISA with heteromer-specific antibodies nevertheless reported MOR/DOR co-expression in these regions (Gupta et al. 2010), a discrepancy that could result from very low levels of co-localized receptors undetectable in our approach.

In conclusion, red/green mapping in double mutant mouse submitted to DOR agonist treatment confirms that double-positive MOR/DOR neurons are essentially distributed in midbrain and hindbrain, whereas neurons expressing a single receptor seem mostly restricted to the forebrain. Mining the MOR/DOR atlas, therefore, led us to postulate that functional interactions between MOR and DOR may operate at cellular level mainly in neurons forming midbrain and hindbrain pathways (“Discussion”).

Discussion

Functional interactions between GPCRs via signaling cross talk or heteromerization have long been established in vitro, but the relevance of these mechanisms in vivo is the subject of intense investigation. In this debate, opioid receptors are leading candidates because mechanisms underlying functional interactions across opioid receptors have important implications for opioid physiology and therapy. MOR/DOR interactions in cellular models are well established. However, there is very little evidence to support in vivo co-expression of MOR and DOR within the same neuron, a prerequisite for either signaling cross talk or physical interactions. Using double fluorescent knock-in mice expressing functional MOR and DOR in fusion with eGFP and mcherry, respectively, we produced a MOR/DOR brain atlas (http://mordor.ics-mci.fr/). This searchable database shows fine mapping of the two receptors throughout the nervous system with cellular resolution, and allows the identification of MOR/DOR neurons co-expressing the two receptors in vivo.

To get further insights into the molecular bases of receptor co-expression, we also investigated potential MOR/DOR physical interactions in the hippocampus where extensive co-localization is observed under basal conditions. Our co-immunoprecipitation experiments indicated MOR/DOR physical proximity in this structure, and in-depth analysis is now required to extend this finding to other regions showing MOR/DOR neuronal co-localization.

Methodological considerations

Detection of MOR/DOR neuronal co-expression relies on the concomitant visualization of the two fluorescent signals but also on our ability to fit their distribution with identifiable neuronal structures. In most brain regions, DOR and MOR expression levels range from 10 to 60 fmol/mg (Slowe et al. 1999; Lesscher et al. 2003; Kitchen et al. 1997; Goody et al. 2002), which raises the possibility of overlooking areas with the lowest expression levels. However, DOR and MOR fluorescent constructs were detected in all regions with previously identified wild-type DOR or MOR expression. Indeed, MOR-mcherry was readily observed owing to the intracellular accumulation of the red fluorescence even in low expressing neurons.

**Fig. 6** MOR and DOR neuronal co-expression in dorsal root ganglia and spinal cord. a In dorsal root ganglia, MOR-mcherry and DOR-eGFP are co-expressed under basal conditions in small- and medium-size neurons in addition to large neurons (shown in Fig. 5b) (arrows). Scale bars 20 μm. b Neurons co-expressing MOR-mcherry and DOR-eGFP are visualized in the different layers of the spinal cord following treatment with the delta agonist SNC 80 (10 mg/kg, s.c., 2 h). General view (top panel) and individual neurons (bottom panel). Scale bars 10 μm.
Accordingly, subsequent amplification with mcherry specific antibodies did not significantly improve MOR-mcherry detection. On the contrary, the green fluorescence associated with DOR was often weak, which required amplification with eGFP-specific antibodies for proper visualization. In addition, DOR-eGFP did not accumulate in the soma as for MOR-mcherry and the green fluorescence was often associated with passing fibers. Therefore, identification of neuronal cell bodies was hampered because of the lack of visual landmarks in particular in structures where the tissue organization was very dense. This limitation was overcome by treating animals with the DOR agonist SNC 80 that concentrated the green fluorescence in the soma in a manner similar to MOR-mcherry. Combining fluorescence amplification with agonist treatment significantly enhanced the sensitivity of our approach and drastically improved identification of DOR-eGFP neurons and, hence, MOR/DOR neurons (Fig. 5a). Nonetheless, the latter may still have escaped detection in regions of very low expression.

Analyzing MOR/DOR co-localization throughout the entire brain brought interrogations about the possible implications of neuronal co-expression. To address MOR/DOR functional role, we identified specific neuronal circuits in which neurons co-expressing the two receptors were located. For this purpose, networks were built that encompass regions of MOR/DOR co-localization with previously documented anatomical connections and behavioral outcome. Though this approach remains speculative and needs experimental validation, it offers a novel frame to investigate the in vivo implications of MOR/DOR co-expression.

**Eating and sexual behaviors**

A most remarkable observation from the MOR/DOR atlas is the widespread distribution of MOR/DOR co-expressing neurons in the brainstem. Receptor co-expression may reflect ancestral expression that was preserved across evolution owing to successful contribution to survival. Opioid receptors have been identified throughout vertebrates including frogs and fishes, and their expression likely results from initial duplication of an ancestral single MOR/DOR gene (Stevens 2009). MOR and DOR may therefore cooperate within neurons to regulate primitive aspects of animal behavior such as sensitivity to somatosensory stimuli and subsequent motor reflexes. Notably, modifications in binding and signaling properties observed in heterologous systems suggest that MOR/DOR co-expression enhances opioid-induced inhibition of neuronal...
activity (Rozenfeld and Devi 2011). Endogenous opioid peptides, therefore, may efficiently control primal behaviors through additive, synergistic or other mechanisms that differ from activation of a single receptor.

Another striking feature is the lack of MOR/DOR neurons in the telencephalon with the notable exception of the piriform cortex that integrates odorant stimuli (Wilson and Sullivan 2011). This may reflect the importance of odorant stimuli as key sensory inputs contributing to food search and recognition, identification of sexual partners or predator avoidance.

Neurons co-expressing MOR and DOR are detected in neuronal circuits that process food intake (Reis 2007; Shin et al. 2011; De Luca et al. 2007), NaCl and water uptake (Shin et al. 2011) or regulation of the sexual activity in both males (Hanson and Watson 2004) and females (Komisaruk and Whipple 2005). These include the intramygdaloid part of the bed nucleus of the stria terminalis, the anterior, lateral and posterior hypothalamus, the hippocampus, and the lateral parabrachial nucleus (Fig. 8a). Noteworthy, MOR/DOR neurons in the lateral hypothalamus overlap with orexin-positive neurons and may modulate the orexigenic component

Fig. 8 MOR/DOR neurons concentrate in networks essential for survival. a MOR/DOR co-expressing neurons are detected in pathways essential for survival. Areas belonging to pathways classically related to memory (filled blue circle), sex and food and water consumption (filled dark green), motor function (filled purple circle), nociception (filled black circle) and audition (filled light green circle) are indicated. Regions belonging to two networks are presented as a two-color circle. b In addition to MOR/DOR-containing neurons (filled black circle), brain regions activated by painful stimuli also included neurons expressing MOR only (filled red circle) See list for abbreviations
of eating behavior by exerting inhibitory controls (Harris and Aston-Jones 2006). Also MOR/DOR neuronal co-expression is found in amygdaloid areas, where lesions produce weight gain and obesity in female rats (King et al. 2003).

Taken together, MOR/DOR cross talk at cellular level may operate in neural circuits involved in behaviors associated with body homeostasis and sexual activities.

Perception and processing of aversive stimuli

Another fascinating observation is the presence of MOR/DOR neurons in subcortical networks responding to presentation of noxious or non-noxious aversive stimuli (Fig. 8a), as described in a recent translational study, that integrates functional neuroanatomy in rodents and meta-analysis of PET and fMRI data in humans (Hayes and Northoff 2011, 2012). Subcortical areas involved in these core aversion-related networks that also show MOR/DOR co-localization include the hippocampus, hypothalamus, nuclei from the midbrain such as the red and pontine reticular nuclei, several areas from the pons including the parabrachial nucleus and the rostral ventral medulla.

Regarding pain processing, co-expression of the two receptors is high throughout nociceptive pathways, including the rostral ventral medulla, lateral parabrachial nucleus, spinal cord and DRGs (Fig. 8b). Interestingly, we were not able to identify concomitant MOR and DOR expression in neurons of the periaqueductal gray, a brain area central to pain control (Basbaum et al. 2009). MOR and DOR may function independently at this level of pain processing, although we cannot exclude that fluorescent signals remained below detection thresholds.

Finally, MOR/DOR neuronal co-expression is also observed in the memory network involving the hippocampus, some septal areas and mammillary bodies (Fig. 8a). MOR/DOR interactions may therefore modulate hippocampal activity and, in particular, the CA1 area operating as a coincidence detector (Faget et al. 2012; Duncan et al. 2012). Similarly, the detected MOR/DOR neurons may influence odor processing in the piriform cortex considered as another coincidence detector (Wilson and Sullivan 2011).

In conclusion, endogenous opioid peptides may trigger MOR/DOR-specific responses in brain pathways contributing to avoid and/or cope with threatening situations. Interestingly, networks associated to perception of aversive painful stimuli also encompass areas where MOR is detected alone. In these regions, presented in Fig. 8b, MOR-mediated control is likely the predominant mechanism.

Sensorimotor pathways

The surprising observation of MOR/DOR co-expression in brain areas involved in motor activity (Fig. 8a) expands our current understanding of opioid physiology. Food, water or salt intake require mandibular movements involved in masticatory reflexes and jaw movements, where the two receptors may co-modulate somatomotor orofacial activity and influence parasympathetic responses and cardiovascular function associated with feeding (Goto and Swanson 2004; Dong and Swanson 2003; Mascaro et al. 2009). Accordingly, MOR/DOR neurons are distributed throughout descending projections to hindbrain preganglionic parasympathetic nuclei and orofacial motor pattern generators. We also observed MOR/DOR neurons in brainstem networks associated to motor aspects of sexual activity, including the (para) gigantocellular reticular formation and lateral vestibular nucleus that participate to the regulation of penile reflexes (Hamson and Watson 2004).

Detection of aversive stimuli, need for food, water or sexual attraction also requires appropriate motor responses. The strong link between aversion-related networks and the autonomic nervous system is especially remarkable and suggests that MOR/DOR neuronal co-expression is essential for the modulation of both ascending somatosensory information and corresponding descending reflex responses intended to protect the individual. Accordingly, MOR/DOR neurons are present in areas of the basal ganglia contributing to integration of information that control motor responses to auditory, visual or olfactory stimuli (Fig. 8a). In addition, we observed MOR/DOR neurons in the auditory complex and the vestibular system that participate in body balance (Sturnieks et al. 2008) (Fig. 8a). Finally, MOR/DOR neuronal co-expression in the auditory system may modulate the processing of auditory inputs and hence impact on male copulation through strong connections to the (para) gigantocellular reticular formations (Bellintani-Guardia et al. 1996).

Therapeutic implications

Targeting MOR/DOR-mediated signaling mechanisms, which would be distinct from single receptor signaling, may lead to develop innovative therapeutic approaches. The identification of neural networks with potential intracellular MOR/DOR interactions provides valuable hints toward selected therapeutic effects.

Mu opioid receptors represent a major target for analgesics, but progressive loss in opioid drug efficacy constitutes a key challenge for clinicians. On the other hand, the notion that DORs significantly contribute to the development of morphine tolerance has often been put forward, but the molecular mechanisms of this particular MOR/DOR interaction remain elusive (Cahill et al. 2007). Specific trafficking and signaling properties of MOR/DOR heteromers were reported in heterologous systems, and the therapeutic potential of receptor heteromers is being
considered to reduce opioid tolerance (Berger and Whistler 2011; Gomes et al. 2013). The high co-localization of MOR and DOR in neurons from nociceptive pathways supports this view and designates the putative heteromers as an attractive target in pain management. In addition, the absence of neuronal co-localization in the pre-Bötzinger complex suggests that MOR/DOR specific targeting may produce analgesic effects devoid of respiratory depression side effects.

Neurons co-expressing the two receptors are also abundant in brainstem nuclei tightly connected with the autonomic nervous system. At these sites, the two receptors may functionally cooperate in the generation of somatic and autonomic symptoms during drug withdrawal. The rostral ventromedial medulla, especially the raphe magnus and nucleus paragigantocellularis are engaged in the expression of several aspects of physical opioid withdrawal, via their efferent projections to autonomic and somatic motor neurons. In addition, the nucleus paragigantocellularis represents the major source of excitatory drive to the locus coeruleus during withdrawal (Williams et al. 2001). Targeting MOR/DOR heteromers may therefore represent an attractive strategy to reduce opioid withdrawal, and possibly withdrawal signs associated with other drugs of abuse. As such, MOR/DOR heteromers have been proposed as a promising molecular entity for the development of selective antagonists to treat alcoholism (van Rijn and Whistler 2009).

Finally, neuronal co-expression of the two receptors in the lateral hypothalamus has interesting implications. MOR/DOR co-localization occurs partly in orexin-positive neurons. Hence, receptor heteromers may represent a potential target for novel strategies to treat obesity. Also, orexin-positive neurons are critical for both food and drug reward. MOR/DOR-specific mechanisms may therefore be targeted to reduce drug-seeking behavior (Aston-Jones et al. 2009).

Conclusion

Overall, MOR/DOR co-expressing neurons are extremely scarce in forebrain networks responsible for higher-order processing, and are detectable mainly at the level of mid- and hindbrain regions with connections to the autonomic nervous system. Mining the brain atlas therefore suggests that functional interactions between MOR and DOR operate predominantly at circuitry level for mood control, reward processing and cognition, whereas the two receptors may cooperate intracellularly in neural networks essential for survival.

Close physical proximity strongly supports the existence of in vivo mu–delta heteromers in the hippocampus. MOR/DOR physical association in neural networks associated with abnormal nociception, aversive aspects of drug withdrawal or eating disorders represents an attractive option for drug design. The identification of neurons co-expressing the two receptors in the nervous system will now initiate in-depth in vivo investigations to understand molecular mechanisms underlying MOR/DOR cooperativity in selected neural networks, and their functional significance in complex behaviors. Ultimately, the MOR/DOR atlas resource provides a proof-of-principle approach to address the challenging issue of GPCR interactions and heteromerization in physiology and disease.

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Conflict of interest

The authors declare no conflict of interest.

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